DEVELOPMENT OF *DIPLOCARPON ROSAE* IN LEAVES OF ROSES DIFFERING IN THEIR SUSCEPTIBILITY

by

JANUSZ JÓZEF KUKLINSKI

B.Sc (King's, London)

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Department of Botany,

Imperial College of Science and Technology,

Silwood Park,

Sunninghill,

Ascot

Berkshire

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ABSTRACT

Isolates of the blackspot fungus, *Diplocarpon rosae* Wolf, from the rose cultivar Frensham germinated equally well on Frensham and Allgold leaf disks. Blackspot lesions usually developed on Frensham but only occasionally on Allgold. The extent of the reaction was partly determined by the temperature at which the plants were grown and the tests were made but an examination of conidial collections from Frensham indicated also some heterogeneity of inoculum.

New staining techniques were developed to study the penetration and development of the fungus in intact and sectioned leaf disks. The number of penetrations was similar on both cultivars but on Allgold fewer penetrating hyphae succeeded in infecting epidermal cells. At c. 48 h after inoculation, there was no visible change in infected epidermal cells on Frensham but on Allgold these often browned rapidly and in these instances the fungus appeared to be restricted to small groups of such cells. However, the reaction of Allgold in this way was not consistent. There was no evidence of such a response on Frensham even in the late stages of infection.

Ultrastructural studies indicated that subcuticular (runner) hyphae, inter- and intracellular hyphae and haustoria, apparently similar in type and organisation, could develop on both cultivars (though to different extents) but that on resistant Allgold haustoria in the palisade cells failed to expand or when they did they were surrounded by a convoluted extrahaustorial membrane.

The reaction of some new cultivars to *D. rosae* was also tested. Only an un-named cultivar, K97E, was resistant but the mechanism appeared different to that of Allgold.

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INTRODUCTION

Blackspot, caused by the ascomycete fungus *Diplocarpon rosae* Wolf, is common in most areas where roses are grown. As the disease name indicates, the main symptom is a black lesion which develops on the upper surface of the rose leaf. This is associated with the growth of fungal hyphae in strands beneath the cuticle ('runner' hyphae) on which acervuli develop and give rise to 2-celled, hyaline conidia which are dispersed by rain. Affected leaves turn yellow and drop prematurely often leaving bare shoots thus further disfiguring the plant.

Rose cultivars visibly differ in their susceptibility to blackspot in terms both of the number and size of lesions which develop on them and the severity of defoliation which subsequently ensues. At present virtually nothing is known about the relationship between lesion development and defoliation except in the most general sense that the two are not necessarily related. On some cultivars small lesions evoke considerable defoliation and on others large lesions develop with little defoliation. Most studies of resistance have been concerned with lesion development on either detached leaflets (Jenkins, 1955; Palmer & Semenuik, 1960; Palmer *et al.*, 1966a) or leaf disks (Saunders, 1970; Knight & Wheeler, 1978) and resistance has been attributed variously to cuticle thickness (Green, 1931; Dodge, 1931), accumulation of polyphenols (Saunders, 1967) and phytoalexins (Saunders, 1967; Knight, 1976).

The variability of the pathogen is a major obstacle in such studies. Collections of *D. rosae* from different localities or even from different cultivars within one locality often induce quite diverse reactions when tested on a standard range of rose cultivars (Frick, 1943; Jenkins, 1955; Palmer *et al.*, 1966a). Also the fungus grows relatively poorly in culture

and soon loses its pathogenicity (Palmer *et al.*, 1966b). The discovery that viable conidia could be recovered from acervuli on infected rose leaves stored at $-15^{o}C$ partly overcame these problems and led to the development of a standard leaf disk test to assess lesion development (Knight, 1976). Even here the results were not always consistent suggesting that the susceptibility of leaf tissue was partly affected by the conditions under which the roses were grown, a feature found also by Palmer *et al* (1966b) in their experiments with roses grown in glasshouses. They obtained two peaks of susceptibility (early spring and late summer) and suggested temperature and day length as two possible factors influencing the susceptibility of rose leaves. Nevertheless, Knight's tests established that generally when isolates of *D. rosae* from the cultivar Frensham were used as inoculum lesions developed on leaf disks of Frensham but not on those of Allgold. This seemed a promising experimental system to investigate resistance and studies, initiated by Knight, first concentrated on the behaviour of conidia on the two cultivars. He established that slightly fewer conidia germinated on Allgold and produced evidence that germinating conidia induced an antifungal substance though not exclusively on Allgold.

The aim of the present investigation was to extend Knight's work and examine by light and electron microscopy the development of *D. rosae* on Frensham and Allgold in the hope that the resistance of Allgold could be characterized. It includes also a preliminary investigation of the resistance of some newer rose cultivars.

I. CONIDIAL GERMINATION OF *D. ROSAE* AND LESION DEVELOPMENT ON SELECTED ROSE CULTIVARS

INTRODUCTION

The experiments reported here first re-examined the behaviour of some Frensham isolates on Frensham and Allgold (Part A) and then (Part B) investigated the effects of temperature and light on the development of lesions, lesion formation on Frensham and Allgold leaf disks cut from plants growing at different temperatures and the variability of isolates collected from roses, mainly Frensham, at Silwood Park.

A. CONIDIAL GERMINATION AND LESION DEVELOPMENT

METHODS

The methods used were essentially those described by Knight (1976) and are thus only given in outline.

(i) Disease-free leaf material

Roses in 30.5 cm pots were pruned and kept at 5°C to arrest shoot development. Three weeks before leaf material was required they were sprayed with dodemorph (2.5 g/l; BASF Ltd), fertilized with 'Baby Bio' (Pan Britannica Ltd) and transferred to a sandbench within a filtered air cabinet (Finney, 1973) in a glasshouse with a 16 h photoperiod.

(ii) Inoculation of leaf disks

Disks (1 cm^2) were cut from the penultimate fully expanded leaves on young shoots, washed for 0.5-1 h in running tap water, blotted dry and placed, adaxial surface uppermost, on damp blotting paper. Each disk was inoculated centrally with 0.005 ml of a suspension containing 200,000 conidia/ml of *D. rosae* using an Agla microsyringe. The inoculated disks were floated on sterile distilled water in polystyrene boxes (5.5 x 3.5 x 2 cm) and incubated at 20°C.

(iii) Assessment of germination and lesion formation

Conidia were removed by pressing small pieces of polypropylene tape (Sellotape E1201) onto the inoculated leaf disks. These pieces were mounted in cotton-blue lactophenol and at least 100 conidia were assessed per leaf disk. Lesion diameters were measured to the nearest 0.5 mm after 14 days.

(iv) Isolates

In the following experiments, conidia were derived from three

collections of *D. rosae* from Frensham, two collected from the field in 1976 and 1977 respectively (F-76-Ash and F-77-Ash) and the third from an infected plant in the glasshouse (F-78-G.H.) inoculated with field isolate (F-77-Ash). All three collections were stored at -15°C.

Isolate F-76-Ash

Leaf disks of Frensham and Allgold were inoculated on June 1977 and germination on ten disks was assessed after 24, 48 and 72 h. There were small but significant differences in germination on the two cultivars at 24 and 48 h but not at 72 h (Table 1).

In another experiment with this isolate ten leaf disks of Frensham, Allgold, Masquerade, Orange Sensation and Iceberg were inoculated on December 1976 and then lesions were measured after 14 days. Only two leaf disks of Allgold, and one of Masquerade became infected compared with ten disks of Iceberg and Orange Sensation and eight of Frensham (Table 2). Lesion size averaged over all ten disks indicated a significant difference between the reactions of Allgold and Masquerade on the one hand and Frensham, Iceberg and Orange Sensation on the other as described by Knight (1976) in his experiments.

Isolate F-77-Ash

Eighteen leaf disks of Frensham and Allgold were inoculated on July 1977. Germination was assessed on five disks after 24 h and 48 h and lesions were measured on the remaining disks after 14 days. There was no difference in germination or mean lesion development on the two cultivars (Table 3). Lesions developed on five leaf disks of Allgold but not on the remaining three (Appendix Table 4).

In another experiment fourteen leaf disks of Frensham and Allgold

TABLE 1 : Mean % germination of *D. rosae* (F-76-Ash) on Frensham and Allgold leaf disks*.

	24 h	48 h	72 h
Frensham	7.6	16.7	17.1
Allgold	4.0	14.1	15.6
Probability (P) that means differ	0.001	0.05	n.s.

* Details in Appendix Table 1.

TABLE 2 : Lesion development (mean diameter in mm) on rose cultivars inoculated with *D. rosae* (F-76-Ash)*.

Masquerade	Allgold	Orange Sensation	Frensham	Iceberg
0.6	0.8	4.8	5.3	6.3

Underscored groups: Masquerade–Allgold; Orange Sensation–Frensham; Frensham–Iceberg.

Cultivars not underscored by the same line differed significantly ($P < 0.01$).

* Details in Appendix Table 2.

TABLE 3 : Development of *D. rosae* (F-77-Ash) on leaf disks of Frensham and Allgold.

	Mean % germination*		Mean lesion size (mm)†
	24 h	48 h	14 days
Frensham	13.2	16.2	5.6
Allgold	11.0	11.8	4.2

* Details in Appendix Table 3.

† Details in Appendix Table 4.

were inoculated on April 1978 and lesions were measured after 14 days. No lesions developed on seven Allgold disks and two of Frensham. Lesion size averaged over all fourteen disks indicated a significant difference between the two cultivars (Appendix Table 5). In an experiment carried out on January 1978 (Appendix Table 21), no lesions developed on any Allgold disks.

In one further experiment with this isolate on September 1978, ten leaf disks of Frensham and Allgold were inoculated and examined for the development of lesions after 14 days. All disks of both cultivars developed lesions which were not significantly different (Table 4).

Isolate F-78-G.H.

Disks of Frensham and Allgold were inoculated in May 1978 and lesions were measured after 14 days. No lesions developed on Allgold but only one Frensham disk remained uninfected (Table 5).

In another experiment in September 1978 disks of Frensham and Allgold were inoculated with the same isolate and examined after 14 days for the development of lesions. All Frensham disks developed lesions but only one Allgold disk became infected (Table 5).

In one further experiment thirty disks of Frensham and Allgold were inoculated in October 1978. Germination was assessed on ten disks at 24 h and 48 h and lesions were measured on the remaining disks at 14 days. Germination was similar on both cultivars (Table 6) and was unusually high (cf. Table 1). Also lesions developed on all leaf disks of Allgold and they were as large as those on Frensham.

TABLE 4 : Lesion development (mean diameter in mm) by *D. rosae* (F-77-Ash) on Frensham and Allgold leaf disks inoculated at different times*.

Date of inoculation	Frensham	Allgold	P
July 1977	5.6	4.2	n.s.
January 1978	7.4	0	0.001
April 1978	3.7	2.5	0.05
September 1978	6.1	7.2	n.s.

* Details in Appendix Tables 4, 21, 5 and 6 respectively.

TABLE 5 : Lesion development (mm) on Frensham and Allgold leaf disks inoculated in May and September, 1978 with *D. rosae* (F-78-G.H.).

Replicate	May		September	
	Frensham	Allgold	Frensham	Allgold
1	9.5	0	5.5	5.0
2	7.0	0	6.5	0
3	6.5	0	5.0	0
4	6.5	0	5.0	0
5	9.0	0	5.0	0
6	7.0	0	7.0	0
7	8.5	0	3.5	0
8	6.5	0	6.0	0
9	9.0	0	7.0	0
10	0	0	7.5	0
Mean	7.0	0	5.8	0.5

TABLE 6 : Development of *D. rosae* (F-78-G.H.) on Frensham and Allgold leaf disks*

	Mean % germination		Mean lesion size (mm)
	24 h	48 h	
Frensham	57.5	65.4	6.5
Allgold	62.5	60.4	7.2

* Details in Appendix Table 7.

B. FACTORS INFLUENCING LESION DEVELOPMENT

EXPERIMENT 1

This experiment examined the effect of different temperatures (in some instances, with or without lights) on the development of lesions in the leaf disk test. Ten leaf disks of Frensham and Allgold (both grown in the glasshouse) were inoculated on September 1978 with *D. rosae* (F-78-G.H.) and incubated as follows: 10°, 15° (± light), 20° (± light), 25° (± light) and 30^oC. The light treatment consisted of placing the polystyrene boxes containing inoculated disks in an illuminated incubator (0.2-1.8 lumens/ft^2) at the appropriate temperature. Otherwise the boxes of leaf disks were placed within incubators in dark cardboard containers which excluded light. All disks were examined after 14 days and lesions were measured.

Lesions developed on all Frensham disks. On these the incubation temperature significantly affected lesion size (Table 7a) but light only affected lesion size at 20^oC where lesions on illuminated disks were larger than those on disks kept in the dark. Relatively fewer lesions developed on Allgold leaf disks (Table 7b) especially those illuminated at 20 and 25^oC where some showed browning of cells characteristic of the resistant response (p. 42). There was again a significant effect of temperature on lesion size (particularly for disks that were not illuminated) and possibly an effect of light at 20^oC but this comparison is based on only one infected disk of Allgold in the light treatment (Appendix Table 9, Table 7c). If the overall effects on lesion development on lesion development are compared by averaging lesion size over all disks then clearly the reaction of the two cultivars is markedly different except at 10^oC (Table 7d).

TABLE 7 : Development of *D. rosae* (F-78-G.H.) on leaf disks incubated with or without light at different temperatures*.

(a) Mean lesion size: Frensham.

	°C				
	10	15	20	25	30
Without light	1.3[a]	5.2[b]	5.5[b]	7.0[c]	9.9[e]
With light	-	5.6[b]	8.3[d]	6.7[c]	-

Means with a different letter differ significantly ($P < 0.05$)

(b) Number of disks infected (of 10): Allgold.

Without light	10	9	8	8	9
With light	-	8	1	1	-

(c) Mean lesion size: infected disks, Allgold.

Without light	1.2[a]	2.1[a]	1.9[a]	5.3[b]	8.6[c]
With light	-	1.4[a]	(5.5)†	(6.0)†	-

Means with a different letter differ significantly ($P < 0.05$)

† One infected disk only

(d) Mean lesion size: all disks.

	Without light					With light		
	10	15	20	25	30°C	15	20	25°C
Frensham	1.3	5.2	5.5	7.0	9.9	5.6	8.3	6.7
Allgold	1.2	1.9	1.6	4.2	7.7	1.1	0.6	0.6
Probability (P) that means differ	n.s.	0.001	0.001	0.001	0.05	0.001	0.001	0.001

* Details in Appendix Tables 8 and 9.

EXPERIMENT 2

This experiment investigated whether the susceptibility of Frensham and Allgold was altered by growing plants at different temperatures. Pots of both cultivars, pruned and fertilized as described in section A, were placed in controlled temperature rooms at 10°, 15°, 20°, 25° and 30°C with a 16 h photoperiod (using six 125 watt daylight fluorescent tubes - Thorn). As the growth of these plants differed considerably (and was extremely slow at 10°C) it was 6 wk before enough leaf material was available for a standard laboratory test and even then it was not possible to have the same number of replicates.

Disks were inoculated with isolate F-78-G.H. on October 1978 and examined at 7 and 14 days. After 7 days, lesions on Frensham grown at 20°, 25° and 30°C were clearly visible; those on Frensham grown at 10° and 15°C were less distinct and there were no visible lesions on Allgold. The results at 14 days can be considered in two ways. In terms of numbers of disks on which lesions developed there appeared to be a marked effect of temperature in respect of Frensham but relatively little effect with Allgold (Table 8a). Thus lesions developed on only about a third of the Frensham leaf disks at 10° and 15°C whereas at 30°C lesions appeared on all disks. On Allgold, lesions developed on approximately 20% of the disks at 10°, 15°, 20° and 25°C and on 50% at 30°C. If the size of the lesions on these infected disks is averaged (Table 8b) then it is apparent that on Frensham lesion size increases with temperature whereas on Allgold it does not, but as few lesions developed on this cultivar the results must be viewed with caution. The overall effects of temperature on lesion development can be obtained by averaging lesion size over all disks (i.e. scoring as 0 those on which no lesions appeared). This indicates (Table 8c) that there were significant differences in the reactions of the two cultivars

TABLE 8 : Lesion development of *D. rosae* isolate (F-78-G.H.) on Frensham and Allgold leaf disks from plants grown at different temperatures*

(a) % leaf disks with lesions.

	°C				
	10	15	20	25	30
Frensham	36.4	30.8	78.6	75	100
Allgold	20	27.8	16.7	16.7	50

(b) Mean lesion size (mm): infected disks only

Frensham	5.5	6.1	5.7	8.0	8.7
Allgold	7.0	5.8	5.0	6.0	7.1

(c) Mean lesion size (mm): all disks

Frensham	2.0	1.9	4.5	6.0	8.7
Allgold	1.4	1.6	0.8	1.0	3.6
Probability (P) that means differ	n.s.	n.s.	0.01	0.01	0.001

* Details in Appendix Table 10.

at 20°, 25° and 30°C.

EXPERIMENT 3

This experiment (which has three parts) investigated the variability of *D. rosae* isolates taken mainly from the Frensham planting at Silwood Park. Tests of these isolates on Frensham and Allgold leaf disks were done between 10 November and 7 December 1977 so that variation in host physiology would be minimal.

(a) Two isolates from Frensham were tested: one (A) collected on 31 October, the other (B) on 7 November 1977. Disks of Frensham and Allgold were inoculated on 7 November and examined for lesions 14 days later. No lesions formed on Allgold inoculated with isolate A but lesions developed on most disks inoculated with isolate B and the mean size of lesions on these disks was somewhat larger, but not significantly than that on Frensham disks (Table 9a).

(b) Two isolates were collected on 24 November 1977, one (C) from Frensham adjacent to bushes of Masquerade, the other (D) from Frensham some 10-15 m away from these bushes. Disks of Frensham and Allgold were inoculated with these isolates on 28 November and examined for lesions 14 days later. No lesions formed on any Allgold leaf disks inoculated with isolate D but with isolate C lesions developed on all disks and these were as large as those on Frensham (Table 9b).

(c) Three isolates were collected on 28 November 1977, one (E) from bushes of Masquerade and two from Frensham either near to (F) or some 10-15 m from (G) Masquerade. Leaf disks of Frensham and Allgold were inoculated with these isolates on 7 December and assessed 14 days later. Lesions did not develop on all disks even those from Frensham (Appendix

TABLE 9 : Development of *D. rosae* isolates* on leaf disks.

	Isolates*	Mean lesion size (mm)†		Probability (P) that means differ
		Frensham	Allgold	
(a)	A	7.3	no lesions	-
	B	6.7	7.7	n.s.
(b)	C	7.2	8.0	n.s.
	D	7.9	no lesions	-
(c)	E	4.9	6.5	0.05
	F	4.7	5.6	n.s.
	G	5.2	5.6	n.s.

* See text and Appendix Tables 11, 12 and 13 for details.

† Infected disks only.

Table 13) but there was little indication, except possibly with isolate G, that more disks of Allgold remained uninfected. The mean lesion size (infected disks only) was similar for all three isolates on Allgold but on Frensham the lesions produced by isolates E and F were smaller (but not significantly) than those produced by isolate G (Appendix Table 13).

C. DISCUSSION

The results presented in this section will be discussed later in relation to the main themes of this thesis but a few points require some consideration now. Clearly the temperature at which roses are grown influences the susceptibility to isolates as measured by a leaf disk test (B. Expt. 2. Table 8) so that some variation in the results of such tests might be expected throughout the year. Also, varying the conditions of light and temperature under which isolates are tested on leaf disks markedly affects the results (B. Expt. 1. Table 7) but in this instance these are factors which can be adequately controlled. The main problem seems to be the variability of the *D. rosae* isolates. For example, in Expt. 3b (Table 9) the two isolates were taken either near to (C) or some distance from (D) Masquerade because Knight (1976) showed that isolates from Masquerade could infect Allgold (as well as Frensham) whereas those from Frensham usually did not affect Allgold. It was not entirely unexpected, therefore, that isolate C infected both cultivars - it could well be a 'Masquerade isolate' - whereas isolate D was the typical 'Frensham isolate' described by Knight (1976). However, in Expt. 3c (Table 9) isolate G might reasonably have been expected to behave like isolate D, since it was collected from a similar location, but it did not. The two tests were also done within 9 days of each other on leaf material produced at the same time of year.

Even so, with Allgold, there are some indications that leaf material raised under similar conditions might vary in its susceptibility since in tests with one conidial preparation some discs become infected while others do not (Table 4). The balance between a resistant and a susceptible reaction on this cultivar thus seems to be a fine one.

II. GROWTH OF *D. ROSAE* IN LEAVES OF FRENSHAM AND ALLGOLD

INTRODUCTION

The results of the previous section indicated that *D. rosae* could infect leaf disks of Frensham and Allgold though to different extents. Whilst germination of conidia on the leaf surface also varied it did not seem, in itself, sufficiently different to account for the observed difference in susceptibility of the two roses but suggested rather that the resistance seen in Allgold was operating at a later stage.

Several workers have suggested that the cuticle plays a significant role in the resistance of roses to blackspot, either because its surface waxes act as repellents to water drops charged with conidia (Aronescu, 1934; Frick, 1943) or because a thick cuticle acts as a physical barrier which the fungus cannot penetrate successfully (Green, 1931; Dodge, 1931).

The main events in the penetration of rose leaves by *D. rosae* have been described most clearly by Aronescu (1934). She demonstrated that the conidia, even non-germinated ones, adhere closely to the leaf surface suggesting that they are surrounded by some mucilaginous substance.

Following conidial germination, the germ-tubes swell to form appressoria which stain darker with cotton blue-lactophenol at their points of contact with the cuticle. The cuticle is then penetrated by a fine hypha (penetration peg) which arises from the base of the appressorium. Attempts by Knight (1976) to examine penetration of *D. rosae* quantitatively by light microscopy and by scanning electron microscopy proved unsuccessful. This lack of data prompted the present study of penetration, the basic aim of which was to determine the role of the cuticle in the apparent resistance of Allgold.

In order to understand a mechanism of resistance it is important to

characterize the sequence of events during infection of a susceptible cutlivar and to determine the success or failure on a resistant one. With this in mind, the further development of *D. rosae* in Frensham and Allgold was next compared by examining sectioned material in the light and electron microscopes. Ducomet (1907) was the first to attempt a detailed scientific study of *D. rosae*. More comprehensive work by Wolf (1912a, b; 1913), Chifflot (1914), Dodge (1931), and Frick (1943) followed. However, the most detailed and complete description of *D. rosae* infection on susceptible rose cultivars is probably that by Aronescu (1934).

These studies indicate that penetration is followed by the formation of an infection hypha which grows through the sub-cuticular layers apparently by both mechanical and enzymic processes. After forming haustoria in the epidermal cells the infection hyphae branch to produce intercellular hyphae and also "runner-hyphae" which travel in the upper wall and serve to spread the infection by providing new sources for the development of haustoria. Haustoria in the palisade cells are next formed. A circular lesion is eventually produced on which the spore bearing structures or acervuli develop.

The present studies and those of penetration involved both the appraisal of published techniques (not all of which were successful), and the development of new ones.

A. TECHNIQUES

1. Removal of the leaf cuticle to study penetration

The aim of this part of the work was to determine whether the cuticles of rose leaves infected with *D. rosae* could be removed satisfactorily so that the penetration points of the fungus could be counted. Several chemical techniques for removing cuticles were tried in which infected leaf disks (1 cm^2) of Allgold and Frensham were floated on varying concentrations and mixtures of the following: pectic enzymes (Johnston *et al.*, 1965; Preece, 1962), ammonium oxalate / oxalic acid solution (Huelin & Gallop, 1951), zinc chloride and hydrochloric acid (Holloway & Baker, 1968), cellulase and sulphuric acid. Healthy leaf disks of apple and broad bean (*Vicia faba*) were also treated with these materials because some of the published methods apparently removed the cuticles of these plants satisfactorily (Appendix Table 14).

The best results with the infected two rose cultivars were obtained when disks were left in a zinc chloride - hydrochloric acid solution (5 g $ZnCl_2$, 8.5 ml conc. HCl) for 6 h but epidermal cells were often found adhering to the detached cuticles and this made it difficult to observe penetration by germinating conidia after staining with cotton blue-lactophenol. Also, many such conidia were dislodged during the detachment of the cuticle. The addition of 2% pectinase to the zinc chloride - hydrochloric acid solution helped slightly in removing epidermal cells from the cuticle. The cuticles of apple and broad bean (*Vicia faba*) were more easily detached than those of Frensham and Allgold. The cuticles on the upper (adaxial) surface of uninfected rose leaf disks proved to be easier to remove than the cuticles on infected disks, suggesting that the subcuticular mycelium may have been anchoring the cuticle to the epidermal cells.

Treatment of infected rose leaf disks for 2-3 min. in concentrated sulphuric acid yielded only fragments of cuticle which, when stained in cotton blue-lactophenol showed holes possibly caused by penetrating germ tubes of *D. rosae* but all conidia were removed by this process.

The difficulties experienced in removing cuticles from rose leaves suggested that this approach was unlikely to provide a reasonable method for examining quantitatively penetration by *D. rosae* so various clearing and staining techniques were developed not only to study penetration but also to follow the development of the fungus within leaves. These techniques were applied to intact leaf disks and to leaf sections.

2. Clearing and staining of leaf disks

Clearing leaf disks by boiling in 95% ethanol for 15 min. followed by staining in cotton blue-lactophenol as described by Aronescu (1934) did not stain either penetration points or sub-cuticular fungus. Similarly an attempt to demonstrate the fungus in tissue by using potassium hydroxide and Grams iodine solution (Gurr, 1965) which has an affinity for chitin, a principal component of the cell walls of higher fungi, failed to stain sub-cuticular mycelium. The use of various methods to clear infected leaf tissue was next examined systematically. The specific techniques are listed in Appendix Table 15. Of these, only immersion in absolute ethanol for 24 h (Method 1) consistently gave good results as judged by the ability to view internal tissues of whole disks when used in conjunction with various stains.

The staining techniques attempted are listed in Appendix Table 16. The methods examined by Knight (1976, p. 129) which failed to give satisfactory results were not examined. Of the dyes used, two appeared particularly promising: crystal violet and thionin. These formed the basis of

the following methods which were subsequently adopted for most work.

(i) Infected leaf disks (1 cm^2) were cleared for 24 h in absolute ethanol and then, without washing, were immersed in 0.1% aqueous crystal violet for 10-15 min. (Corlett & Kokko, 1975). Temporary mounts were made in 50% glycerol. This method, the first to be developed, was particularly useful for viewing haustoria which stained violet. Its main disadvantages were that it also stained host tissue to some extent but did not adequately stain either the penetration points of the fungus or subcuticular hyphae. However, when cleared disks were immersed for 10 min. in 5% aqueous phenol and then transferred without washing to 0.1% aqueous crystal violet for 5-10 min., the runner hyphae of the subcuticular fungus stained reddish-violet. The phenol treatment proved to be an essential step in the process: without it, the subcuticular fungus was not stained.

(ii) The penetration points of the fungus were more readily identified by substituting phenolic thionin (0.1 g thionin, (Solmedia Ltd) in 100 ml 5% aq. phenol) for the separate treatments of phenol and crystal violet of the above method (Stoughton, 1930). Either cleared or uncleared (green) leaf disks were immersed in the phenolic thionin, usually for 5-10 min., although the length of this period did not appear critical and could be extended to several hours. Disks were then mounted in 50% glycerol. Penetration points were identified by dark blue haloes. The subcuticular hyphae stained light blue (Plate 4) and so did the haustoria (Plate 5 & 6). The haloes retained their stain for at least one year but the hyphae soon lost their stain so for studies of these the preparations were examined immediately.

3. Preparation and staining of sectioned material

The clearing and staining techniques developed in section 2 were first

used to study the fungus in thick sections (12 µm) of fresh, infected tissue cut on a Cambridge freezing microtome. Better results were obtained using thin sections (0.5-2.5 µm) of material prepared for electron microscopy as follows.

Small squares (1-2 mm) of infected tissue were cut from leaf disks under glutaraldehyde (2% v/v) in 0.1 M sodium cacodylate buffer at pH 7 (Perera & Gay, 1976), to which caffeine (0.5% w/v) was usually added (Mueller & Greenwood, 1978). They were left for 24 h at 18-22°C, and then washed three times in buffer. This was followed by 2-3 h in osmium tetroxide (1% w/v) in the buffer at 18-22°C and dehydration in an ascending series of acetone solutions including three ½ h washings in 100% acetone. Resin (Spurr, 1969) was infiltrated using acetone as a link reagent and after several changes in undiluted resin the speciments were transferred to flat embedding dishes and incubated at 70°C for 8 h. The embedded material was cut, in predetermined planes, on a Reichert microtome ("Om U2" Ultra microtome) using glass knives.

For light microscopy, sections were secured to a glass slide by passing it over a flame, after which a stain was applied. Excess stain was washed off in a stream of water and, after drying, the sections were covered with 50% glycerol to give semi-permanent preparations.

The staining procedures were adopted after extensive trials, the details of which are given in Appendix Table 17. With 2.5 µm sections, applying aqueous azure blue (Pfaltz & Bauer inc, 0.1% w/v) gave the best results. Intracellular and intercellular fungus stained bluish-brown, collars around the haustorial neck light blue and haustoria brown with blue borders (Plate 8 & 24). With thinner sections (0.5-1 µm), staining with aqueous toluidine blue (BDH Chemicals Ltd., 0.1% w/v) was slightly

better. Haustorial collars and fungal cell walls, including those of the haustoria and subcuticular hyphae stained bluish-purple (Plate 23), so did young conidia developing in the acervulus (Plate 28), but otherwise the fungal tissues were not so extensively stained. For electron microscopy, gold and silver sections (150 Å - 60 Å thick) were cut and picked up on uncoated Cu/Rh grids 3.05 mm wide with a mesh size of 300. These sections were stained with aqueous uranyl actate (2% w/v) for 25 mins. and/or lead citrate (Reynolds, 1963) for 10-15 min. at 18-22°C. They were examined at 60 keV in a Philips EM300 microscope.

B. PENETRATION OF FRENSHAM AND ALLGOLD

1. Histology

Studies with the light microscope indicated that usually penetration of the cuticle by *D. rosae* occurs directly beneath the larger of the two cells in the conidium (Plate 1). Occasionally, staining with thionin (p. 33) showed the dark blue haloes associated with penetration beneath both cells or at the ends of short germ tubes but the absence of even small haloes beneath conidia with long germ tubes suggests that here no penetration occurs. The diameter of the haloes varied between 2 and 8 µm and frequently on leaf disks with 10 day old infections both small and large haloes were seen (Plate 2). On a few occasions circular, browned deposits (1-5 µm in diameter) were observed at the tips of Allgold spores presumably near to sites of penetration (Plate 30) and the underlying epidermal cells were not penetrated.

An electron-dense material surrounds the short 3 µm germ-tube (appressorium) which develops from the germinating conidial cell and this probably aids in adhesion (Plate 38). Penetration itself is effected by a fine peg, less than ½ µm wide, and the leaf cuticle is distinctly depressed at this point but with no clear indication of any chemical changes. There is, however, a massive deposition of a well structured material on the cell wall of the epidermal cell immediately beneath the point of penetration which seems to extend into the upper section of the epidermal cell. The nature of this material was not determined but collars around haustoria which developed in epidermal cells stained light blue with lacmoid, BDH Chemicals Ltd, (Gurr, 1965) suggesting that callose was present in these (Plate 13) and might also, therefore, be associated with the material deposited on the cell wall.

2. Comparison of penetration on the two cultivars

Leaf disks of Frensham and Allgold were each inoculated with 1000 conidia of *D. rosae* contained in a 0.005 ml drop using the techniques and conditions of the standard laboratory test (p. 11). Penetration was assessed on ten disks of each cultivar, stained with thionin (p. 33), by counting the number of haloes associated with at least 100 conidia in traverses of the inoculum drop on each disk. In the two experiments carried out, disks were examined at 24, 48 and 72 h after inoculation (Tables 10 & 11).

Several points of interest emerged from these experiments. Penetration ranged from 20% to 57% after 72 h, with an overall average for the two cultivars just below 40%. These figures are generally higher than those for germination given in Part I (p. 13 & 15) and by other authors (Knight, 1976; Palmer *et al.*, 1966a).

In Expt. 1 (Table 10) haloes were less readily seen in material prepared at 24 and 48 h after inoculation than at 72 h, though during these early stages there was a more intense reaction on Allgold than on Frensham. However, at 72 h there was no overall difference in the number of penetrations on the two cultivars. With the experience of this experiment, stained disks were examined particularly closely in Expt. 2 for penetration points and counts obtained for all disks (Table 11). Again, at 72 h there was no overall difference in the number of penetrations on the two cultivars nor at 24 h, but at 48 h there were significantly more penetrations on Frensham. An analysis of penetration on the two cultivars (Table 12) showed that between 24 and 48 h there were apparently significant increases in penetration on both cultivars but during the next 24 h penetration increased significantly only on Allgold. This suggests that pene-

TABLE 10 : Penetration of Frensham (F) and Allgold (A) leaf disks by *D. rosae* (isolate F-76-Ash) : Experiment 1.

Time		Penetration (no./100 conidia)	Mean
24 h	F	22, 22, *, *, *, *, *, *, *, *.	
	A	19, 28, 20, 23, 26, 20, *, *, *, *.	
48 h	F	23, 22, *, *, *, *, *, *, *, *.	
	A	33, 34, 32, 41, 27, 33, 24, 40, *, *.	
72 h	F	44, 25, 32, 34, 41, 42, 37, 49, 30, 39.	37.3
	A	27, 30, 47, 29, 41, 38, 48, 39, 42, 39.	38.0

* No 'haloes' associated with penetrations.

TABLE 11 : Penetration of Frensham (F) and Allgold (A) leaf disks by *D. rosae* (isolate F-76-Ash) : Experiment 2.

Time		Penetration (no./100 conidia)	Mean	P*
24 h	F	10, 24, 22, 24, 10, 11, 15, 21, 16, 17.	17.0	n.s.†
	A	10, 17, 13, 15, 11, 12, 18, 14, 16, 18.	14.4	
48 h	F	35, 34, 31, 36, 34, 33, 38, 26, 37, 37.	34.1	<0.001
	A	24, 24, 18, 24, 26, 33, 28, 24, 21, 27.	24.9	
72 h	F	57, 26, 31, 42, 33, 28, 33, 50, 42, 37.	37.9	n.s.†
	A	39, 34, 28, 46, 21, 32, 47, 25, 20, 52.	34.4	

* P = probability that means differ; based on t-test data transformed to degrees of an angle (Fisher & Yates, 1963).

† n.s. = means do not differ significantly.

TABLE 12 : Analysis of penetration on Frensham and Allgold : Experiment 2.

Period	Mean increase in % penetration		't'	P
24-48 h	Frensham :	17.1	8.444	<0.001
	Allgold :	10.5	6.769	<0.001
48-72 h	Frensham :	3.8	1.178	N.S.*
	Allgold :	9.5	2.483	<0.05

* N.S. = means do not differ significantly.

tration occurs more slowly on Allgold. While this might contribute in part to resistance, the similarity in penetrations on the two cultivars after 72 h suggests that the resistance of Allgold operates mainly at the post-penetration stage.

C. POST-PENETRATION STUDIES

1. The overall responses of Frensham and Allgold

Typical blackspot lesions with sub-cuticular runner hyphae developed on leaf disks of Frensham 14 days after inoculation with a 'Frensham isolate' of *D. rosae* (Plate 14), and infected cells were only lightly browned (Plate 3). Examination of Allgold leaf disks, stained with thionin, 14 days after inoculation with the same isolate frequently showed isolated groups of infected cells usually with no runner hyphae and lesions did not develop (Plate 32). On untreated disks these groups were surrounded at their periphery by brown deposits not present on infected Frensham disks (Plate 31).

The reactions of Allgold to inoculations with *D. rosae* isolate (F-76-Ash) were examined more closely by counting the groups of infected cells (associated with browning) and the number of cells per group on cleared and stained disks.

In the first experiment, 4 to 8 disks of Allgold were examined at 48 h, 72 h, 8 days and 14 days after inoculation. This indicated several features of interest. Browned cells, associated with a penetration by the fungus, were detected within 48 h. These were mainly isolated, single cells though a few were in groups of 2-4 cells. By 72 h, the total number of browned cells had increased, due mainly to an increase in the incidence of single cells though there was a small increase in groups with 2 or more cells (Table 13). At 8 days a further increase in the total number of browned cells was associated with an increase to a mean number of c. 3 cells per group. The leaf disks remaining at 14 days differed in their reaction. On 2 disks, small lesions had developed and on these no counts of browned cells were possible; on the remainder, there were fewer such

TABLE 13 : The behaviour of *D. rosae* on leaf disks of Allgold : Experiment 1.

Time after inoculation	Mean number groups/disk of					Mean number brown cells	
	1	2	3	4	5 or more brown cells	per group	per disk
48 h	1.9	0.9	0.4	0.1	0	1.7	5.4
72 h	9.3	1.6	1.3	1.3	0.1	1.4	17.5
8 days	2.8	4.0	3.5	3.0	3.3	3.2	52.8
14 days*	0	1.0	1.3	1.3	1.3	4.5	21.8

* Two disks had small lesions and were not scored.

cells per disk than on the other disks at 8 days but, on average, the groups contained more cells.

In a second experiment, disks of Frensham were also inoculated as controls. Seven disks of Allgold were examined both at 48 h and 72 h, leaving 16 disks for examination and comparison with 16 inoculated leaf disks of Frensham at 14 days.

After 48 h there were groups of browned cells on all 7 disks of Allgold that were examined but the numbers varied considerably from disk to disk (Table 14a). On the Frensham leaf disks there was no such reaction. Although there were small isolated groups of light, brown-yellow cells on some disks, discolouration in these instances was confined to cell contents and there were no brown deposits associated with cell walls as seen on Allgold.

At 72 h, there was a slight increase in the total number of groups of browned cells on Allgold. In view of the studies reported earlier (p. 37) this might indicate further penetrations by the fungus during the 48-72 h period but in the light of the disk - disk variation can not be considered a significant increase. This was confirmed by analyses which are detailed in Appendix Table 18.

However, there was also a change in the distribution of group types (Table 14b), with more groups now containing 2 or more brown cells. A comparison of the distributions of group types at 48 h and 72 h, using a χ^2 test for homogeneity (see Appendix Table 19) shows them to be significantly different ($P < 0.001$), indicating that during the 48-72 h period there was a continuing response to the fungus within discrete loci. This may or may not be associated with further growth of the fungus within the

TABLE 14 : The behaviour of *D. rosae* on leaf disks of Allgold : Experiment 2.

(a) Numbers of groups of browned cells.

Time after inoculation	Groups of browned cells per disk	Totals (7 disks)
48 h	6 , 45, 4 , 33, 8 , 24, 16	136
72 h	23, 14, 14, 21, 19, 26, 44	161
14 days	13, 15, 31, 56, 12, 35, 5	167

(b) Distribution of group types.

Time after inoculation	No. groups (totals of 7 disks) with				
	1	2	3	4	5 or more brown cells
48 h	66	40	17	17	6
72 h	41	55	32	18	15
14 days	56	34	25	15	37

(c) Numbers of browned cells.

Time after inoculation	Totals (7 disks) in groups of 1-5	Nos. in individual groups > 5	Totals (for 7 disks)
48 h	236	6, 6 , 7 , 8	263
72 h	359	6, 6 , 6 , 6 , 6 , 9 , 10	408
14 days	324	6, 6 , 6 , 6 , 6 , 6 , 6 , 6 6, 7 , 8 , 8 , 8 , 8 , 9 , 9 9, 11, 12, 15, 16, 19, 25, 28	570

established infection and the possibility cannot be excluded that this could be a response to a further penetration in close proximity (i.e. within one or two cells) to an established infection, though this seems somewhat less likely. On 8 of the 10 Frensham leaf disks examined at 72 h there were several areas in which many cells were clearly infected by the fungus. Indeed, the extent to which the fungus had become well established on Frensham during the 48-72 h period was in marked contrast to its behaviour on Allgold.

Nevertheless, as in the first experiment, two different reactions were seen in the Allgold leaf disks left for 14 days. On 7 disks, there were no lesions but only groups of browned cells scattered throughout the inoculum area. On 9 disks, lesions developed which were significantly smaller ($P < 0.05$) than those on the Frensham disks (Table 15). With the exception of 2 disks, none of the cells within the lesions of the remaining 7 disks had any of the dark deposits characteristic of the resistant reaction. The total number of groups of browned cells on the Allgold disks with no lesions (167) was similar to that at 72 h (161). The distribution of group types was significantly different to that at 72 h ($P < 0.01$), as again indicated by a χ^2 test for homogeneity (Appendix Table 19). Particularly there were more groups with over 5 browned cells and some of these had between 10 and 28 brown cells (Table 14c).

These two experiments indicated the following three situations regarding infection of leaf disks by *D. rosae*:

(i) Lesions develop consistently on Frensham and are associated with rapid colonization of host tissues between 48 h and 72 h after inoculation.

(ii) Lesions develop on some leaf disks of Allgold after 14 days but

TABLE 15 : Lesion development on Frensham and Allgold at 14 days : Experiment 2.

Cultivar	Lesion size (mm)/disk	Mean
Frensham	7.5, 5.5, 7.5, 7.0, 7.0, 6.0, 4.5, 7.0, 6.0, 5.5, 7.5, 7.0, 6.0, 7.0, 8.0, 4.5.	6.5
Allgold	4.5, 3.5, 3.0, 5.0, 8.5, 3.0, 6.0, 7.5, 6.0.	5.2

S = 1.46

t = 2.14

df = 23

P : <0.05

failure to detect subcuticular fungus in cleared disks at 72 h suggests there is some delay in lesion development at this stage.

(iii) On other leaf disks of Allgold no lesions develop and this is associated with rapid browning of discrete groups of cells suggesting a hypersensitive response. This response is variable even on leaf disks which show it and the continued increase in the number of browned cells within groups suggests that it may not entirely limit fungal growth.

These three situations were next examined in detail by light and electron microscopy. Allgold disks, with 6 to 14 day old infections were selected for this study as a clear differentiation between the susceptible (ii) and resistant response (iii) on Allgold before this time can not be effectively determined. Infected Frensham disks, 6 to 14 days after inoculation were also used in the present investigation.

2. Histology of infection

(a) On Frensham

Following penetration of the cuticle the narrow infection peg enlarges into a subcuticular infection hypha (Fig. 1). In section there was no evidence of enzymic degradation, but distention of the epidermal cell wall and cuticle suggested that the fungus exerts mechanical pressure (Plate 39). The infection hypha grows downwards and proceeds either to move between the epidermal cells as intercellular mycelium (Plate 40), or else penetrates directly through the epidermal cell walls (Plate 39).

Subcuticular runner-hyphae also originate from the point of infection. These tend to be associated in fascicles composed of several parallel filaments (Fig. 1 & 2; Plate 46) which by branching and various criss-cross linkages produce a complex network (Plate 4). Their passage

under the cuticle seems to be accomplished by mechanical pressure but it is also associated with changes in appearance of the fibrillar wall material and may thus be partly enzyme assisted (Plates 41 & 46). As the runner hyphae pass over the epidermal cells, they send down branches which breach a relatively large area of the cell wall during penetration (Plate 9). There does not seem to be any constriction of the penetrating fungus at this site. E.M. pictures show changes in staining properties and fibrillar architecture of the cell walls suggesting that the penetration process is both mechanical and enzymic (Plate 42).

Following penetration both intracellular hyphae and haustoria may be formed. Intracellular septate hyphae are found in the epidermal cells from the earliest stages of lesion development (Plates 7 & 8), often surrounded by electron dense material (Plates 43 & 49). During the present investigation no host membrane was seen near these hyphae but this was probably due to poor fixation and/or staining, rather than rupture of the membrane during penetration. Some intracellular hyphae penetrate adjacent epidermal cells, and these are constricted when passing through the two cell walls and intervening middle lamella (Plate 45). The size and morphology of intracellular hyphae is fairly variable. They may be rotund and occupy up to half the host cell lumen (Plate 43), but more often they remain narrow and elongated taking up a relatively small space within the host cell (Plates 8, 45 & 49).

Haustoria are found in both epidermal and palisade cells, and can be distinguished from intracellular hyphae by their characteristic 'terminal' or determinate growth. Epidermal haustoria are formed from infection (Fig. 1) or runner hyphae (Fig. 2; Plate 6). Papillae (appositions) are frequently associated with these haustoria and are formed

adjacent to sites of penetration on the inside of epidermal cell walls. The semi-circular papillae up to 20 μm at their base, contain a darker staining material embedded within a lighter matrix (Plate 46). Usually the haustorial neck grows through the papilla which remains as a collar surrounding the proximal extremity of the haustorium (Plates 5, 6 & 47). Dark staining particles were seen in one transverse section adhering closely to the fungal wall (Plate 48). The haustorium enlarges prior to its emergence from the collar but tapers at its tip. There was no evidence of an extrahaustorial matrix and the extrahaustorial membrane (of vacuolar and/or host plasmalemma origin) seems to have ruptured (Plate 47). Usually not more than two or three haustoria are formed in the epidermal cells.

Palisade haustoria are sometimes formed from intercellular hyphae, but more frequently from intracellular hyphae. Intracellular hyphae often enlarge at the site of penetration and can be considered as haustorial mother cells because they contain a nucleus, and are rich in mitochondria and lipid bodies suggesting they are sites of intense metabolic activity (Plate 52). Prior to penetration a papilla (of an unidentified material) is laid down in the palisade cell beneath that part of the cell wall adjacent to the fungus. At the site of penetration the fungal wall appears thinner where it passes through the cell wall and judging by the deep staining surrounding this site suggests that penetration is at least partly enzymic (Plate 51). The haustorial mother cell usually penetrates one palisade cell but occasionally two adjacent palisade cells are penetrated (Plate 44). Following penetration the haustorial neck grows through the papilla which then remains as a collar, but much smaller than those formed around epidermal haustoria. Shortly after penetration into the lumen of the cell a cross wall is formed (Plate 51) in the haustorium

which then enlarges. Haustoria in the palisade tend to be slightly wider than those in the epidermis and usually emerge from the upper wall and extend down the cell but otherwise have no distinguishing features (Plates 10 & 53). Occasionally in sections, an extrahaustorial matrix (Plate 54), and extrahaustorial membranes (Plate 55) were observed but the E.M. techniques used did not appear capable of showing this in sufficient detail.

Apart from the palisade haustoria the infection structures so far described are usually fairly well developed within the 48 h period following inoculation. Because of the difficulty experienced in locating fungal structures in sectioned leaf material it was not possible to establish precisely when haustoria first appeared in palisade cells but it seems to be soon after the invasion of the epidermal cells.

The further development of the lesion is primarily associated with the ability of the runner hyphae to grow under the cuticle and invade successive epidermal cells. The energy for this growth is presumably obtained from haustoria already present in the epidermis and palisade cells.

The final stage in the development of *D. rosae* results in the production of acervuli usually 10-14 days after inoculation. They are initiated from specialized areas of the sub-cuticular mycelium which produce side branches (Fig. 3) that become modified as spore bearing structures. As spores are budded off the basal stroma, the cuticle becomes distended and eventually ruptures exposing conidia in many stages of development (Plate 11). The underlying cells are also exposed and eventually perish presumably through the disturbance of transpiration. When mature, the bicellular hyaline conidia are 18-25 μm long by 5-6 μm wide and are loosely attached to the thin basal stroma (Plate 12). Fully

formed acervuli on Frensham attain sizes of 250-350 μm.

(b) On Allgold giving a susceptible response

In situations where *D. rosae* gives rise to lesions on Allgold there are many features in common with its development on Frensham but some also that are different.

Following the penetration of the cuticle, a subcuticular infection hypha is formed which may be up to 40 μm long and 5 μm wide (Plate 18). As in Frensham, continued growth from this hypha can be of several kinds:

(i) Hyphae move downwards between the epidermal cell walls to form a septate intercellular mycelium which push the cell walls apart to a remarkable degree (Plate 56).

(ii) Runner hyphae are very similar to those seen on Frensham and their progress beneath the cuticle (Plate 57) and within the epidermal cell wall appears to be effected by both mechanical and chemical means (Plate 56).

(iii) Other hyphae penetrate the epidermal cell wall and form either intracellular hyphae or haustoria in the lumen of the cell. As on Frensham, prior to penetration a papilla is laid down between the plasma membrane and the wall adjacent to the fungal cell, presumably as a result of local secretory activity by the host. Darkly stained particles at the base of this papilla are a feature not observed on Frensham (Plate 29).

During penetration a relatively large area of epidermal cell wall is breached (Plate 20), although compared with Frensham, the penetrated walls show very little change in staining properties suggesting that the penetration process is largely mechanical (Plates 58 & 59).

Intracellular hyphae are not always like those on Frensham. On one occasion approximately 20 round to elliptical bodies, ranging in size from $\frac{1}{8}$ µm to $\frac{1}{2}$ µm were seen adhering closely to the fungal cell wall and embedded in the electron dense material which surrounds the hypha (Plate 61). Their significance is not clear, although it is possible they are products of hyphal breakdown.

Haustoria in the epidermal cells are not unlike those on Frensham (Plate 63). They arise most frequently from the sub-cuticular hyphae and then either emerge from the upper wall and extend straight down the cell (Plate 21) or emerge from the topmost corner of cells (Plate 23). Occasionally they arise from intercellular mycelium and form in the lower half of the epidermal cell (Plate 24). Papillae laid down before penetration are breached by the haustorial necks, even as much as 15 µm long (Plate 22) and remain as collars surrounding the proximal extremities of the haustoria. Other features of the epidermal haustorium are: a cross-wall laid down soon after penetration of the cell, a region which stains deeply approximately halfway along the haustorial neck (Plate 62) and an extra-haustorial matrix which is distinct from the collar and epidermal cell contents and surrounds the haustorial body. This can be regarded as a 'protective buffer' between host and pathogen. A close examination of its edge reveals the presence of extrahaustorial membranes (Plates 58 & 62) but it is not certain whether these were of vacuolar and/or host plasmalemma origin. Although these were not seen so clearly in Frensham material this probably only reflects the greater difficulty experienced in E.M. work with this cultivar. Following the development of epidermal haustoria side branches can form close to the site of haustorial initiation (Plate 58) and which by continued growth presumably enable the fungus to spread within the host as intercellular mycelium.

Palisade haustoria in Allgold are initiated as in Frensham from both intercellular (Plates 25, 27, 65 & 70) and intracellular hyphae (Plates 26 & 64). Although on occasions they appear to be similar to those on Frensham, they usually have two characteristics by which they can be differentiated. One is the rather large collar which extends well over the whole neck region and even envelops the proximal portion of the expanding haustorium (Plate 65). The other is the diffuse electron dense material which extends over most of the neck region (Plate 65) and is more conspicuous than in epidermal haustoria. In T.S. the electron dense material is seen to adhere closely to the host membrane adjacent to the haustorial neck (Plate 66). As on Frensham, haustoria generally had an extrahaustorial matrix though on one occasion this feature was not observed (Plate 67).

However, even on infected Allgold it was generally difficult to observe ultrastructural details of the host although sections of uninfected controls especially through palisade cells, clearly showed chloroplasts (with starch grains and osmiophilic bodies), cell membranes, a nucleus (containing a nucleolus), endoplasmic reticulum, vacuoles, mitochondria and other unidentified cell organelles (Plate 68). Epidermal cells of Allgold were more difficult to study because they do not show many of the details so clearly seen in palisade cells.

Finally, the ultrastructural features of acervulus development (which were not investigated on Frensham because of the E.M. difficulties encountered with this material) were examined in detail on Allgold.

An acervulus begins its development when the sub-cuticular fungus enlarges, thereby pushing up the cuticle. At the same time cross-walls are apparently laid down which presumably serve to delimit the

specialized spore bearing cells (Plate 69). The epidermal cells immediately beneath the acervulus may contain fungal structures such as haustoria (Plate 72). Within the acervulus spores in various stages of development are seen, the oldest usually being near the middle (Plate 28). Young conidia begin their development as protruberances on 'mother - cells' from which they are delimited by a cell wall early in their development (Plate 73). Occasionally two spores may begin to develop from a single mother cell (Plate 71). The acervulus contains a highly structured matrix and a light structureless material immediately surrounding the developing spores which may be embedding resin (Plates 71 & 73). As the developing spores enlarge another wall is laid down approximately half way along their length giving the spores their characteristic two celled appearance, and a marked constriction appears at the point of attachment to the mother cell (Plate 74). Occasionally, the wall delimiting the two cells of the spore does not seem to form (Plate 75). Fully formed spores are of similar dimensions to those developed on Frensham, although in contrast to Frensham, acervuli rarely exceed a size of 250 µm (Plate 29).

(c) On Allgold giving a resistant response

A close examination of infected cells in the light microscope reveals that the cytoplasm of penetrated cells becomes discoloured, granular and necrotic possibly resulting in the death of the cells, and the (epidermal) walls become birifringent. The growth of *D. rosae* is apparently restricted to a small group of cells and the establishment of and effective relationship is prevented.

Fourty eight hours after penetration a very distinct subcuticular, infection hypha develops. This may be 4 µm wide and up to 60 µm long (Plate 17), or a lot shorter (Plate 18). Rudimentary sub-cuticular hyphae

originate from the infection hypha (Plate 19) as on Allgold giving a susceptible response and on one occasion after 72 h they were seen to form very close to the penetrating spore (Fig. 4). Epidermal haustoria begin developing 24-72 h following inoculation, often at the site where the infection hypha penetrates the cells (Plates 15 & 16). It would seem that the infection strand also gives rise to the intercellular hyphae which in turn produce epidermal haustoria some distance away from the infection strand (Fig. 5).

Fourteen days after inoculation penetrated epidermal cells show haustoria that have apparently been occluded by an unidentified encapsulation (Plates 33, 34 & 35). The presence of papillae on the inside of walls suggests they are sites of unsuccessful attempts at penetration by intercellular hyphae, which are eventually limited from further formation (Fig. 6) probably due to the poor development of runner hyphae and haustoria. The palisade cells seem to be successfully breached by penetrating fungus but fail to develop normal haustoria as seen on Frensham and susceptible Allgold (Plates 36 & 37). It was not possible to detect runner-hyphae except in isolated instances, and as there was some difficulty in deciding by the use of the light microscope what was the exact fate of the fungus after penetration, a parallel study using E.M. was conducted.

Intercellular hyphae where they occur are well developed, and are occasionally seen penetrating into palisade cells (Plate 76). Subcuticular 'runner-hyphae' were observed on a number of occasions (Plates 76 & 77) suggesting that some limited development is possible even where a resistant response occurs on Allgold. Haustoria in the epidermis are not dissimilar from those seen in Frensham and 'susceptible' Allgold, the only distinguishing feature being a well developed extrahaustorial matrix

(Plate 78).

Septate, intracellular hyphae in the epidermis are well developed, and except on one occasion (Plate 79) are usually seen to be surrounded by an electron dense material. As on Frensham, an appressorial-like structure (haustorial mother cell) develops from the intracellular hypha prior to penetration into the palisade (Plate 80). As there is no alteration in the staining properties of the palisade cell wall during penetration it would seem that the process is largely mechanical.

The fact that haustoria develop in the palisade cells probably accounts for the ability of the fungus to persist for many days within the host tissues. Although occasionally palisade haustoria appear to be similar to those on Frensham and Allgold (susceptible response) (Plate 86) there are usually certain features in which they differ:

(i) On two occasions where apparently normal haustoria developed the extrahaustorial membranes were highly convoluted adjacent to a barely distinguishable extrahaustorial matrix (Plates 82 & 85). Presumably the alteration in membrane appearance reflects some reaction directly or indirectly induced by a secretion of the haustorium.

(ii) On three other occasions failure to expand resulted in poor haustorial formation. Haustoria either remained narrow and elongated with relatively large collars (Plate 84), or shorter, slightly wider structures, with smaller collars and a bulbous tip which seemed to be encased in an unidentified matrix (Plates 81 & 83). Compared to haustoria in (i), these showed no evidence of having a wall delimiting them from the mother cell and only Plate 84 showed evidence of an electron dense material on the haustorial neck. However, the absence of these features, and the failure

to detect an extrahaustorial membrane may possibly be attributed to poor preparation of the material for E.M.

The failure of palisade haustoria to form normally and of the sub-cuticular mycelium to undergo satisfactory development are probably the most important features responsible for the inability of acervuli to develop.

In the present investigation it was not possible to determine the behaviour and fate of cell organelles following infection (and therefore, cell death) despite controls indicating the E.M. technique to be adequate. However, a modification of the technique, the use of a diamond knife for cutting sections and variations in staining methods should improve the quality of the electron micrographs.

III. DEVELOPMENT OF *D. ROSAE* ON SOME NEWLY INTRODUCED ROSE CULTIVARS

INTRODUCTION

The aim of the work in this section was to examine in the light microscope the development of Frensham isolates of *D. rosae* on rose cultivars which were shown to be resistant to the fungus in field trials at the Royal National Rose Society Gardens in St. Albans. Leaf disks were inoculated in a standard laboratory test and examined after 14 days for fungal development using the clearing and staining methods developed in Section II.

In particular, the object of the present investigation was to compare the reactions of the new cultivars following inoculation with *D. rosae* with those already observed on resistant Allgold, such as the browning response in the epidermal cells and the absence of sub-cuticular mycelium.

In Part A the cultivars Babylon, Red Rum and Astral were inoculated with *D. rosae* isolate, F-77-Ash in a series of experiments. In Part B, 7 un-named cultivars were inoculated with *D. rosae* isolate F-77-Ash in January and May 1978, and with F-78-G.H. in October 1978. The following cultivars were used:

Cultivar	Abbreviated form used in table and text
J547A	J5
K329F	K3
K444A	K4
K85A	K8
K97E	K9
L105A	L1
L425A	L4

The reactions of each cultivar are outlined under the appropriate headings.

A. BABYLON, ASTRAL AND RED RUM

Experiment 1

In the first experiment (December 1977) leaf disks (1 cm^2) of Babylon, Red Rum and Astral were inoculated with an isolate of *D. rosae* (F-77-Ash) in a standard laboratory test (pg. 11). Lesion formation was examined after 14 days.

Results indicated that despite their observed 'field-resistance' all 3 cultivars developed lesions, except for one disk of Astral (Appendix Table 20). Red Rum developed lesions significantly larger than the other two cultivars, between which there was no difference (Table 16a).

Leaf disks of Babylon and Astral were then examined after they were cleared and stained using the methods developed in Section II (pg. 33). Infected epidermal cells on Babylon were browned, especially in the cell wall region, though this browning was not of the type seen on 'resistant' Allgold (pg. 42).. This, however, did not prevent the growth of sub-cuticular mycelium and the development of small lesions with acervuli. Haustoria with large collars, possibly the result of a more intense host response to fungal penetration were also observed. Astral, though it also formed small lesions, seemed to react like the cultivar, Frensham.

Experiment 2

The aim of experiment 2 (January 1978) was to examine further the reactions of Babylon, and to compare these with the reactions of Frensham and Allgold, and so disks were inoculated with a *D. rosae* isolate (F-77-Ash) and examined after 14 days.

There were significant differences in lesion development on all 3 cultivars (Table 16b). Large lesions developed on Frensham disks, no

TABLE 16 : Analysis* of lesion development on Babylon, Red Rum and Astral (Experiment 1 and 3) and Babylon, Frensham and Allgold (Experiment 2) inoculated with an isolate of *D. rosae* (F-77-Ash).

(a)

Experiment	Mean lesion diameter (mm)†			S.E. ±
1	Babylon	Astral	Red Rum	0.48
	3.0	3.25	6.1	

	Mean lesion diameter (mm)†			
3	Astral	Babylon	Red Rum	0.52
	0	0.38	2.63	

(b)

Experiment	Mean lesion diameter (mm)		
2	Allgold	Babylon	Frensham
	0	2.9	7.35

$s = 4.1$

$df = 38$

$t = 3.44^{**}$

* Details in Appendix Tables 20, 21 and 22.

† Differences between cultivars (Duncan's new multiple range test). Cultivars not underscored by the same line differed significantly at 1% level.

lesions were seen on Allgold and Babylon showed an intermediate response with a mean lesion diamter of 2.9 mm (cf. Expt. 1). An examination of Babylon disks showed that except for 2 disks which had Frensham-like lesions (Appendix Table 21 - disks 1 & 5), the majority had small lesions in which the epidermal cells were deep brown in colour, and often contained large haustoria (cf. Expt. 1). An examination of Allgold disks failed to detect sub-cuticular mycelium and the browning reaction characteristic of the resistant response was observed on 9 disks.

Experiment 3

This experiment was similar in design to Expt. 1, but disks of Red Rum, Astral and Babylon were inoculated in May 1978 using the same *D. rosae* isolate (F-77-Ash).

After 14 days, no lesions developed on Astral and only 4 of 8 disks of Red Rum, and 2 of 8 disks of Babylon had lesions (Appendix Table 22). Disks of the 3 cultivars without lesions were then examined but no fungal structures (sub-cuticular hyphae and epidermal haustoria) or any changes in host cells were observed, indicating that a 'resistant' or 'immune' response may be operating at the leaf surface. It would seem that the decreased susceptibility (cf. Expt. 1) is the result of a change in host physiology as the *D. rosae* isolate used and conditions under which test was carried out were as in Expt. 1.

B. SEVEN UN-NAMED CULTIVARS

J5

In Experiments 1 and 2 few lesions developed on leaf disks (Appendix Tables 23 & 24). However, in Experiment 3 (Appendix Table 25), lesions formed on all leaf disks (mean diameter of 5.85 mm), which were not dissimilar from the typical Frensham lesions.

One feature of interest emerged after disks with no lesions from Experiment 1 were examined. Of the 10 disks inoculated, 4 had groups of browned epidermal cells. This reaction was not observed on any other disks of J5. The browning was most evident in the cell wall region but was not as prounced as, or of the type described for 'resistant' Allgold (pg. 42).

This cultivar proved difficult to work with as disks often curled up after being punched from leaves. This resulted in poor adherence of inoculum droplets, and may explain the apparent resistance (or 'immunity') of some disks to the fungus where no reaction was observed.

K3

Lesions with a mean diameter of 4.95 mm (Experiment 3), 5.35 mm (Experiment 1), and 7.25 mm (Experiment 2) (Table 17), developed on leaf disks that were similar to those on Frensham.

K4

In Experiments 1 and 3 (Appendix Tables 23 & 25), lesions which developed on disks all had a diameter of 5 mm or more, but in Experiment 2 (Appendix Table 24), lesions never exceeded 3.5 mm.

An examination of disks with lesions from all 3 experiments, showed

TABLE 17 : Analysis* of lesion development on 7 'un-named' cultivars, Frensham (F) and Allgold (A) with Frensham isolates of *D. rosae.*

Experiment 1.

Mean lesion diameter (mm)†

K9	A	K8	J5	F	K4	L1	L4	K3	S.E. ±
0.25	0.6	0.8	1.2	2.15	2.95	3.15	3.65	5.35	0.86

Experiment 2.

J5	L4	K9	K4	A	F	L1	K3	K8	
0.25	0.25	0.63	1.5	4.06	5.63	7.06	7.25	8.94	0.58

Experiment 3.

K9	K3	J5	K4	L4	F	K8	L1	A	
0.55	4.95	5.85	6.0	6.35	6.5	6.5	6.7	7.2	0.51

* Details in Appendix Tables 23, 24 and 25.

† Differences between cultivars (Duncans new multiple range test). Cultivars not underscored by the same line differed significantly at 1% level.

that Frensham-like lesions were produced on all disks (including the smaller lesions in Experiment 2).

This cultivar like J5, was difficult to work with due to leaf disks curling, thereby, resulting in the poor adherence of inoculum droplets, and may explain why some disks did not show any signs of infection.

K8

Lesions with a mean diameter of 8.94 mm in Experiment 2 and 6.5 mm in Experiment 3 (Appendix Tables 24 & 25) developed on leaf disks of K8. An examination of infected epidermal cells showed that apart from the presence of haustoria, they did not differ markedly in colour from non-infected cells.

In contrast, in Experiment 1 no lesions developed on 9 of the 10 disks (Appendix Table 23). An examination of these disks showed areas of light browned epidermal cells, but sub-cuticular fungus and haustoria were not observed.

K9

Lesions failed to develop on most disks of this cultivar (Appendix Tables 23, 24 & 25). In Experiments 1 and 2 only one disk became infected and two disks in Experiment 3. Where lesions developed, infected epidermal cells were light brown in colour and did not show any features by which they could be distinguished from 'Frensham' lesions.

Disks on which lesions had not formed, however, showed several interesting features. In Experiment 2, 4 disks showed small groups of browned epidermal cells scattered throughout the inoculum drop areas. These cells contained granular, dark brown deposits and their walls were birifringent. No fungal structures were observed either in or near these

cells. The infected cells were occasionally surrounded by a diffuse yellow-green material which tended to spread into adjacent uninfected cells, but no further. On one other disk in the same experiment, a circular green deposit 10-15 μm in diameter was seen beneath penetrating spores on 7 occasions.

<u>L1</u>

Lesions with a mean diameter of 3.15 mm (Experiment 1), 6.7 mm (Experiment 3) and 7.06 mm (Experiment 2) (Appendix Tables 23, 24 & 25), developed on leaf disks of L1, that were not dissimilar from typical 'Frensham' lesions.

In Experiment 1, no lesions developed on 4 disks which when examined did not show any evidence of a host reaction to the fungus.

<u>L4</u>

In Experiment 3, lesions developed on all leaf disks (Appendix Table 25) which were similar to lesions on Frensham. Lesions that developed in Experiments 1 and 2 (Appendix Tables 23 & 24) frequently had browned epidermal cells with haustoria often larger than seen in infected Frensham cells (cf. Babylon, pg. 61). On disks with no lesions no host response was observed in epidermal cells.

DISCUSSION

When leaf disks of Frensham are inoculated with isolates of *D. rosae* from Frensham lesions develop within 14 days under the standard conditions first described by Knight (1976) and indicated earlier in this thesis (p. 11 Indeed the response of this cultivar is remarkably consistent. Knight (1976) inoculated a total of 160 leaf disks in fourteen tests which, over 2 years, involved three different collections of *D. rosae* and lesions developed on 150 of them (93.8%). In the present investigation, 122 leaf disks were inoculated in eleven tests involving nine different collections of *D. rosae* and lesions developed on 102 disks (83.6%). Even when the incubation conditions of the inoculated leaf disks were varied, lesions still developed on Frensham though they differed in size (Appendix Table 8). Only when the host material was grown at relatively low temperatures (10° and $15^{o}C$) was there any indication that fewer disks became infected (Table 8, Appendix Table 10).

By contrast, the response of the rose cultivar Allgold to these isolates is variable and this has been the basic problem in this investigation as indeed it was in some of Knight's work. Some of this variation appears to be associated with the inoculum of *D. rosae*. Thus in Knight's tests with isolate F-74-Ash no lesions were observed on any of the 55 leaf disks of Allgold which were inoculated in five tests between November 1974 and May 1975 whereas lesions developed on 29 of 75 disks inoculated with isolate F-73-Ash in seven tests between December 1973 and January 1975 (Table 19). Similarly, in the present experiments no lesions developed on leaf disks of Allgold inoculated with isolates A and D whereas they did so on all disks inoculated with isolate C and on 9 of 10 disks inoculated with isolate B (Table 3; Appendix Tables 11, 12 & 13).

TABLE 18 : Lesion development (mean diameter in mm) by *D. rosae* on Frensham and Allgold leaf disks.

Isolate	Date	Lesion diameter		*P*
		Frensham	Allgold	
F-76-Ash	Dec. 1976	5.3	0.8	0.01
F-77-Ash	July 1977	5.6	4.2	N.S.
	Jan. 1978	2.2	0.6	N.S.
	Jan. 1978	7.4	0	0.001
	April 1978	3.7	2.5	0.05
	May 1978	5.6	4.1	N.S.
	Sept. 1978	6.1	7.2	N.S.
F-78-G.H.	May 1978	7.0	0	0.001
	Sept. 1978	5.8	0.5	0.001
	Oct. 1978	6.5	7.2	N.S.
	Oct. 1978	8.3	0.6	0.001

TABLE 19 : Lesion development on Frensham and Allgold inoculated with isolates of *D. rosae* from Frensham (Data from Knight, 1976).

Isolate	Date of test	No. disks of each cv. inoculated	No. disks with lesions	
			Allgold	Frensham
Fr-72-Ash	23 May 1973	20	0	20
	14 June	10	0	9
Fr-73-Ash	5 December 1973	10	0	10
	26 February 1974	10	9	8
	6 May	10	9	10
	22 June	15	3	15
	23 July	10	5	10
	28 August	10	3	10
	8 January 1975	10	0	10
Fr-74-Ash	20 November 1974	15	0	14
	8 January 1975	10	0	10
	5 February	10	0	9
	22 April	10	0	9
	24 May	10	0	6

However, variability in lesion development can not be attributed solely to inoculum. In two of Knight's tests with isolate F-73-Ash no lesions developed on the 20 Allgold leaf disks that were inoculated whereas in the remaining five tests lesions appeared on 29 of 55 disks, although inoculum of this isolate was derived throughout from infected rose leaves stored at $-15^{o}C$. In this instance it could be argued that the conditions under which the host material was grown partly affected its response to this isolate. This may be so though in the present experiments growing Allgold plants at different temperatures had relatively little effect on subsequent lesion development on leaf disks (Table 8). Moreover leaf disks of Allgold vary in their response to a single collection of *D. rosae* within individual experiments, for example in Knight's tests with isolate F-73-Ash in June, July and August 1974 (Table 19) and tests here with isolates F-76-Ash (Appendix Table 2) and F-77-Ash (Appendix Tables 4 & 5). The interesting feature is that within the limited amount of host material used there is such variability and that it is expressed as an 'all or nothing' response. Lesions either develop or they do not and where they do develop on Allgold they are often of comparable size to lesions on Frensham.

What appears to emerge from such considerations is that the balance between resistance and susceptibility of Allgold is a fine one and any hypothesis regarding possible mechanisms of resistance must take account of this. Viewed in this light, comparisons of conidial germination and penetration of the fungus on Frensham and Allgold now appears somewhat irrelevant since at these stages there can be no indication of the final outcome of such processes in terms of lesion development on the Allgold leaf disks examined. Also there is no guarantee that if other similar leaf disks of Allgold are kept for 14 days, their response to inoculation will necessarily indicate what this final outcome would have been.

As it happened, in the experiments reported here there were few differences either in conidial germination on or penetration of the two cultivars (Tables 1, 3, 6, 10 & 12) which perhaps makes it the more unlikely that the reaction of Allgold is determined at these stages. Knight (1976) did obtain, consistently, small but significant differences in conidial germination on the two cultivars. However, it now appears from the present study that successful penetrations occur close to and often beneath the conidium and that long germ tubes on the host surface are not effective in this process. Thus although Knight's results are not in question, they can not solely account for a resistant reaction on Allgold nor did Knight suggest that they could (Knight, 1976; p. 145).

This leads to a consideration of differences at the cellular level. Visually the most striking of these is the presence of browned epidermal cells in resistant Allgold and their absence in susceptible Allgold and in Frensham (Tables 13 & 14). These browned cells, which are indicative of a hypersensitive response, appear within 48 h after inoculation either singly or in groups. They are associated with a limited intercellular mycelium (Fig. 5) and also limited development of the fungus within these cells. The number of browned cells within groups increases with time but there appears to be little change in the total number of groups after 72 h (Table 14a). Since the processes of germination and penetration take some 48 h, this suggests that generally the development of lesions is determined on Allgold between 48 h and 72 h after inoculation.

On Frensham, colonization of the host proceeds rapidly at this time. Runner hyphae originate from the point of infection and their growth enables the fungus to spread radially beneath the cuticle. Subsequently branches from these runner hyphae penetrate epidermal cells forming limited

structures (haustoria) and intracellular hyphae. Haustoria are also formed in the palisade cells from inter- and intracellular hyphae (Plates 53 & 54). The function of these haustoria is a key question. Structurally they appear not dissimilar to the haustoria of biotrophic fungi such as the rusts and smuts and thus might have the same function of absorbing essential food materials. That they develop at all from the runner hyphae suggests that these either cannot absorb nutrients from the underlying epidermal cells or their ability to do so is limited and insufficient to support extensive growth and sporulation. Several parasitic fungi which initially develop a subcuticular mycelium, e.g. *Venturia inaequalis*, eventually disrupt and kill the epidermal cells and presumably so gain access to nutrients. *D. rosae* does not. There may be colour changes in the infected rose leaf and it may drop prematurely but there is usually no necrosis. This in itself suggests a balanced relationship between parasite and host like that of fungi generally accepted as biotrophs. The logical extension of this argument is that the proper development of haustoria is essential to *D. rosae* and particularly during the early stages of its development, that is 48 h-72 h after inoculation.

The E.M. studies in particular showed no striking differences between epidermal haustoria in resistant Allgold and those in Frensham. However, there were differences in haustoria within palisade cells. On resistant Allgold some of these haustoria failed to expand after penetrating the cells wall (Plates 81, 83 & 84) and those which appeared to be fully expanded were seen to be bounded by a convoluted extrahaustorial membrane (of host plasmalemma and/or vacuolar membrane origin). Because membranes play a part in biochemical specialization, permeability, transport and also act as binding sites such an alteration in their structure may disrupt one or more of these functions and prevent the haustoria from functioning

effectively as absorbing organs. However, this poses other questions, notably the extent to which *D. rosae* depends on the palisade haustoria. In most powdery mildew fungi, for example, haustoria are limited to the epidermal cells. These are not photosynthetic units but, nevertheless, these fungi are able to direct the flow of nutrients from the palisade cells. Why should the epidermal haustoria of *D. rosae* not do the same? Is it not more likely that the unusual development of the palisade haustoria is the result of the hypersensitive reaction in the epidermal cells of resistant Allgold rather than its cause? It seems unlikely that such questions will be readily answered until we know much more about the parasitism of this fungus on a susceptible cultivar.

On a practical level, the type of resistance shown in Allgold appears too unstable to be of value to rose breeders and other types need to be sought. In this respect cultivar K97E is worth further investigation though more promising approaches might be to examine the resistance of some species roses and those features of existing cultivars which militate against the retention of inoculum in the field.

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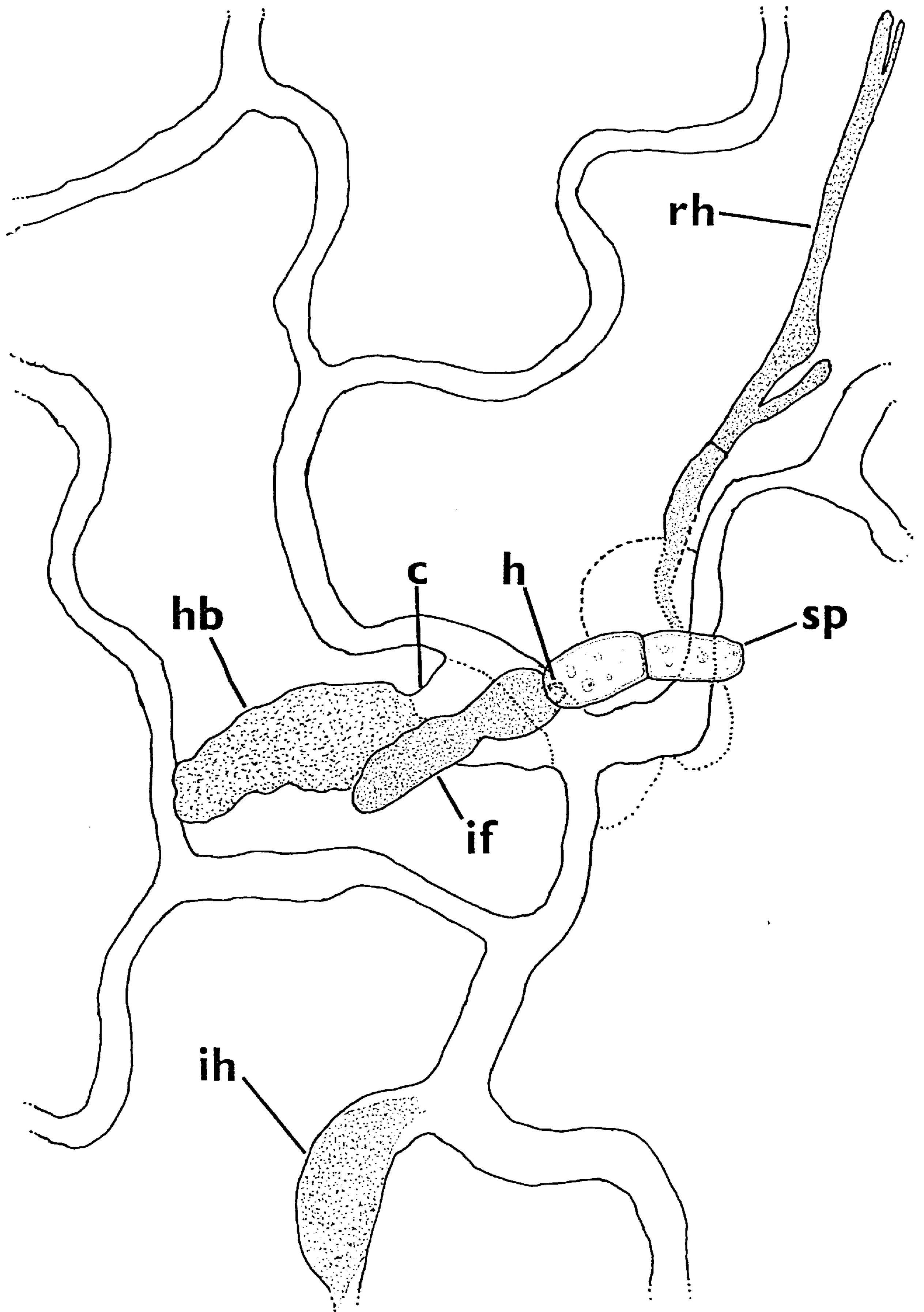
Abbreviations used in Figures and Plates

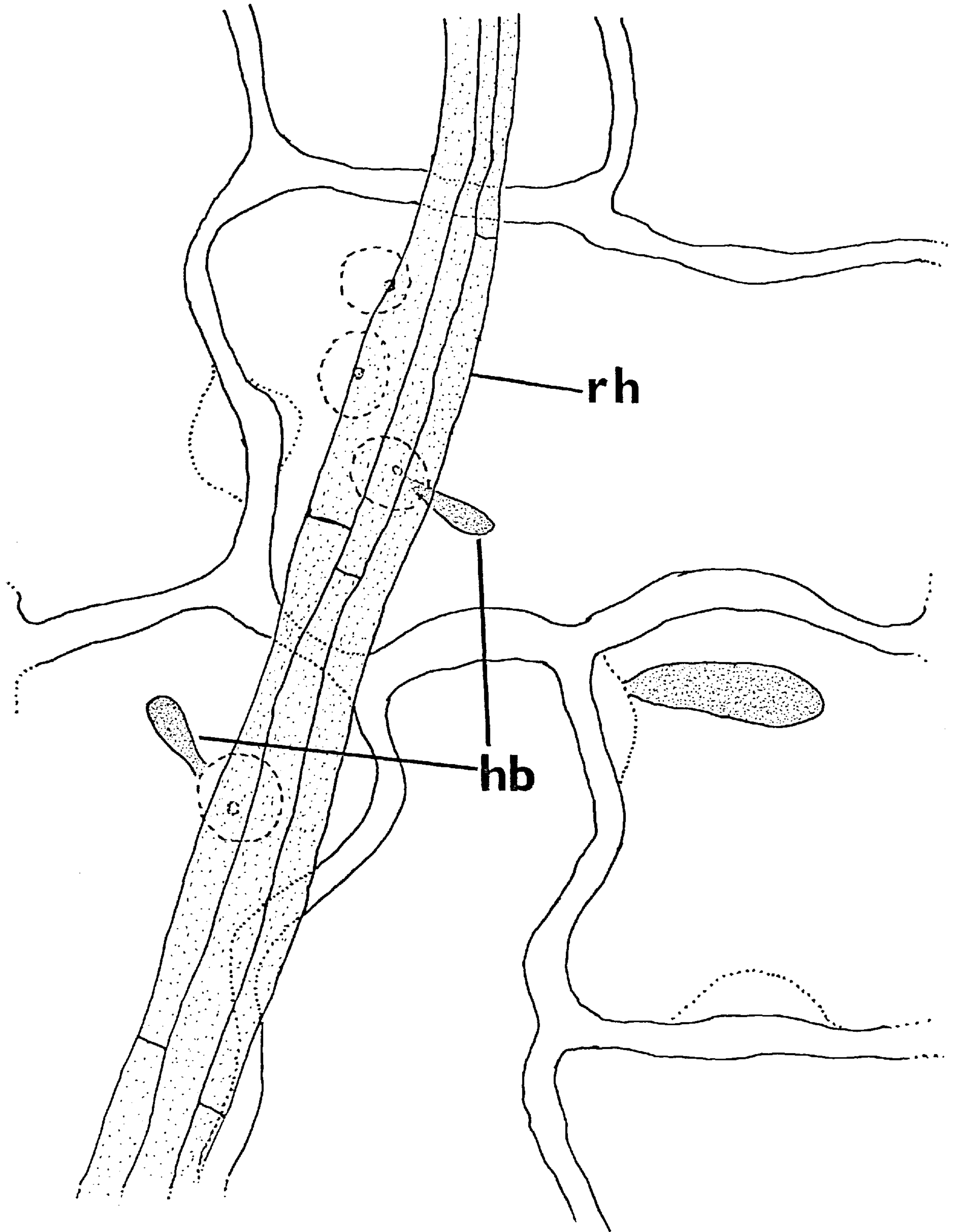
a - acervulus
c - collar
cl - chloroplast
cu - cuticle
cw - cell wall
e - electron dense material/ particles
ec - epidermal cell
eh - extrahaustorial membrane
em - extrahaustorial matrix
er - endoplasmic reticulum
fp - fungal plasmalemma
fw - fungal wall
h - halo
hb - haustorial body
hm - haustorial mother cell
ia - intracellular hypha
if - infection hypha
ih - intercellular hypha
k - crystal
l - lipid
m - mitochondria
mo - spore mother cell
n - haustorial neck
nl - nucleolus
nu - nucleus
ob - osmiophilic bodies
p - papilla
pc - palisade cell
ph - penetration hypha
pp - penetration peg
rh - sub-cuticular runner hyphae
s - septum
sa - dark staining areas
sd - structured deposit
sg - starch grain
sp - spore
st - structured matrix
v - vacuole
w - cross-walls

Plates 38-86: electron photomicrographs (details of material preparation are given on p. 34; caffeine used during fixation except in Plates 40, 41, 42, 49, 52, 53, 61, 77, 78, 79, 82, 83, 85, 86).

FIGURE 1 : Infection on Frensham (48 h after inoculation with *D. rosae* isolate, F-76-Ash); surface view. Note the spore (sp) with a halo (h), subcuticular infection hypha (if), runner hyphae (rh), haustorium (hb) with collar (c) in the epidermal cell and intercellular hyphae (ih) some distance away from penetration site.

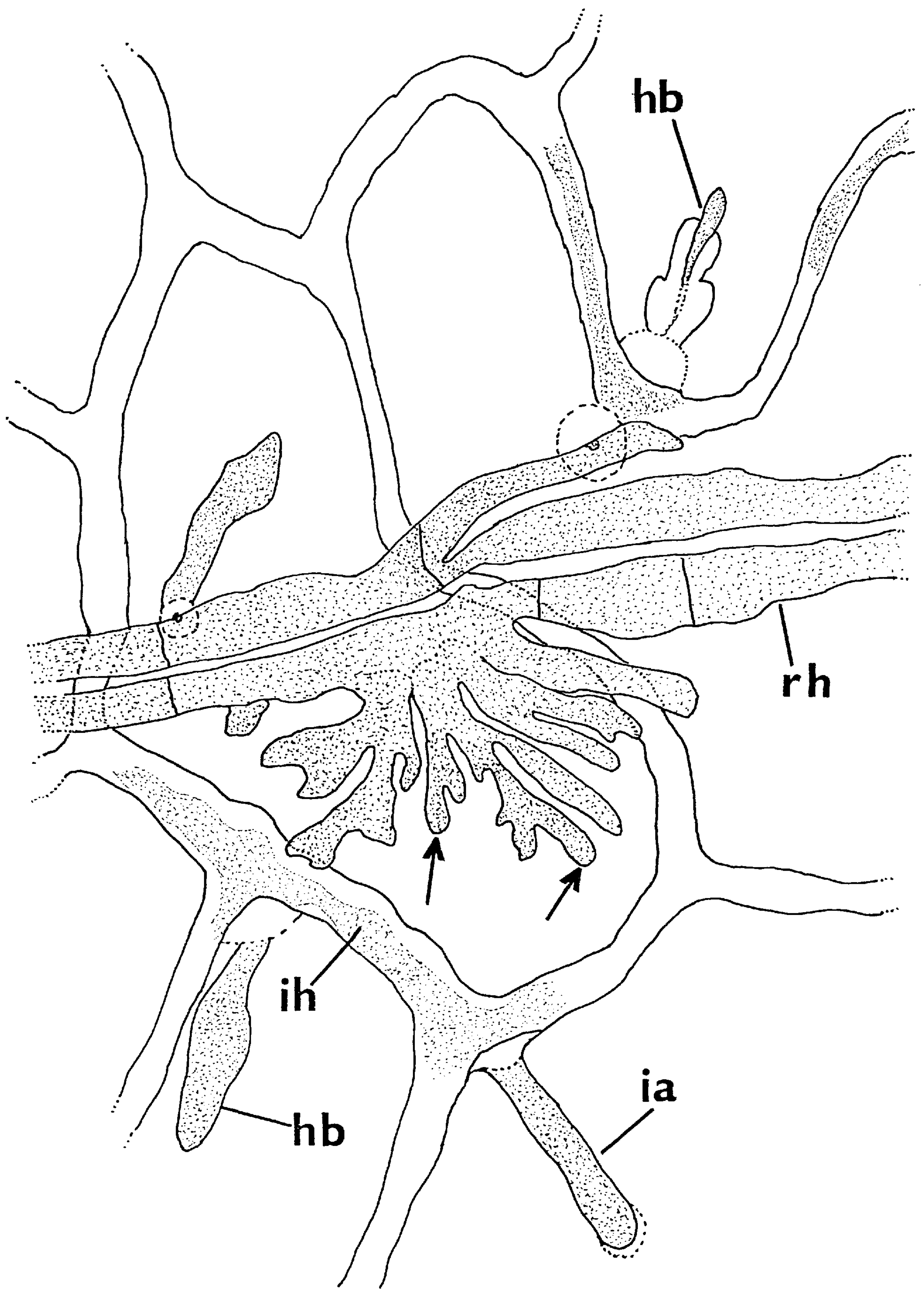

15µm
rh
c
h
hb
sp
if
ih

<u>FIGURE 2</u> : Infection on Frensham (14 days after inoculation with *D. rosae* isolate, F-76-Ash); surface view. Note the parallel strands of the runner hyphae (rh) which give rise to haustoria (hb) in the epidermal cells.



15µm
rh
hb

FIGURE 3 : Early stage in the development of an acervulus on Frensham (14 days after inoculation with *D. rosae* isolate, F-76-Ash); surface view. Side branches (arrowed) are seen growing from a specialized region of the runner hyphae (rh). Note the haustoria (hb) in the epidermal cells, intercellular hyphae (ih) and what may be an intracellular hypha (ia).



15µm
hb
rh
ih
hb
ia

FIGURE 4 : Infection on Allgold-resistant reaction (72 h after inoculation with *D. rosae* isolate, F-76-Ash); surface view. Note the spore (sp) with a halo (h) and well developed runner hyphae (rh) originating near the site of penetration (arrowed). The epidermal cell (ec) has a brown deposit in its cell walls (cw), (not indicated on Figure) and the haustorium (hb) in its lumen seems to be totally occluded by collar material (c).

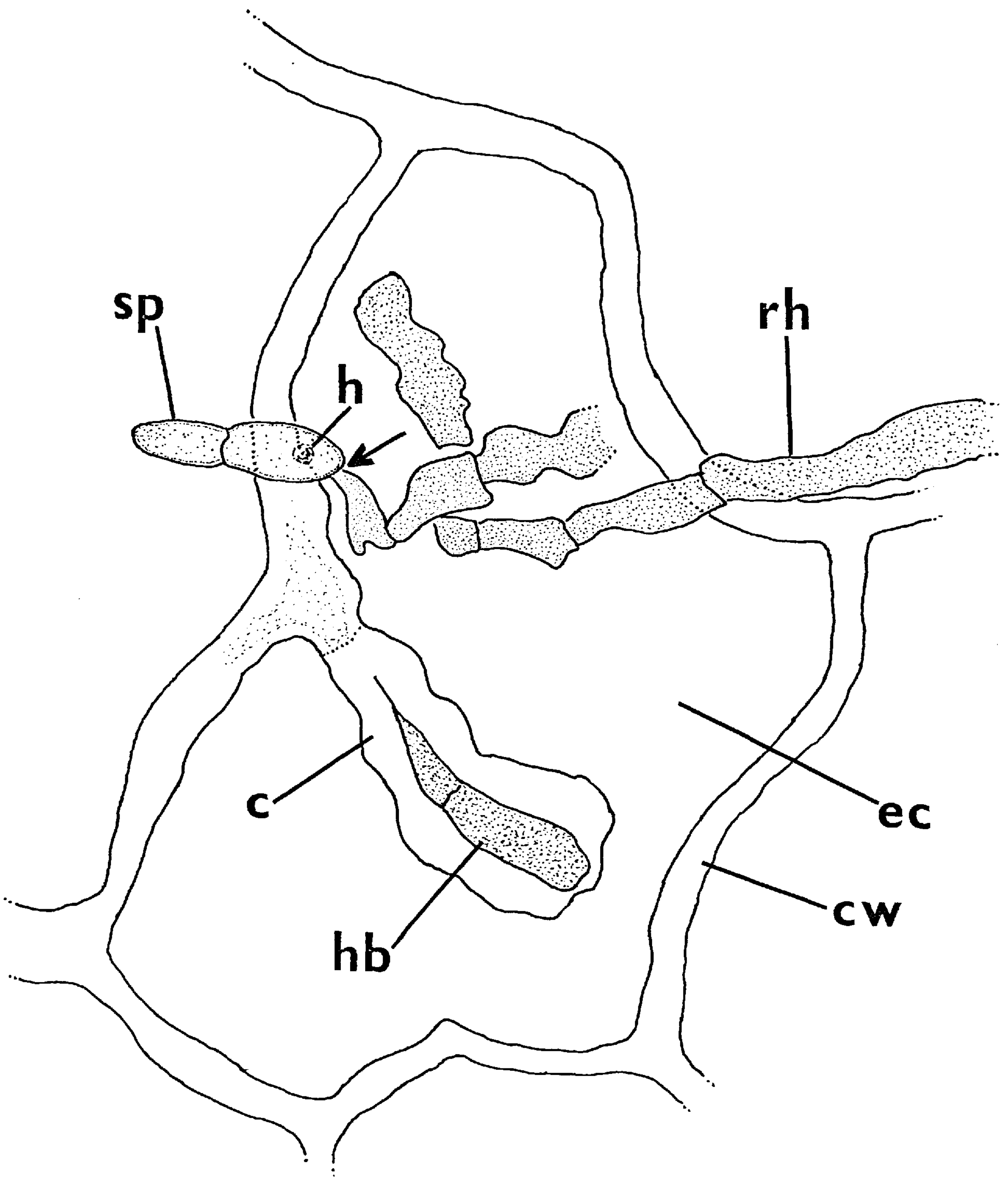

15µm
sp
h
rh
c
ec
cw
hb

FIGURE 5 : Infection on Allgold-resistant reaction (48 h after inoculation with *D. rosae* isolate, F-76-Ash); surface view. Note the spore (sp) with a halo (h), infection hypha (if) and the intercellular hypha (ih) which seems to give rise to a haustorium (hb) in the epidermal cell some distance away from the infection hypha. Note also the distention of the epidermal cell walls (arrowed) due to growth of the intercellular hyphae and the absence of runner hyphae.

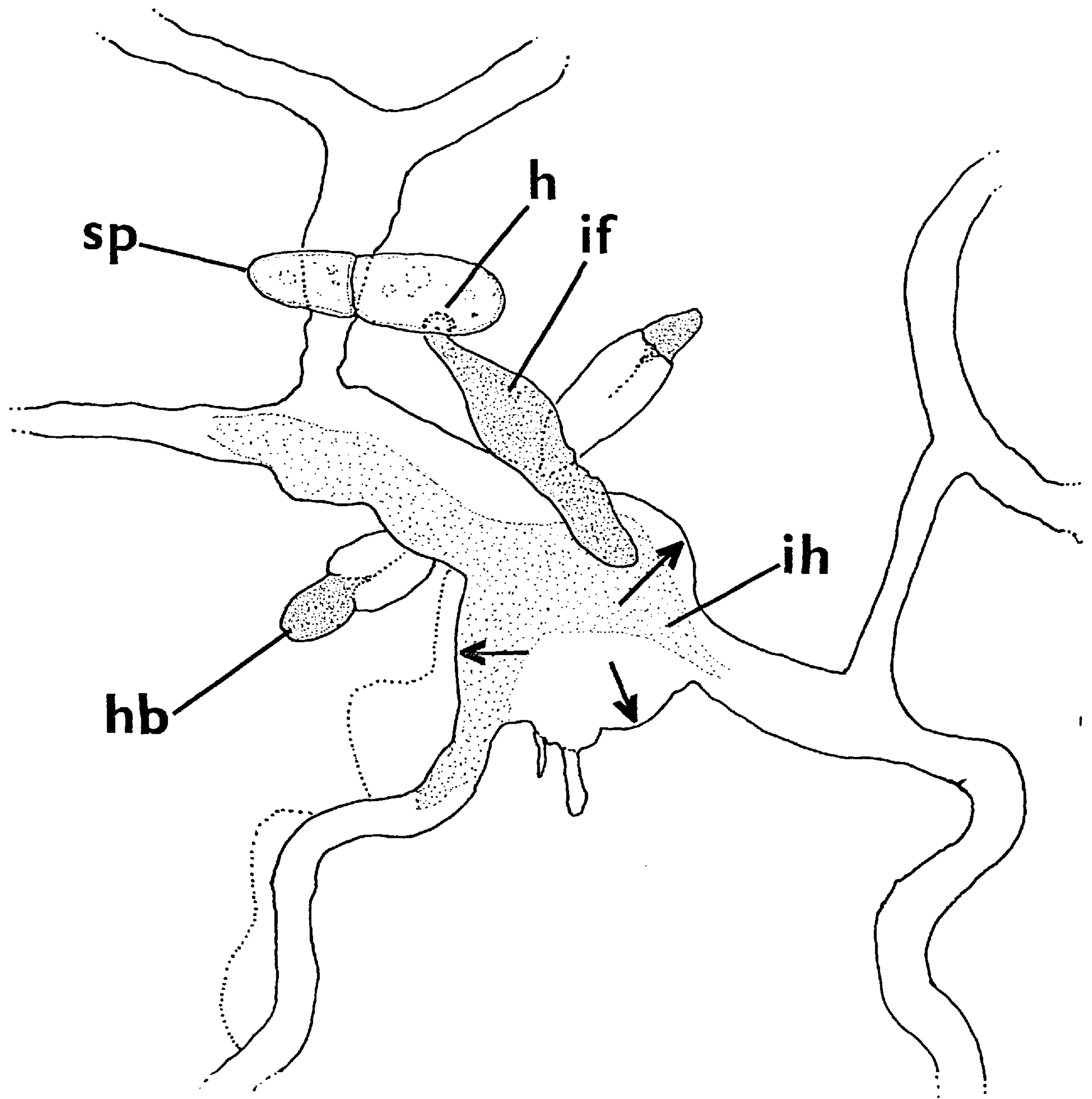

15 µm
sp
h
if
ih
hb

FIGURE 6 : Infection on Allgold-resistant reaction (14 days after inoculation with *D. rosae* isolate, F-76-Ash); surface view. Note the spore (sp) with a halo (h), intercellular hypha (ih), haustorium (hb) with collar (c), and papillae (arrowed) indicating sites of attempted penetration into epidermal cells. Note also the absence of runner hyphae and the distention of the epidermal cell wall (cw).

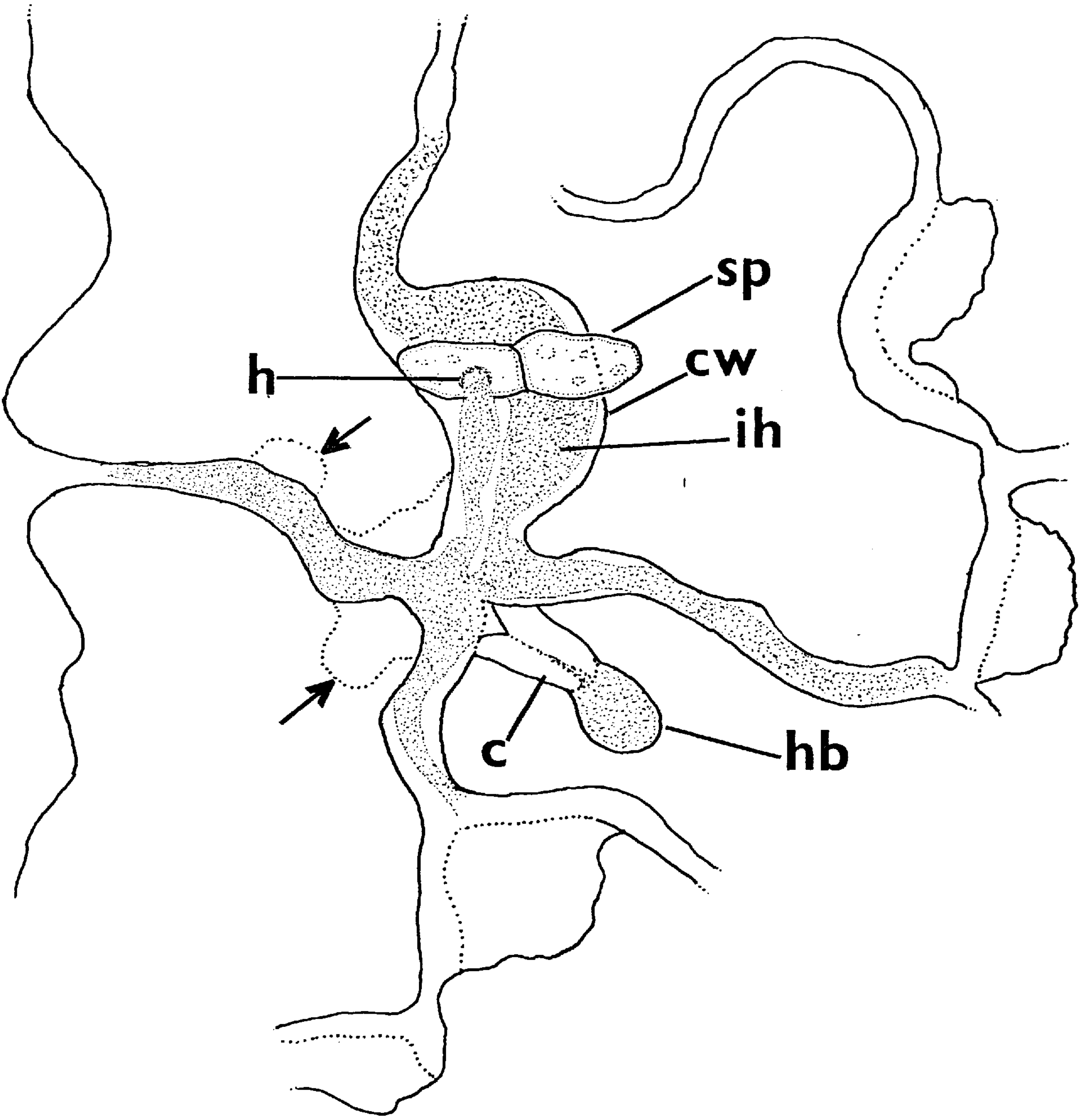
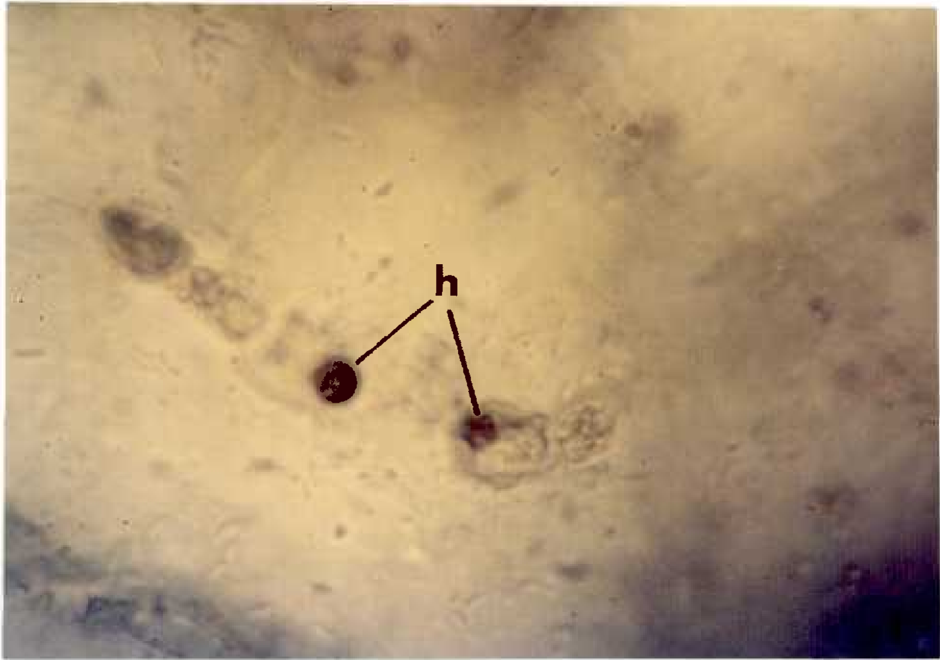

15µm
sp
h
cw
ih
c
hb

PLATE 1 : Frensham, 10 days after inoculation; surface view. Part of leaf disk cleared in absolute ethanol and stained in phenolic thionin. Note the halo (h) at the site of penetration beneath the larger of the two cells in the spore. (X1471)

PLATE 2 : Frensham, 10 days after inoculation; surface view. Part of leaf disk cleared in absolute ethanol and stained in phenolic thionin. Note the differences in halo size (arrowed) at sites of penetration. (X1471)


h

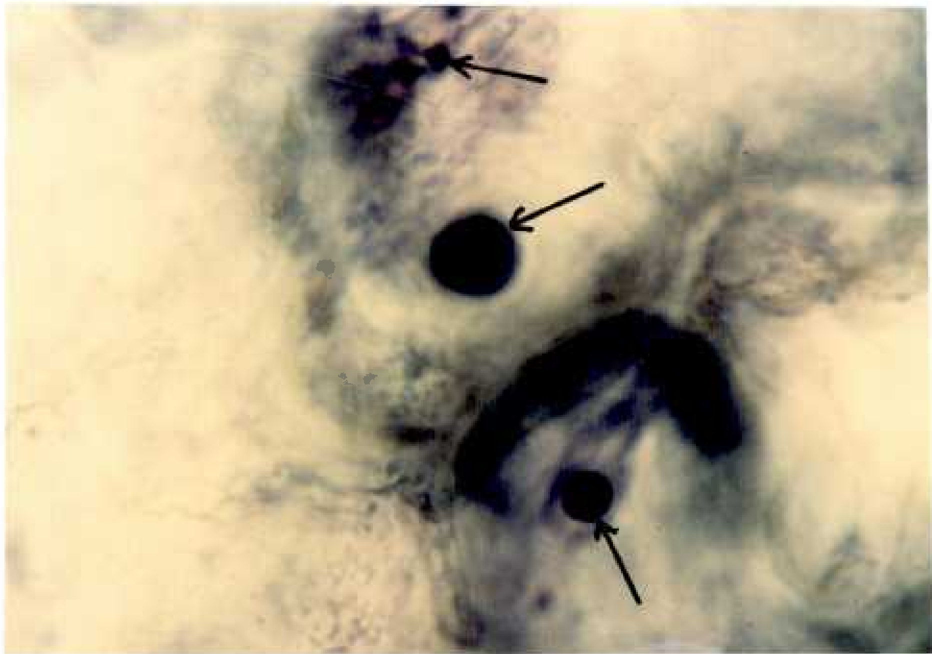
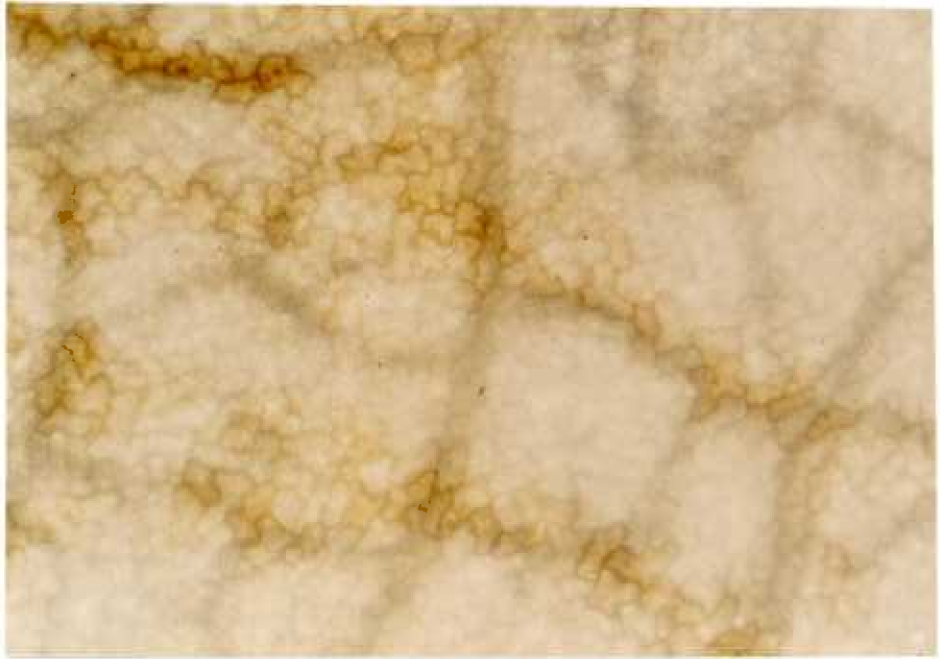


PLATE 3 : Frensham, 14 days after inoculation; surface view. Part of leaf disk cleared in absolute ethanol to show infected cells (yellow-brown in colour - cf. Plate 31). (X93)

PLATE 4 : Frensham, 7 days after inoculation; surface view. Part of leaf disk cleared in absolute ethanol and stained with phenolic thionin. Note the parallel strands of the sub-cuticular runner hyphae (rh) and acervuli (a). Infected cells stain more deeply than non-infected cells. (X149)



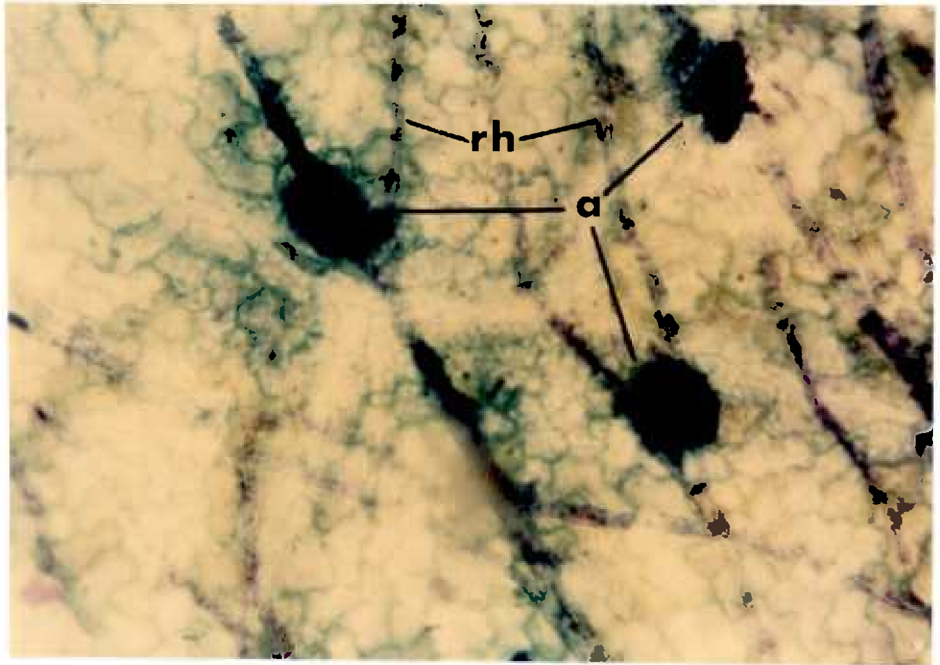
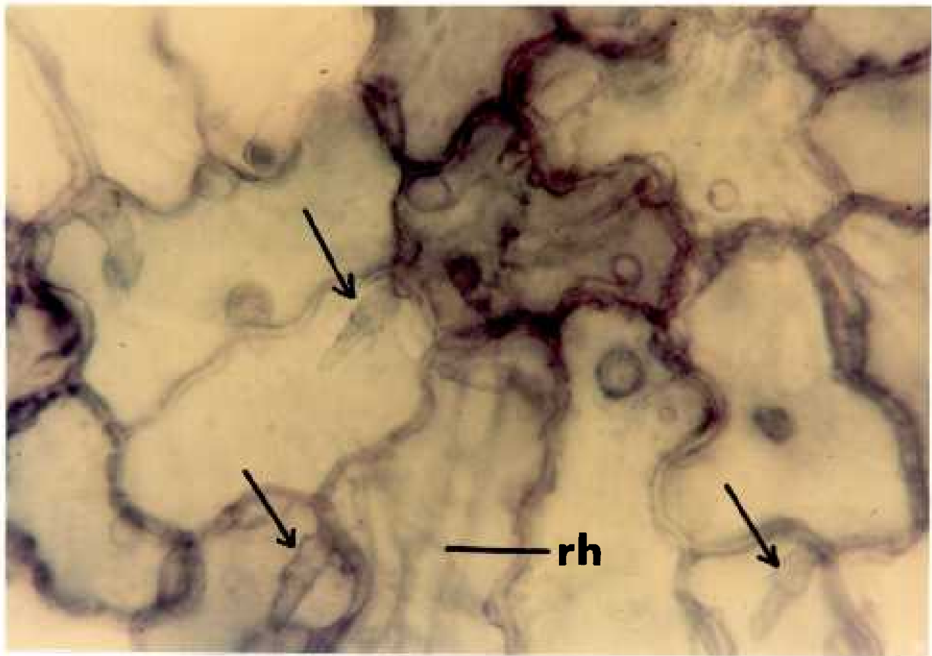

rh
a

PLATE 5 : Frensham, 7 days after inoculation; surface view. Part of leaf disk cleared in absolute ethanol and stained with phenolic thionin. Note the runner hyphae (rh) and haustoria with collars (arrowed) in the epidermal cells. (X595)

PLATE 6 : Frensham, 7 days after inoculation; surface view. Part of leaf disk cleared in absolute ethanol and stained with phenolic thionin and aq. crystal violet. Note the sub-cuticular hypha (rh) giving rise to a haustorium (hb) with a collar (arrowed) in an epidermal cell. A papilla (p) is seen in the adjacent cell. (X1471)


rh

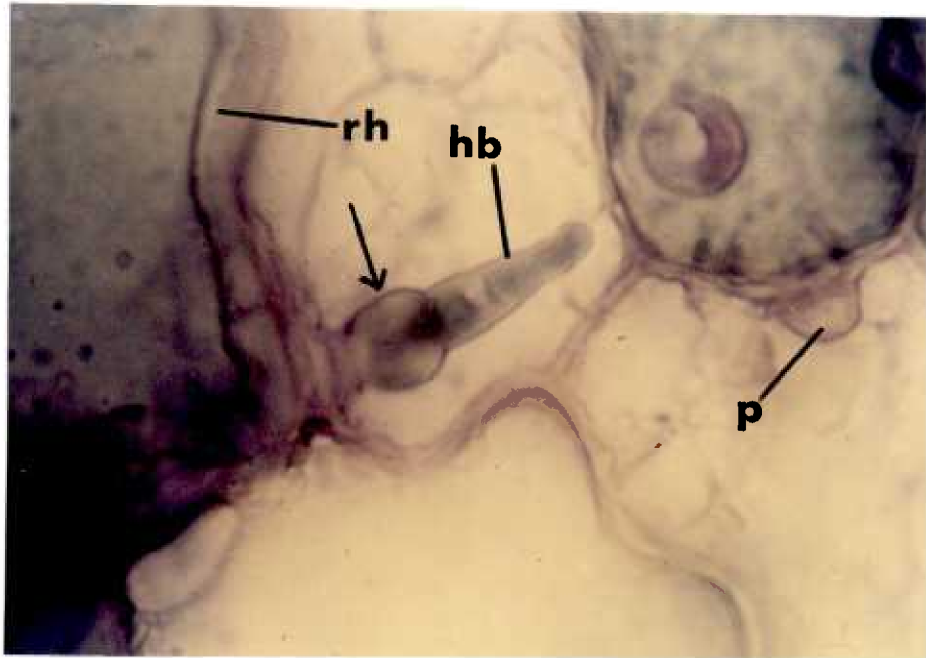
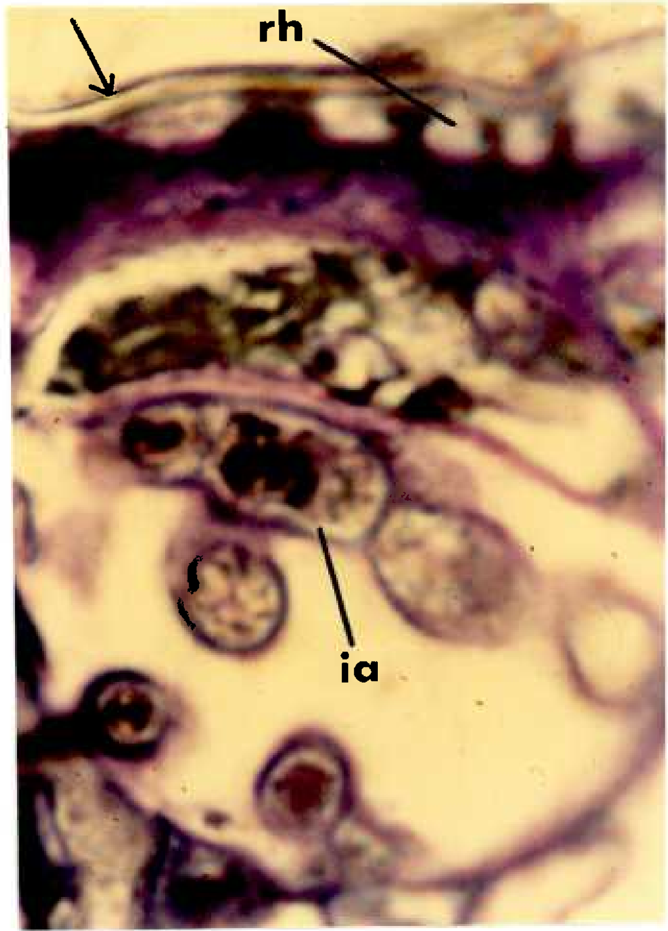

rh
hb
p

PLATE 7 : Frensham, 7 days after inoculation. T.S. (10 μm) of leaf epidermal cell stained in aq. crystal violet. Note the runner hyphae (rh) under the cuticle (arrowed) and septate intracellular hypha (ia) in epidermal cell. (X2273)

PLATE 8 : Frensham, 14 days after inoculation. Oblique section (2.5 μm) of leaf through epidermis stained with aq. azure blue. Note the intracellular hypha (ia) and section through browned haustorium (hb). (X1471)


rh
ia

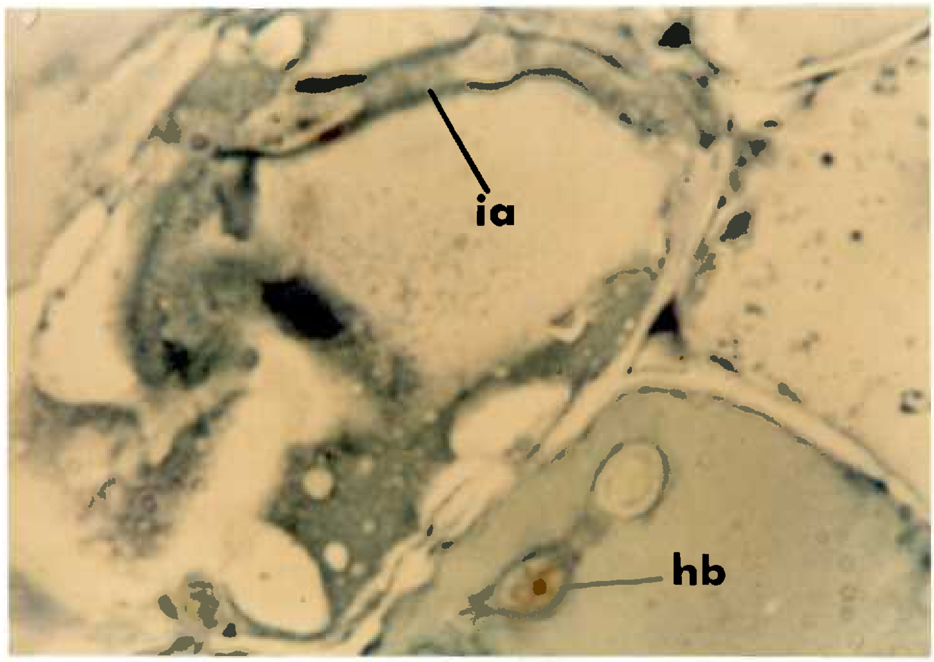
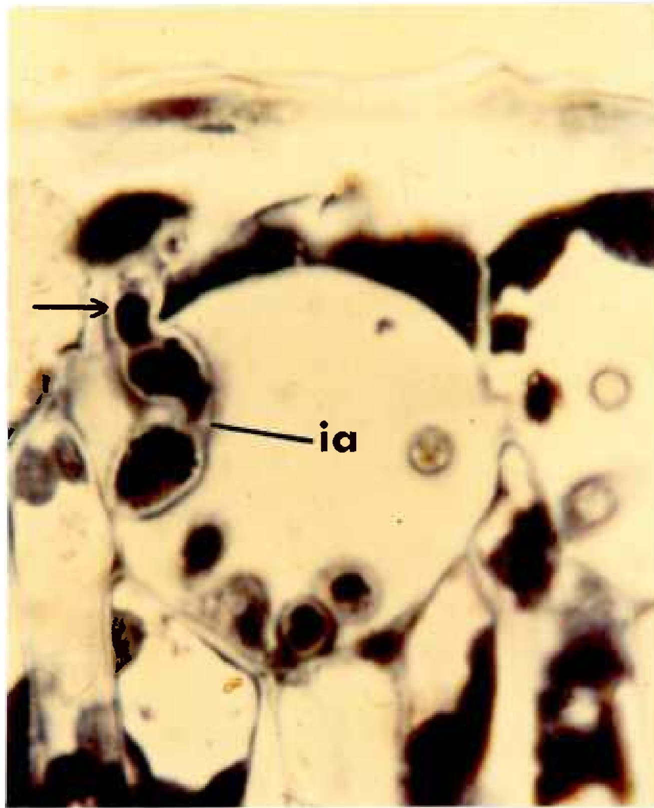

ia
hb

PLATE 9 : Frensham, 14 days after inoculation. T.S. (2.5 μm) of leaf through epidermis and part of palisade stained in aq. toluidine blue. Note the large area of cell wall breached (arrowed) by the penetrating fungus. An intracellular hyphae (ia) is seen in the epidermal cell. (X1471)

PLATE 10 : Frensham, 14 days after inoculation. T.S. (1.0 μm) of leaf through epidermis and part of the palisade stained in aq. azure blue. Note the haustorial mother cell (hm) in the epidermal cell (ec) which gives rise to a haustorium (arrowed) in a palisade cell. (X1471)


ia

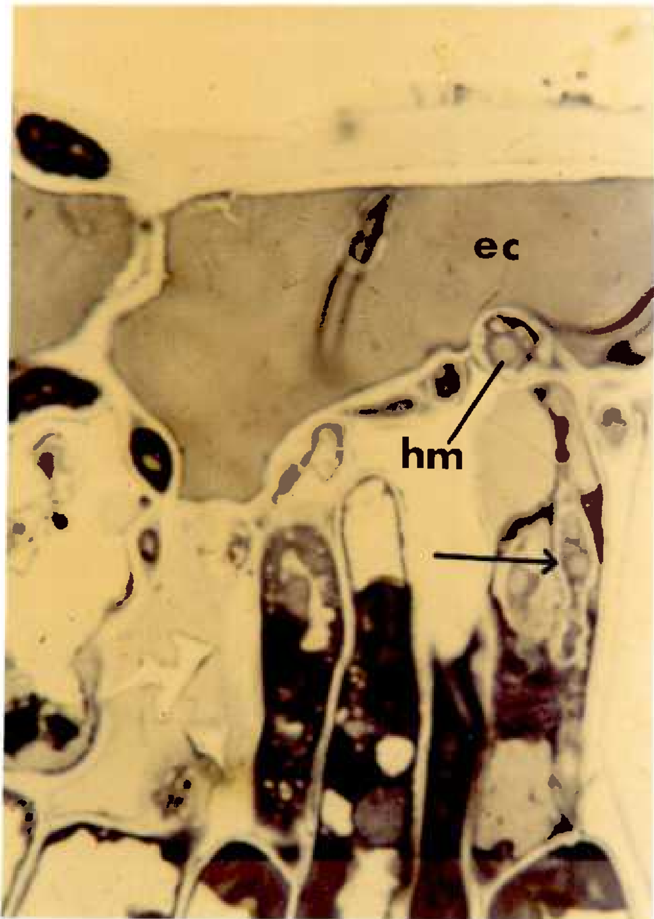

ec
hm

PLATE 11 : Frensham, 14 days after inoculation. T.S. (2.5 μm) of leaf through epidermis and palisade stained in aq. azure blue. Spores in many stages of development (some arrowed) are seen in the ruptured acervulus (a). (X595)

PLATE 12 : Frensham, 14 days after inoculation. T.S. (2.5 μm) of leaf surface stained in aq. azure blue. Note the ruptured cuticle (arrowed) at the edge of the acervulus (a). Note also the fully developed spore (sp) still attached to the basal stroma. (X1471)

a

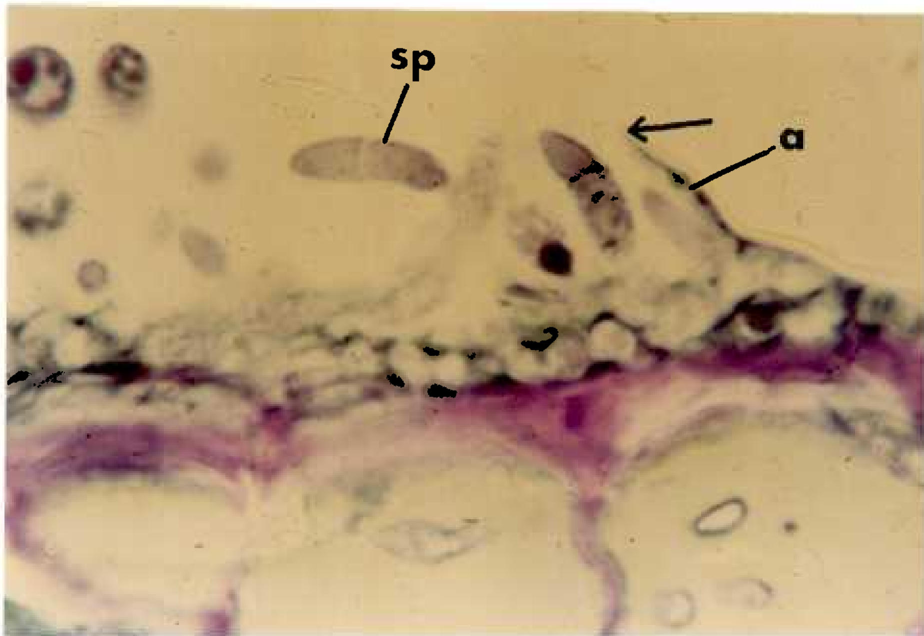
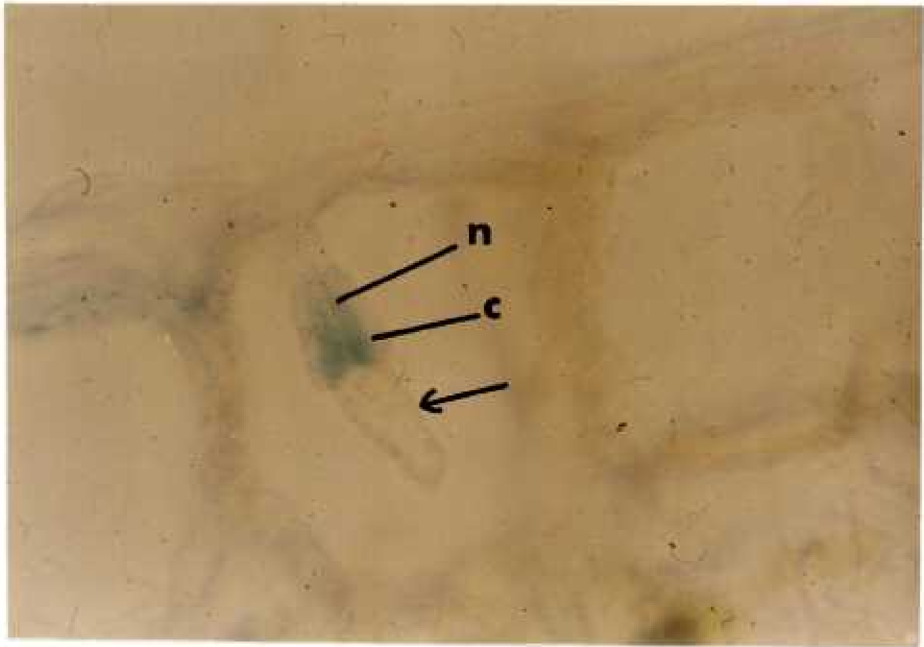

sp
a

PLATE 13 : Frensham, 14 days after inoculation. T.S. (12 μm) of leaf through epidermis containing a haustorium (arrowed) stained with lacmoid. The collar (c) around the haustorial neck (n) in the epidermal cell has stained light blue indicating that callose may be present. (X1471)

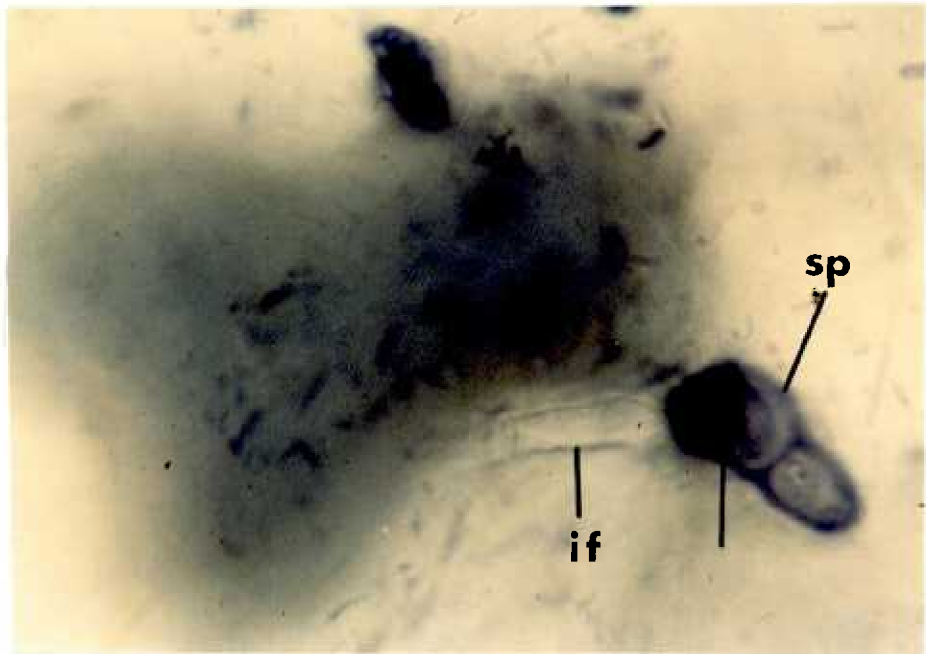
PLATE 14 : Incubated leaf disks (1 cm^2) in plastic boxes. Lesions (14 days old) are seen on Frensham but not on Allgold. Small lesions developed on Babylon but are not clearly visible. (X1)


n
c

FRENSHAM
BABYLON
ALLGOLD - OLD

PLATE 15 : Allgold, 72 h after inoculation; surface view. Part of leaf disk cleared in absolute ethanol and stained with phenolic thionin. Note the halo (arrowed) at the site of penetration beneath the spore (sp) and the sub-cuticular infection hypha (if) growing towards intercellular region between two epidermal cells. The infected cell has stained deeply. (X1471)

PLATE 16 : Detail of infected epidermal cell from Plate 15. A papilla (p) has developed in the stained epidermal cell and a haustorium (arrowed) has formed in the adjacent cell. Note the intercellular hypha (ih). (X1471)


sp
if

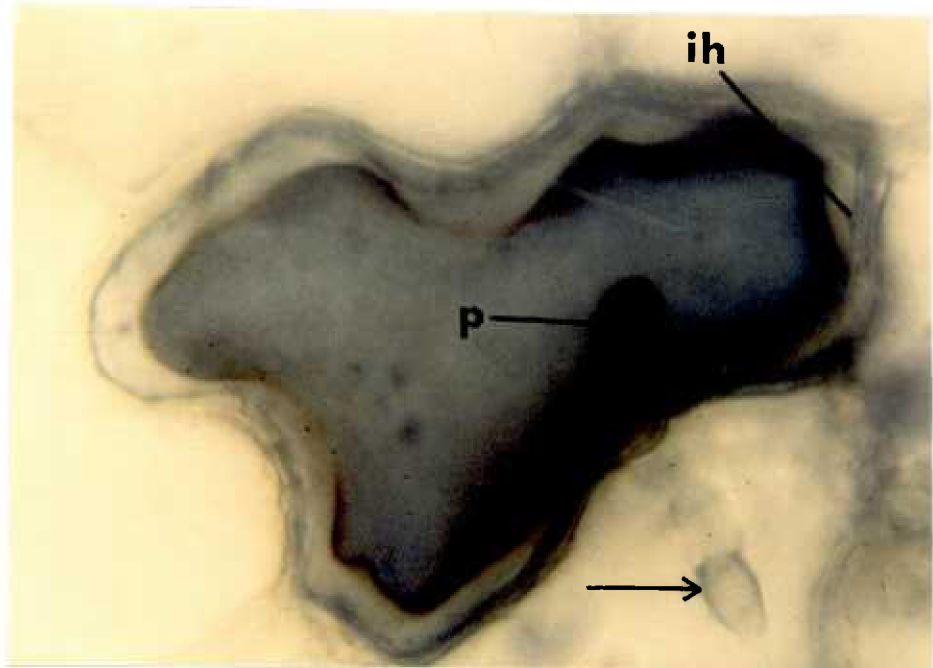
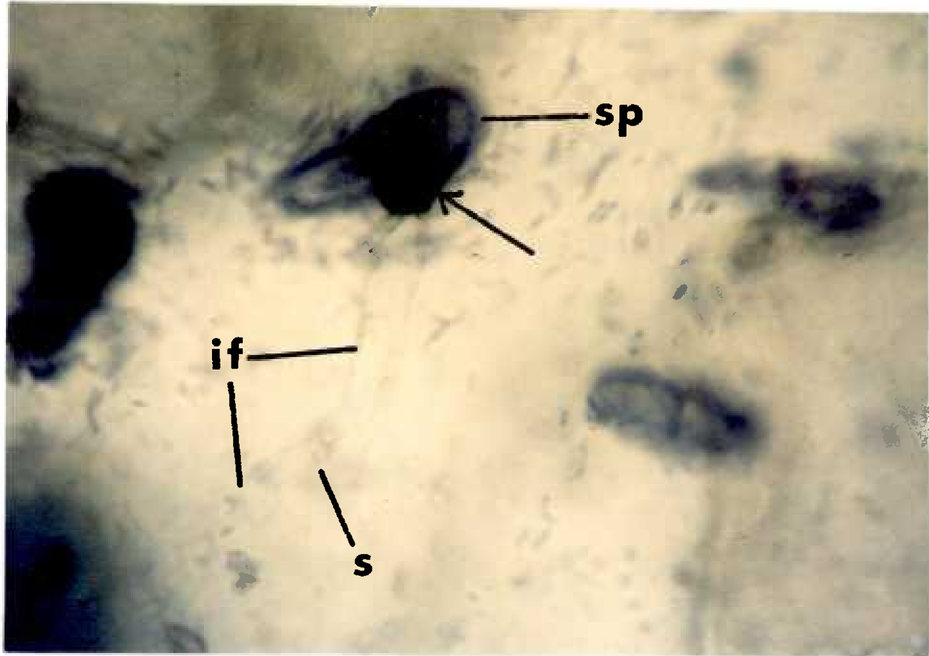

ih
p

PLATE 17 : Allgold, 72 h after inoculation; surface view. Part of leaf disk cleared in absolute ethanol and stained with phenolic thionin. Note the halo (arrowed) at the site of penetration beneath the larger of the two cells in the spore (sp) and the long sub-cuticular infection hypha (if) with a septum (s). (X1471)

PLATE 18 : Allgold, 48 h after inoculation; surface view. Part of leaf disk cleared in absolute ethanol and stained with phenolic thionin. Note the halo (arrowed) at the site of penetration beneath the spore (sp) and the well developed sub-cuticular infection hypha (if). (X1471)


sp
if
s

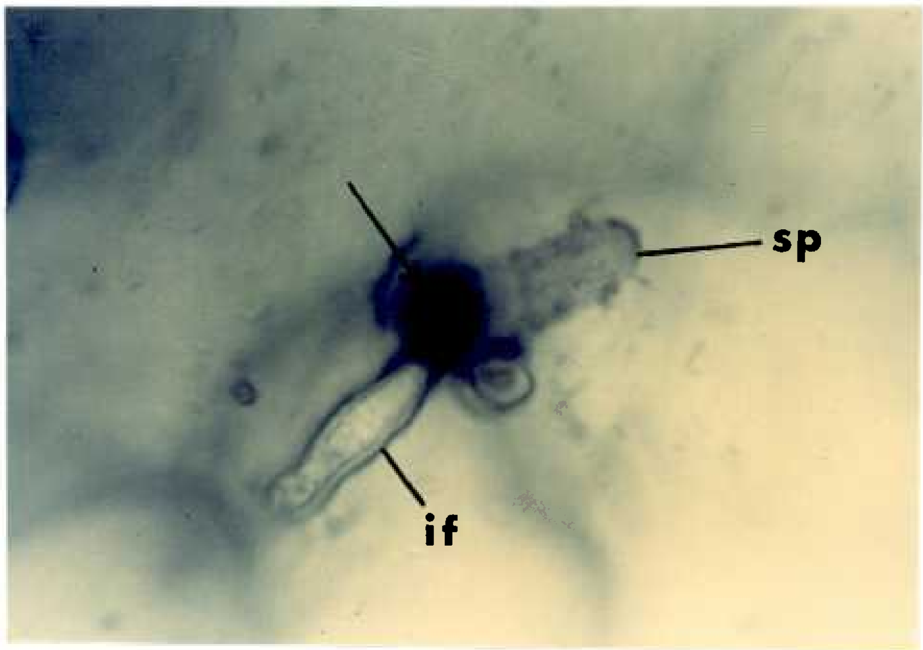
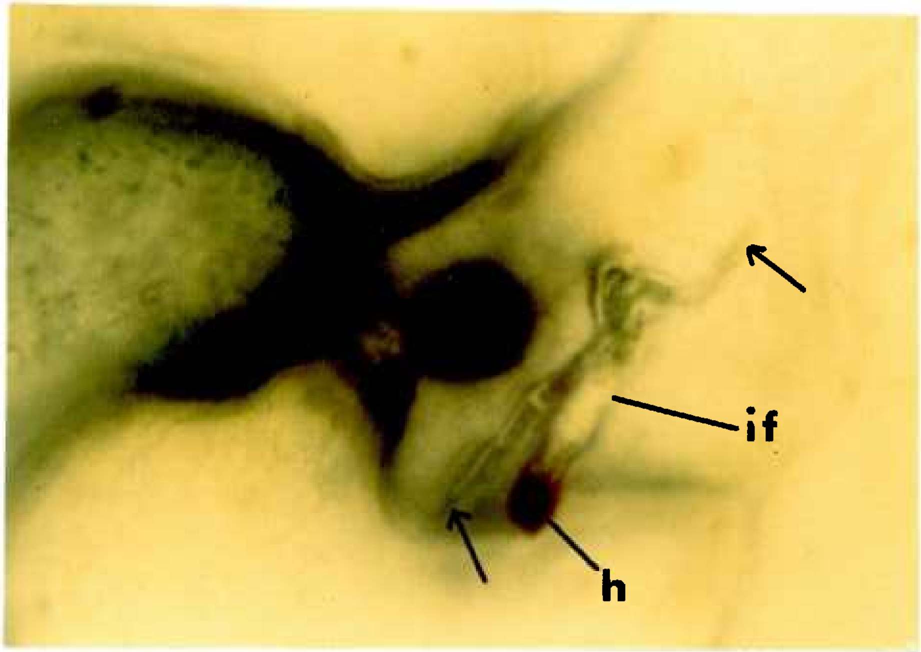

sp
if

PLATE 19 : Allgold, 48 h after inoculation; surface view. Part of leaf disk cleared in absolute ethanol and stained with phenolic thionin (photographed with orange filter). Note the halo (h) and sub-cuticular hyphae (if) from which the runner hyphae (arrowed) seem to grow in two directions. (X1471)

PLATE 20 : Allgold-susceptible reaction, 6 days after inoculation. T.S. (0.5 μm) of leaf through epidermis stained with aq. toluidine blue. Penetration into epidermal cell (ec) from well developed runner hyphae (rh) just under the cuticle (cu). Note the large areas of cell wall penetrated (arrowed) and the absence of fungal constriction at this site. (X1471)


if
h

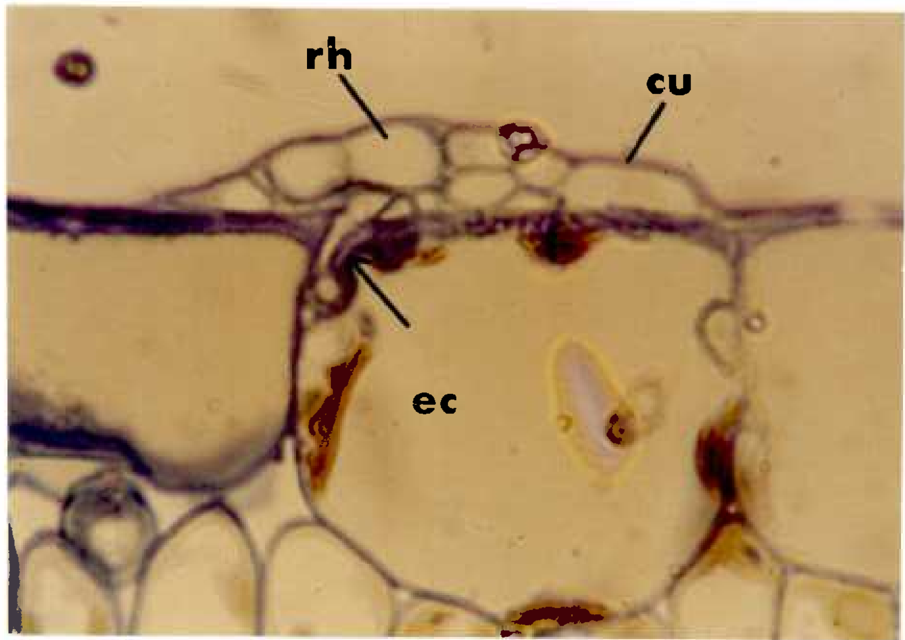
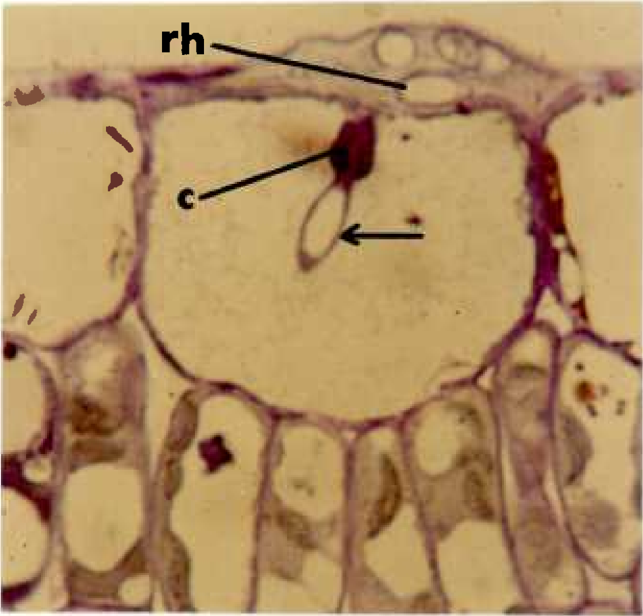
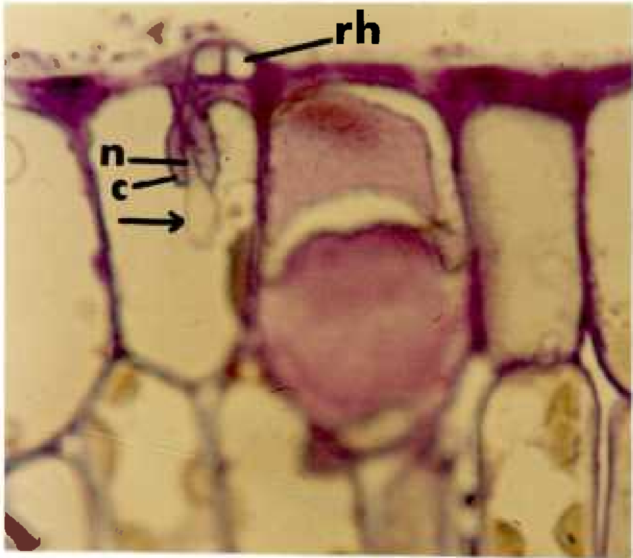

rh
cu
ec

PLATE 21 : Allgold-susceptible reaction, 14 days after inoculation. T.S. (0.5 μm) of leaf through epidermis and palisade stained with aq. toluidine blue. Note the runner hypha (rh) and haustorium (arrowed) with a deeply stained collar (c) emerging from the upper wall of the epidermal cell. (X1471)

PLATE 22 : Allgold-susceptible reaction, 14 days after inoculation. T.S. (0.5 μm) of leaf through epidermis and palisade stained with aq. toluidine blue. Runner hyphae (rh) give rise to a haustorium (arrowed) in the epidermal cell. Note the long haustorial neck (n) and deeply stained collar (c). (X1471)

PLATE 23 : Allgold-susceptible reaction, 14 days after inoculation. T.S. (0.5 μm) of leaf through epidermis stained with aq. toluidine blue. Note the runner hyphae (rh) and haustoria (arrowed) with deeply stained collars (c) arising from the top corners of adjacent epidermal cells. (X1471)


rh
c


rh
n
c

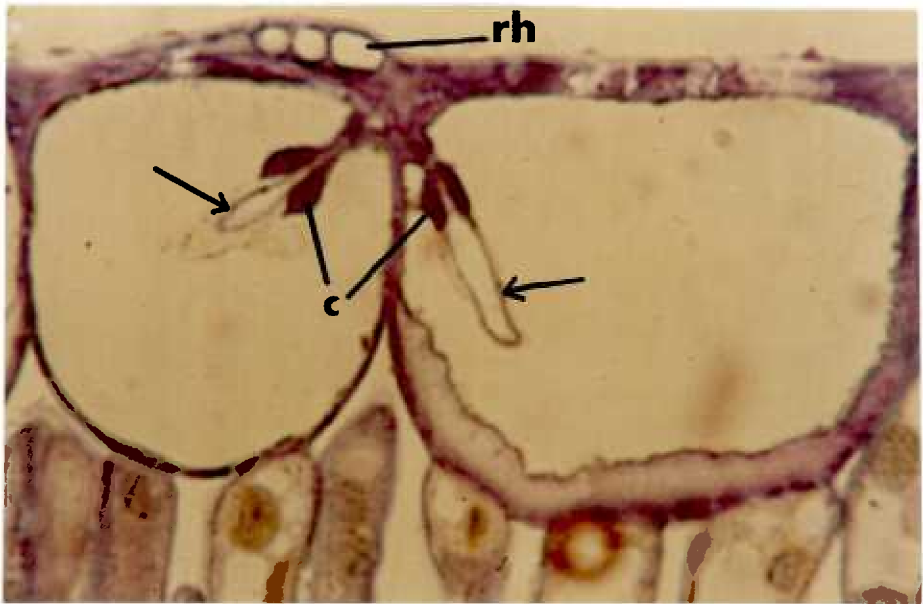
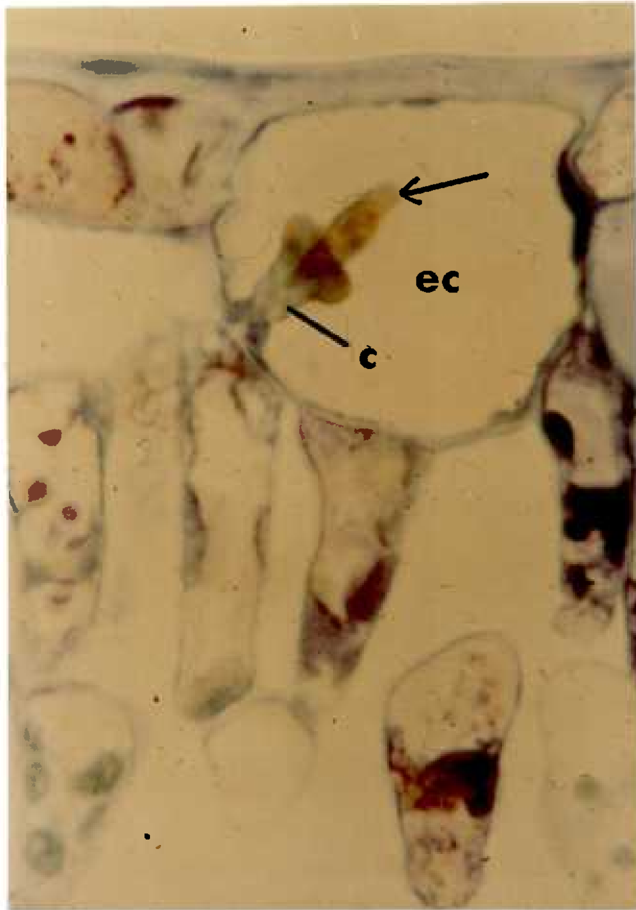

rh
c

PLATE 24 : Allgold-susceptible reaction, 14 days after inoculation. T.S. (2.5 μm) of leaf through epidermis and palisade stained with aq. azure blue. Note the brown haustorium (arrowed) and large light blue collar (c) in lower half of epidermal cell (ec). (X1471)

PLATE 25 : Allgold-susceptible reaction, 14 days after inoculation. T.S. (0.5 μm) of leaf through epidermis and palisade. Note the runner hyphae (arrowed), intercellular hypha (ih) and the haustorium (hb) in a palisade cell. (X595)


ec
c

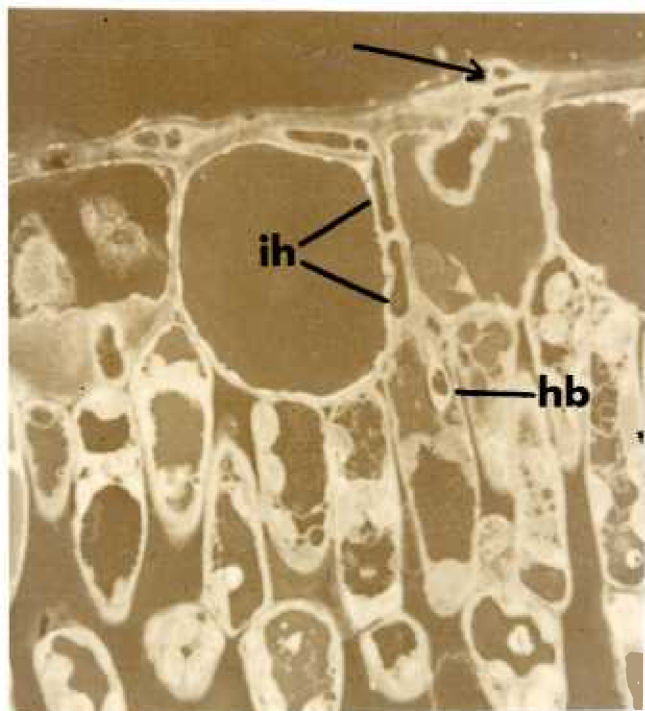
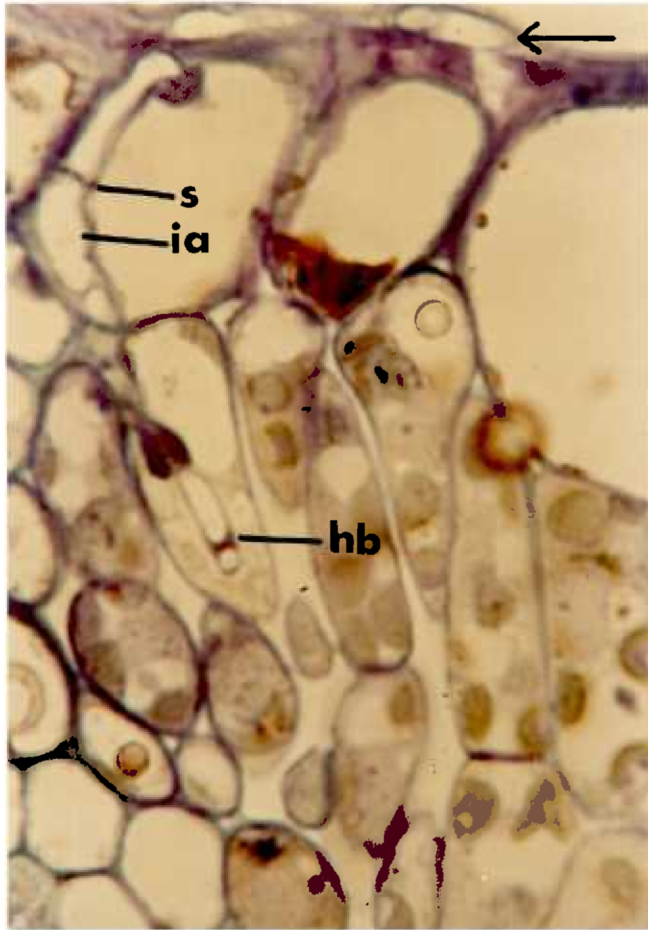

ih
hb

PLATE 26 : Allgold-susceptible reaction, 14 days after inoculation. T.S. (0.5 μm) of leaf through epidermis and palisade stained with aq. toluidine blue. Note the runner hyphae (arrowed) intracellular hypha (ia) with a septum (s) in the epidermal cell giving rise to a haustorium (hb) in a palisade cell. (X1471)

PLATE 27 : Allgold-susceptible reaction, 14 days after inoculation. T.S. (0.5 μm) of leaf through epidermis and palisade stained with aq. toluidine blue. Note the haustoria (arrowed) with deeply stained collars (c) in adjacent palisade cells. One at least seems to have been formed from an intercellular hypha (ih). (X1471)


s
ia
hb

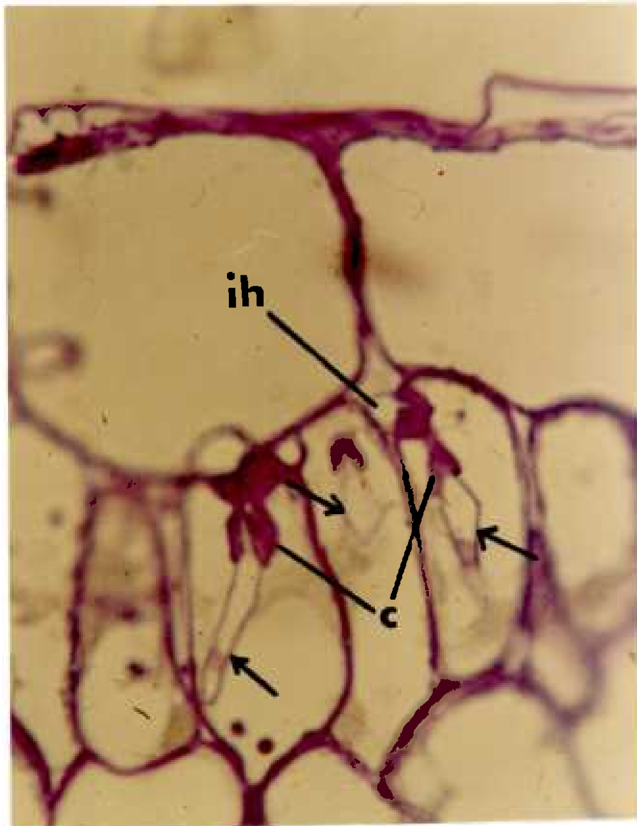
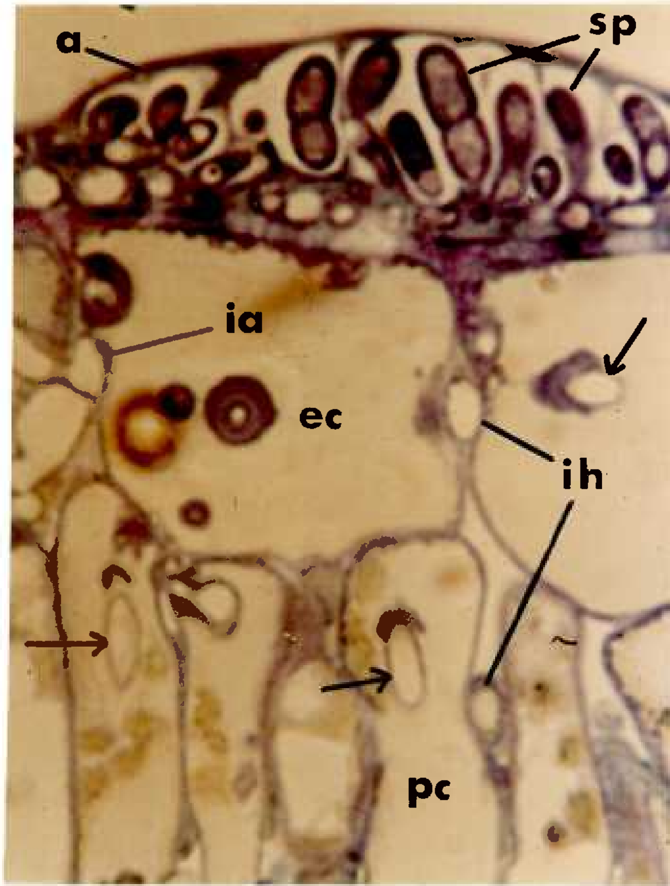

ih
c

PLATE 28 : Allgold-susceptible reaction, 14 days after inoculation. T.S. (0.5 μm) of leaf through epidermis and palisade stained with aq. toluidine blue. Note the acervulus (a) with developing conidia (sp). Haustoria (arrowed) are visible in the epidermis (ec) and palisade (pc). Intracellular hyphae (ia) and intercellular hyphae (ih) are also present. (X1471)

PLATE 29 : Allgold-susceptible reaction, 14 days after inoculation. T.S. (0.5 μm) of leaf through epidermis and palisade stained in aq. toluidine blue. Note the small acervulus (a) with developing conidia (arrowed) and total absence of fungal structures in epidermal and palisade cells. (X1471)


a
sp
ia
ec
ih
pc

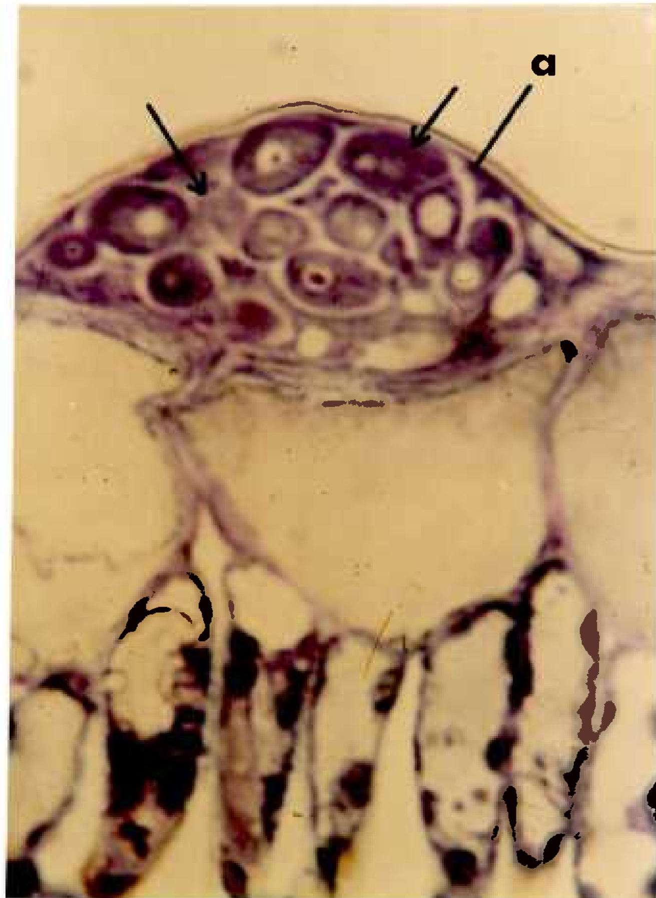
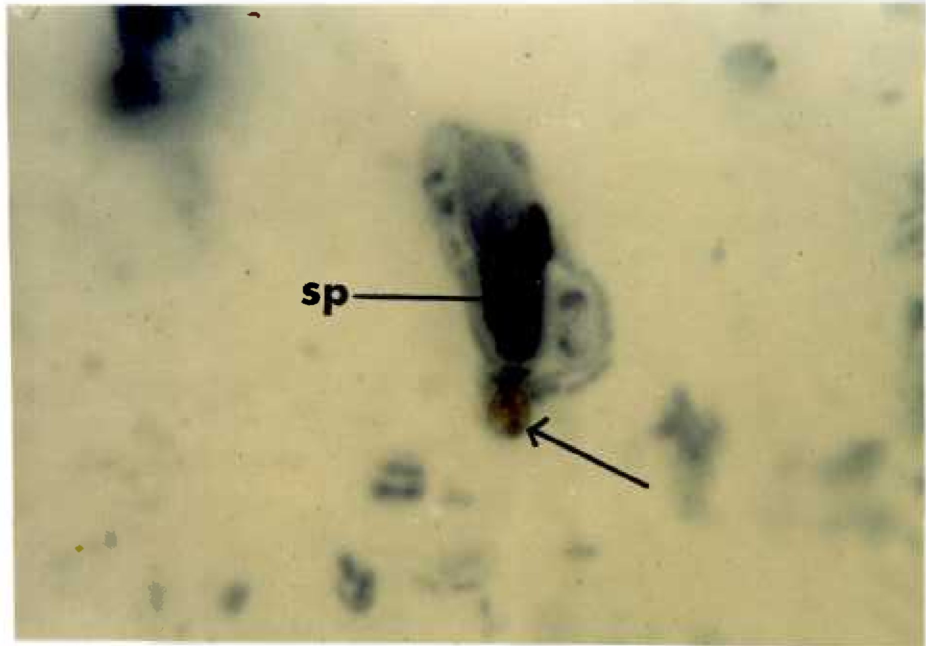

a

PLATE 30 : Allgold-resistant reaction, 7 days after inoculation; surface view. Part of leaf disk cleared in absolute ethanol and stained in phenolic thionin. Note the browned deposit (arrowed) at the tip of a spore (sp) which has failed to initiate infection in epidermal cell beneath it. (X1471)

PLATE 31 : Allgold-resistant reaction, 14 days after inoculation; surface view. Part of leaf disk (not cleared or stained) showing a dark-brown substance (arrowed) surrounding a group of infected epidermal cells. (X227)


sp

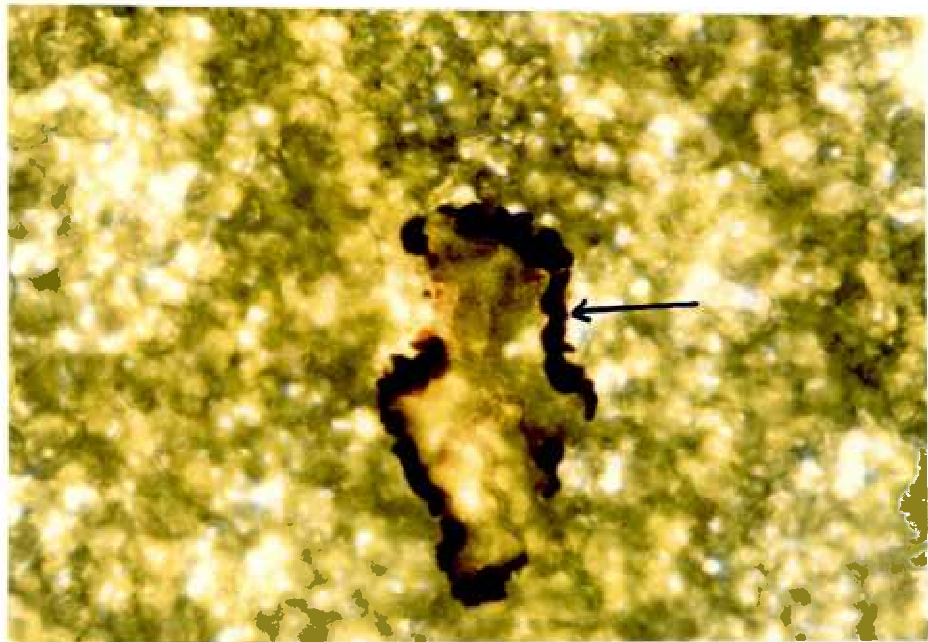
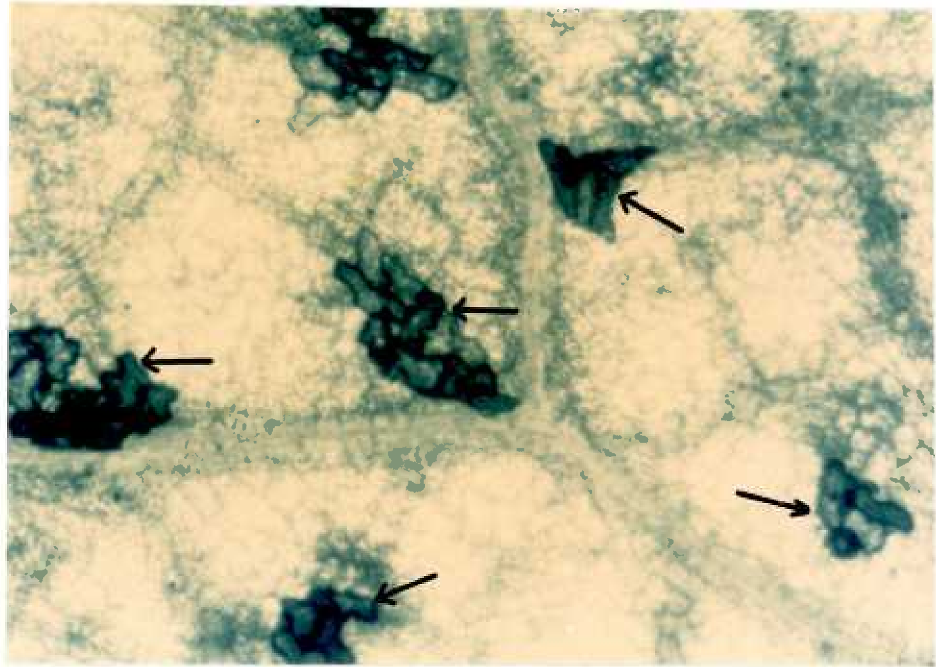


PLATE 32 : Allgold-resistant reaction, 14 days after inoculation; surface view. Part of leaf disk cleared in absolute ethanol and stained in phenolic thionin. Note the deeply stained isolated groups of infected cells (arrowed) and the absence of runner hyphae. (X93)

PLATE 33 : Allgold-resistant reaction, 14 days after inoculation; surface view. Part of leaf disk cleared in absolute ethanol and stained in aq. azure blue. The two infected epidermal cells stain blue-green and each contains a fungal structure (hb-haustoria?) but runner hyphae are absent. Note the deep browning (arrowed) which does not surround the whole infected group. (X595)



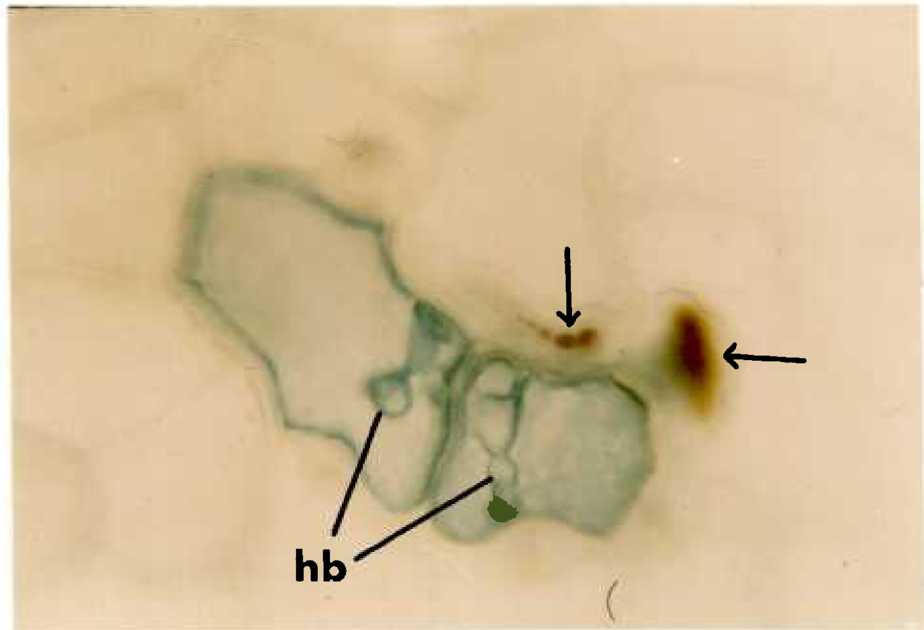
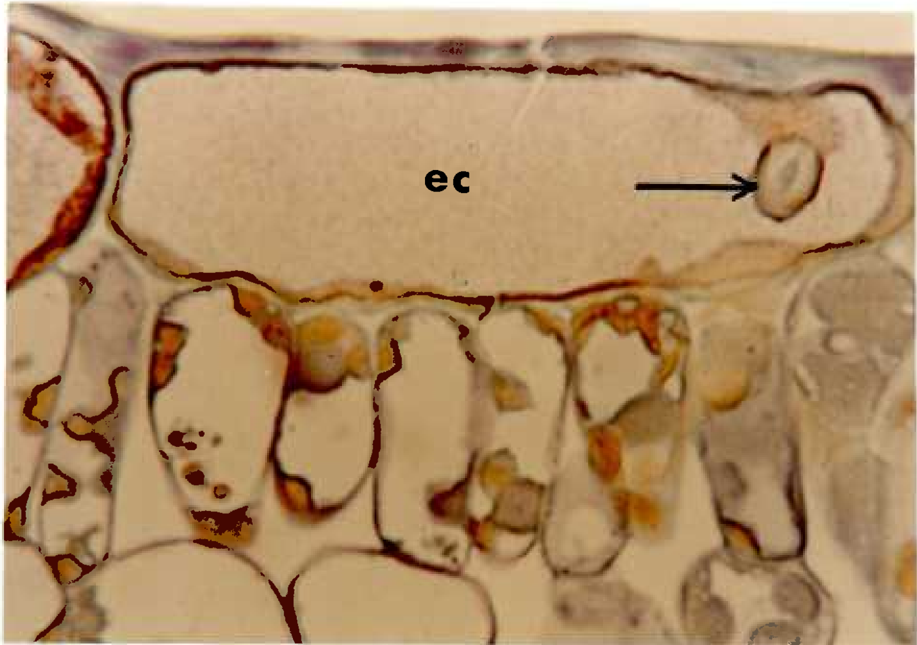

hb

PLATE 34 : Allgold-resistant reaction, 14 days after inoculation. T.S. (0.5 μm) of leaf through epidermis and palisade stained with aq. toluidine blue. Note the apparently occluded haustorium (arrowed) in the epidermal cell (ec). No other fungal structures are visible. (X1471)

PLATE 35 : Allgold-resistant reaction, 14 days after inoculation. T.S. (0.5 μm) of leaf through epidermis and palisade stained with aq. toluidine blue. Note the apparently occluded haustoria (arrowed) in the epidermal cells. No other fungal structures are visible (cf. Plate 34). (X1471)


ec

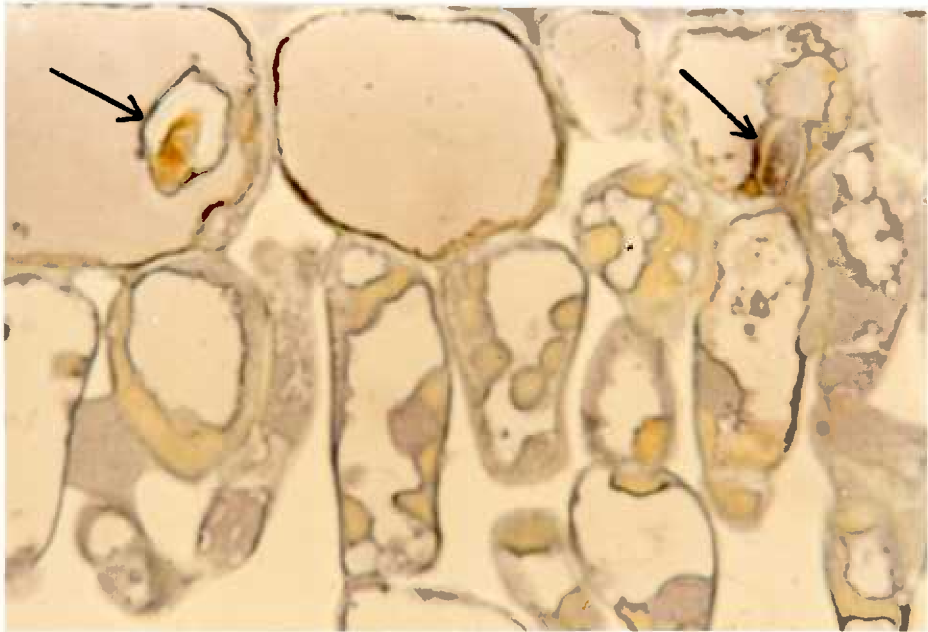
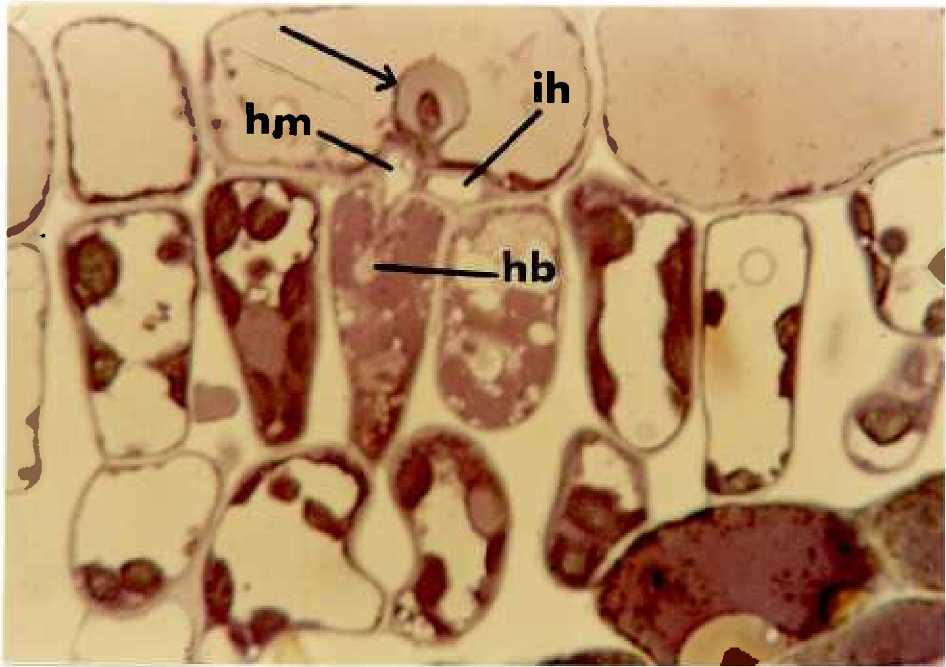


PLATE 36 : Allgold-resistant reaction, 14 days after inoculation. T.S. (0.5 μm) of leaf through epidermis and palisade stained with aq. toluidine blue. Note the fungal structure (arrowed) in the epidermal cell, intercellular hypha (ih) and intercellular haustorial mother cell (hm) giving rise to a poorly developed haustorium (hb) in a palisade cell. (X1471)

PLATE 37 : Allgold-resistant reaction, 14 days after inoculation. T.S. (2.5 μm) of leaf through epidermis and palisade stained with aq. azure blue. The haustorium (arrowed) in the palisade cell (pc) has failed to expand and remains as a narrow shrivelled structure. (X1471)


ih
hm
hb

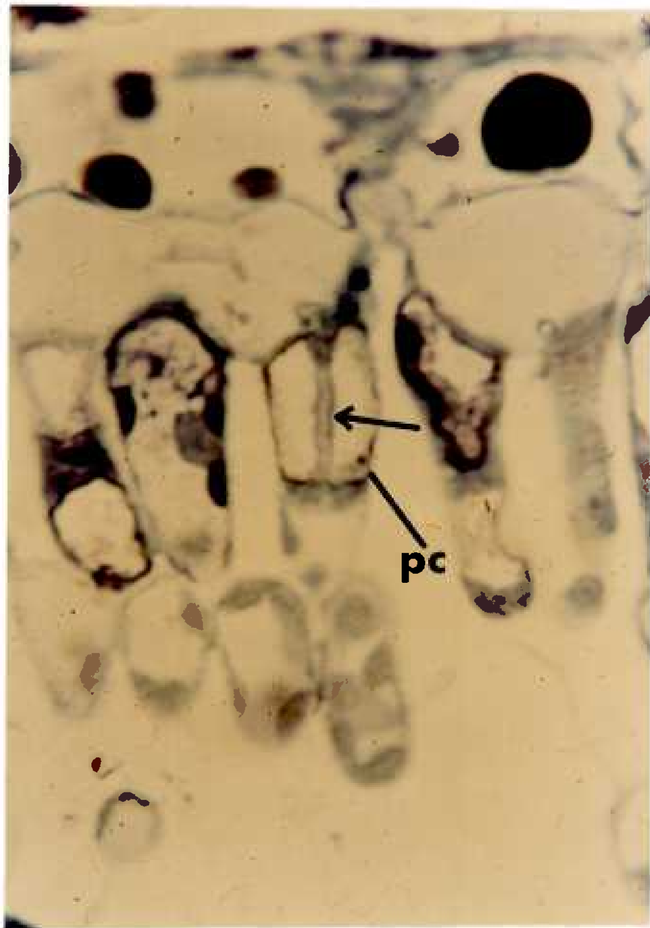

pc

PLATE 38 : T.S. through spore (sp) showing early penetration stage on Frensham leaf. Electron dense material (e) surrounds the germ tube/appressorium (arrowed). Note the identation of the cuticle (cu) at the point of penetration by a fine peg (pp). Note also the structured deposit (sd) under cuticle to right of penetration peg and massive deposition of material on the cell wall (cw) of the epidermal cell immediately beneath the point of penetration. (X18016)

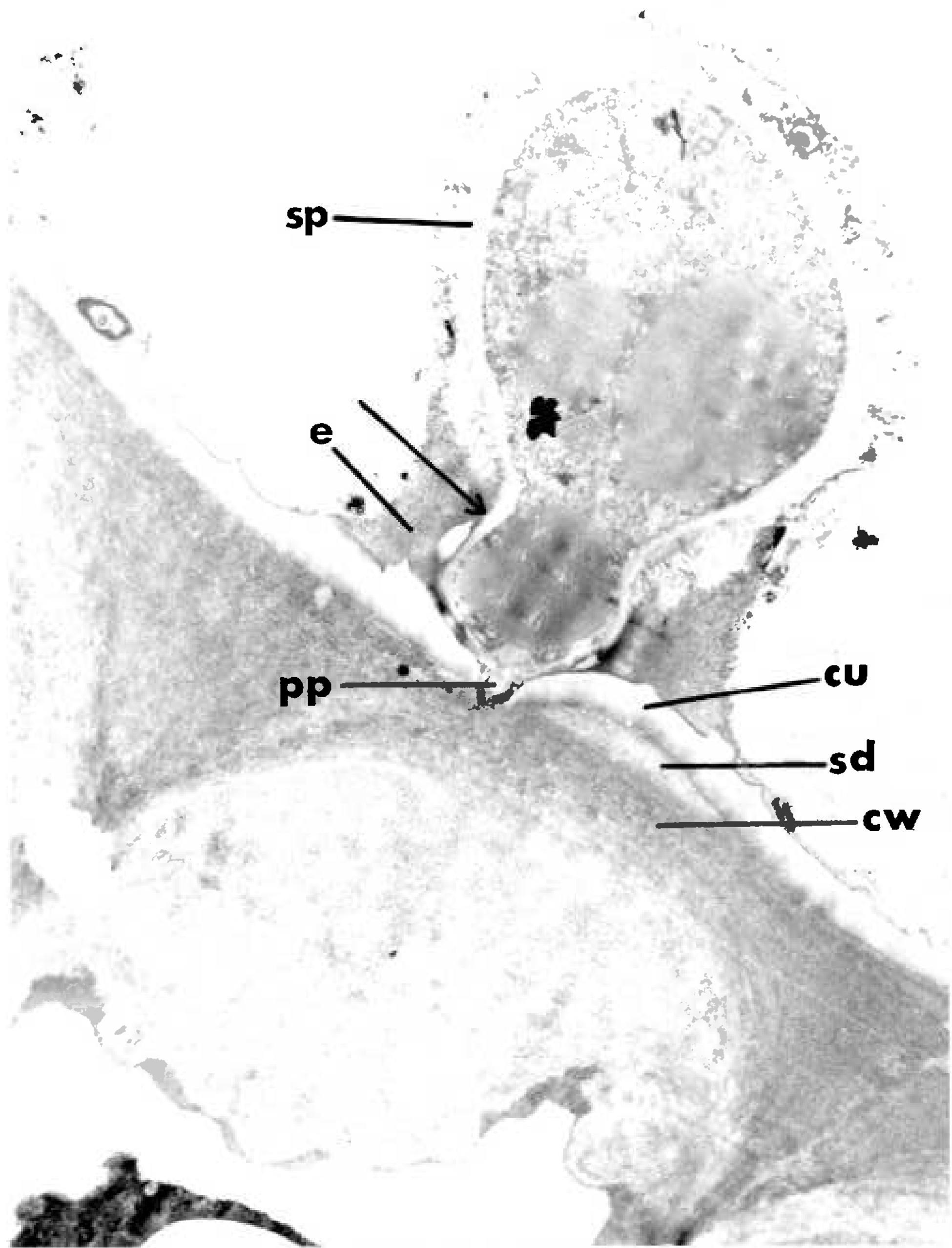
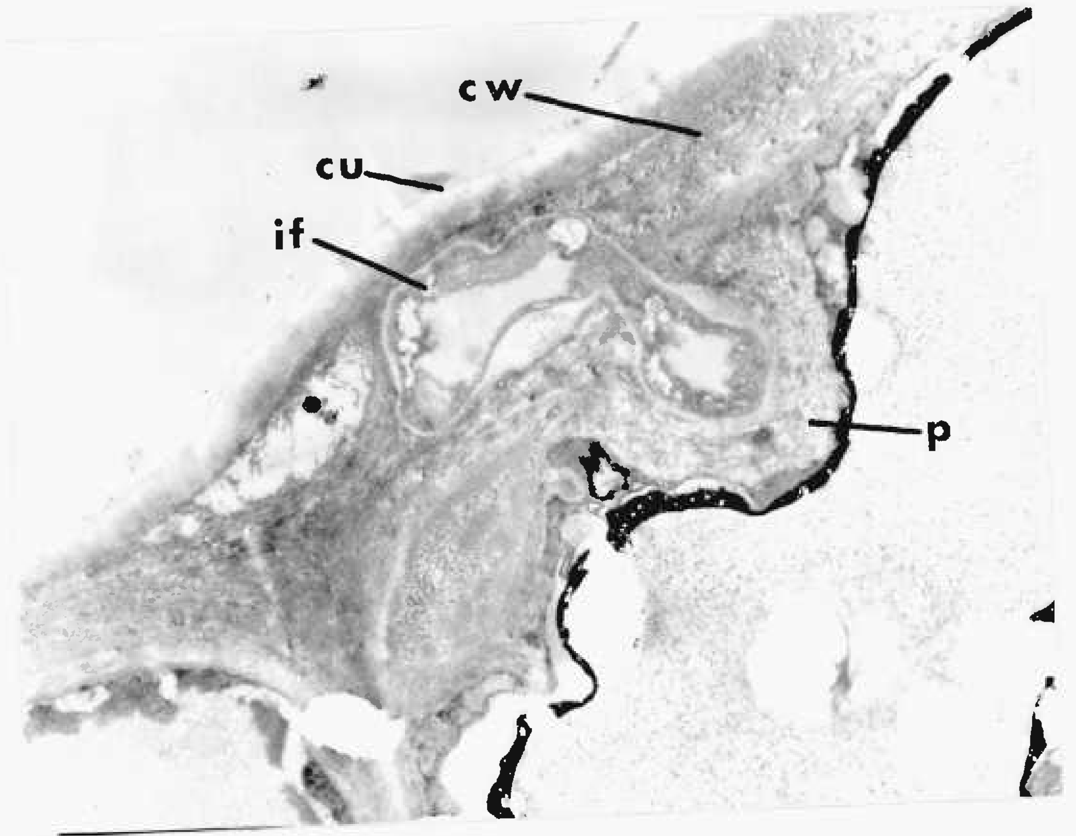

sp
e
pp
cu
sd
cw

PLATE 39 : Sub-cuticular infection hyphae (if) penetrating cell wall (cw) of Frensham leaf. Note displacement of cuticle (cu) and papilla (p) around penetrating infection hyphae. (X6064)

PLATE 40 : Section through the junction of two epidermal (ec) and a palisade cell (pc) of Frensham leaf. Note the intercellular hyphae (ih) between epidermal and palisade cells. (X5215)


cw
cu
if
p

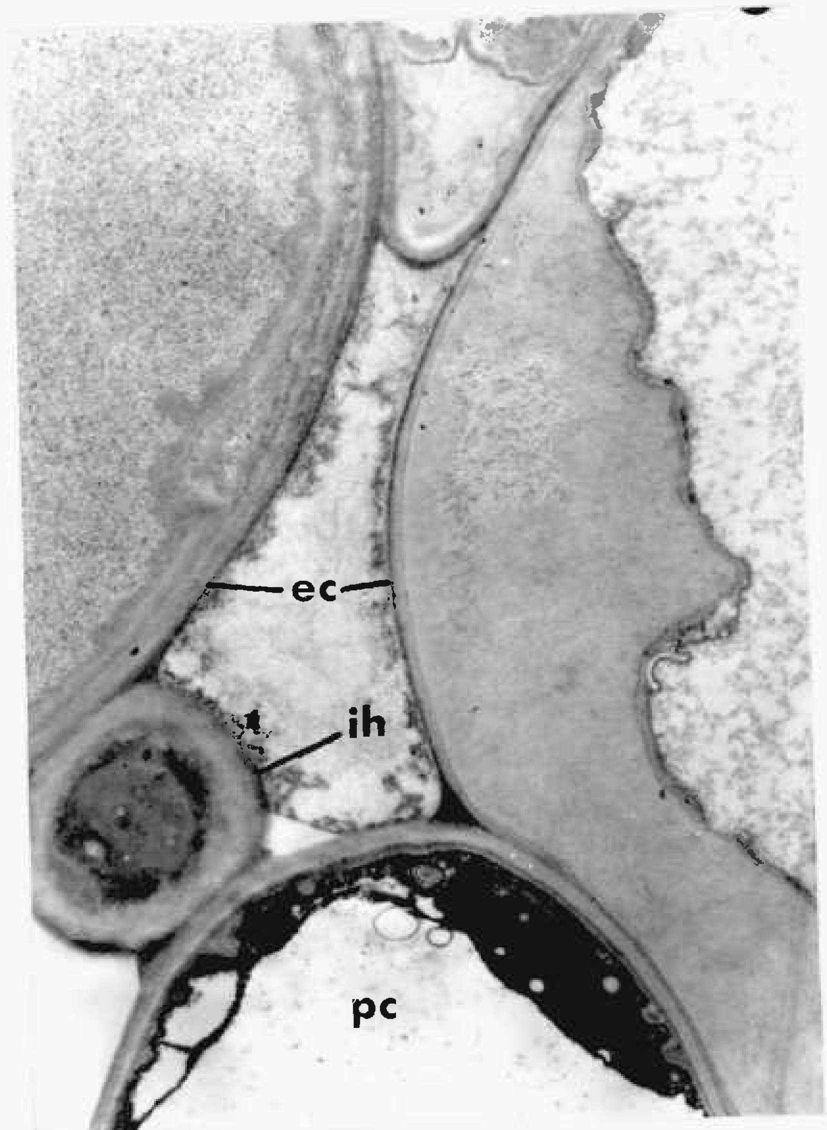

ec
ih
pc

PLATE 41 : Sub-cuticular runner hyphae (rh) on Frensham leaf. Note the cuticle (arrowed) and dark staining areas (sa) within the cell wall (cw). (X15632)

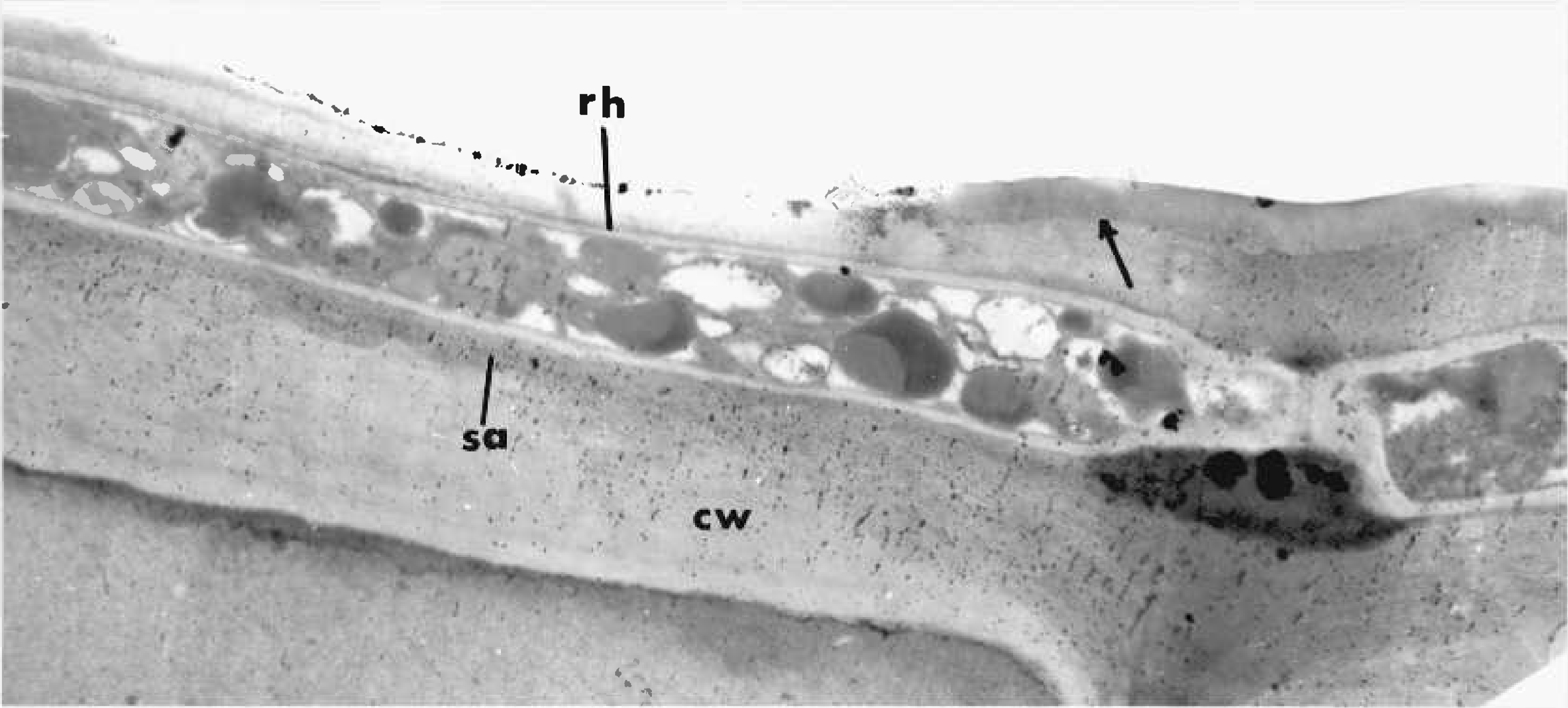

rh
sa
cw

PLATE 42 : Superficial hyphae (arrowed) on Frensham leaf with penetration of epidermal cell wall (cw). Note the dark staining areas (sa) in the cell wall associated with the penetration hyphae (ph). An intracellular hyphae (ia) forms below the cell wall in the cell lumen. (X28880)

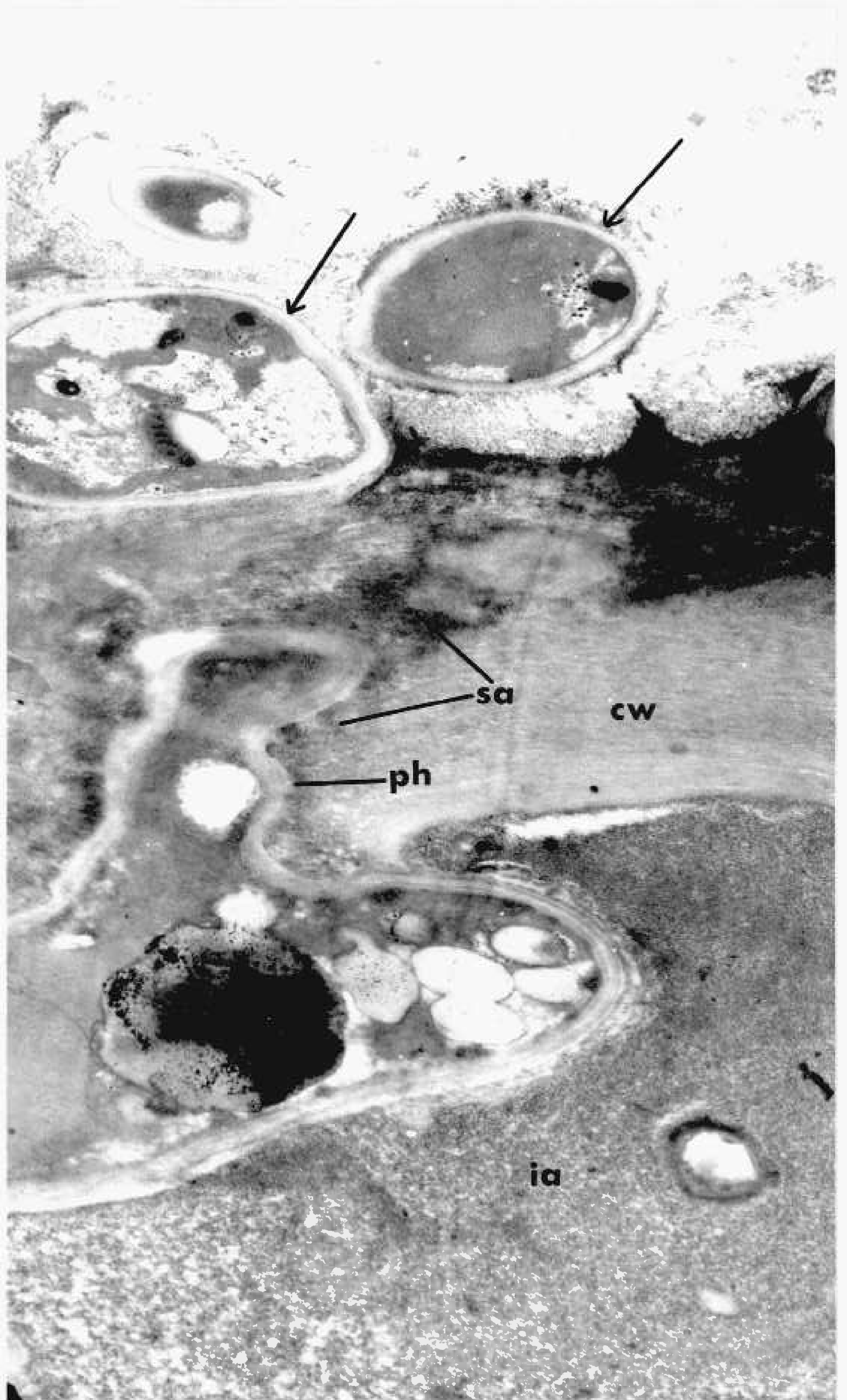
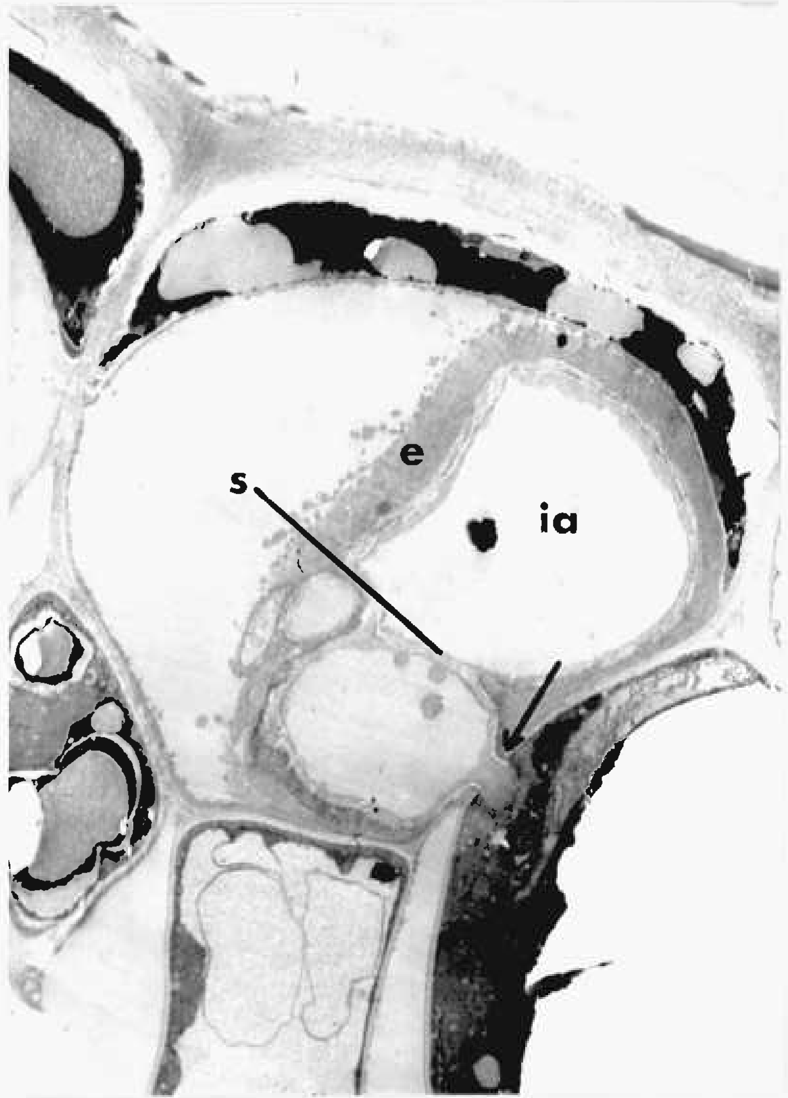

sa
cw
ph
ia

PLATE 43 : Intracellular hyphae (ia) in epidermal cell of Frensham leaf. Note the septum (s), the electron dense material (e) that surrounds the intracellular hyphae and penetration (arrowed) into adjacent palisade cell. (X2420)

PLATE 44 : Intracellular haustorial mother cell (hm) in an epidermal cell of Frensham leaf, which adheres to the wall and has penetrations into two adjacent palisade cells (pc). Note the large penetration hyphae (ph) and the papilla (p) surrounding each one. (X12897)


e
s
ia

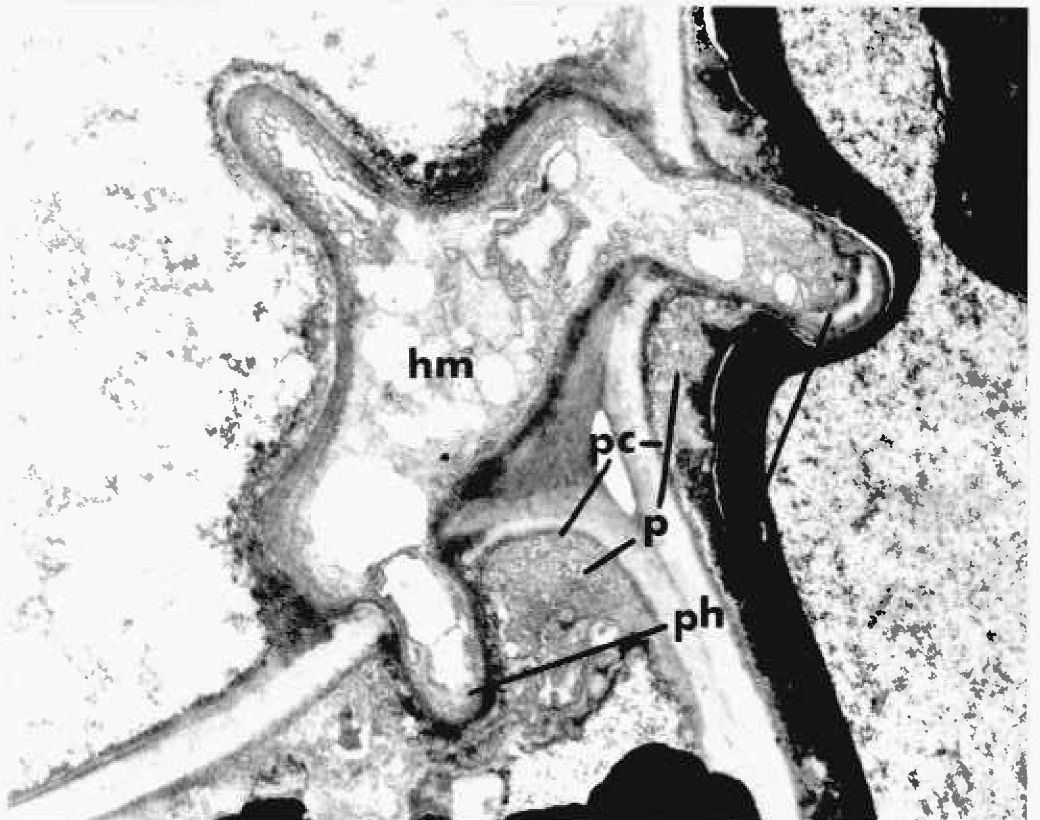
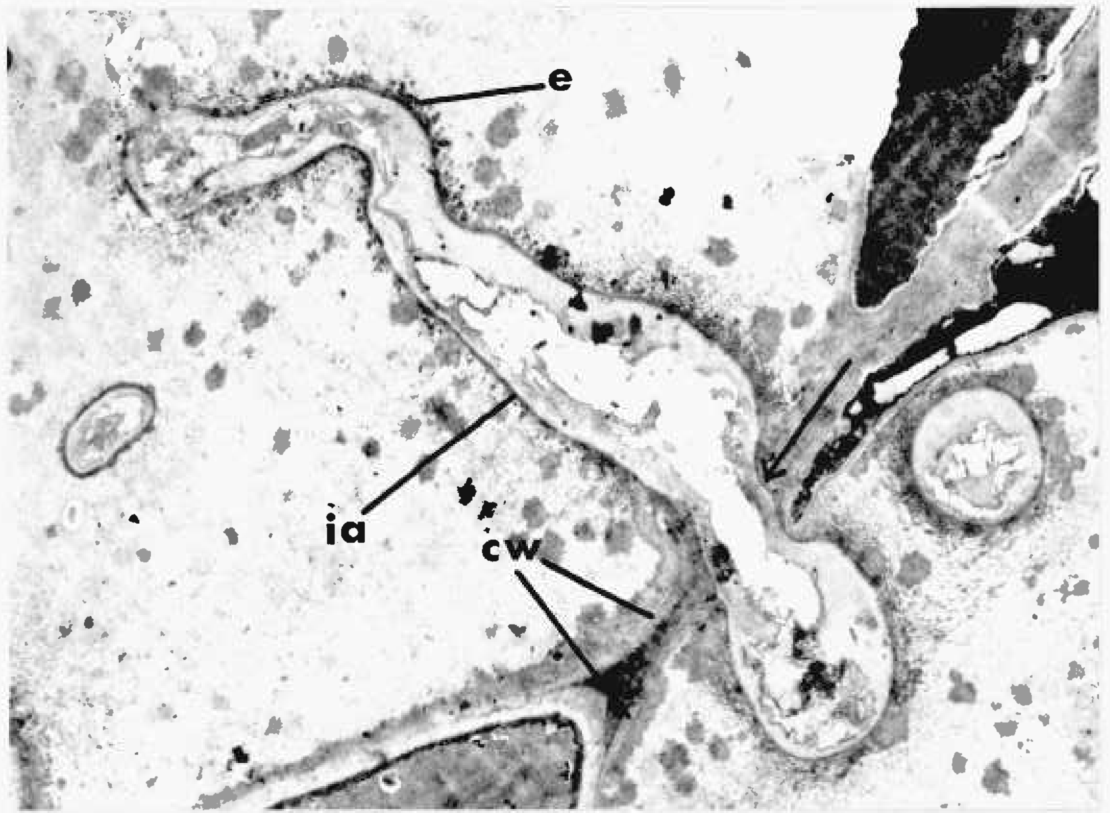
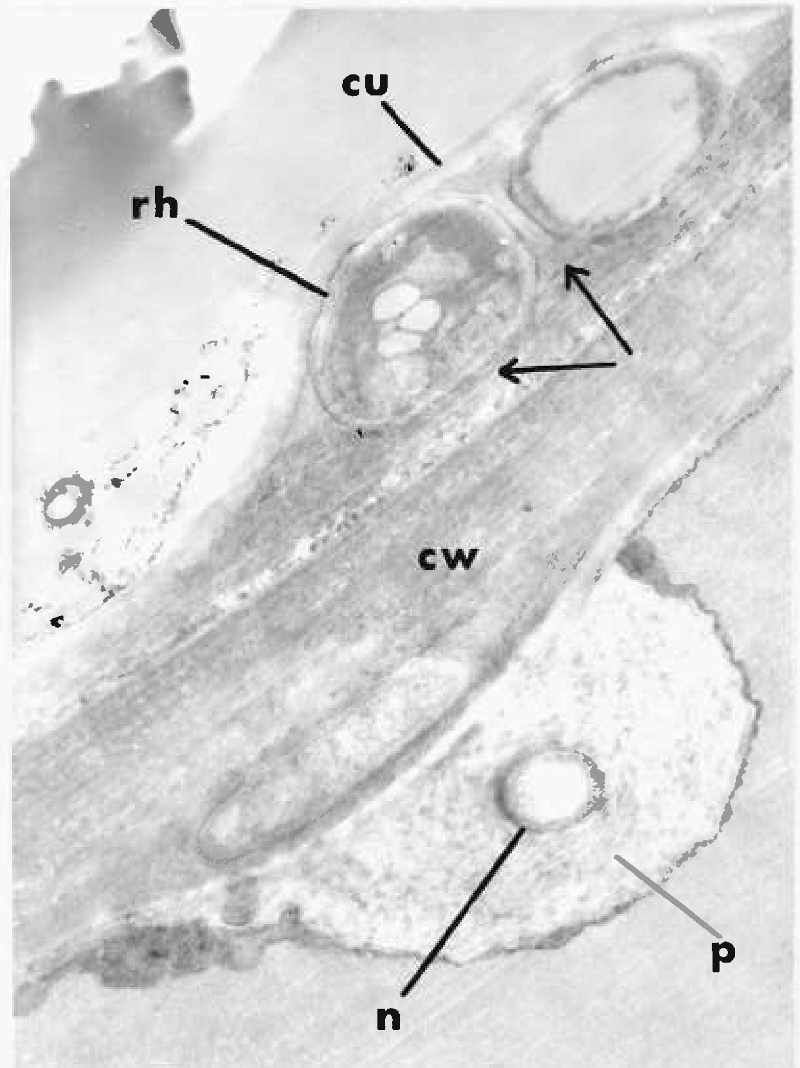

hm
pc
p
ph

PLATE 45 : Intracellular hyphae (ia) passing through two epidermal cells of Frensham leaf. Note the constriction (arrowed) as it passes through the walls (cw). An electron dense material (e) surrounds the intracellular hyphae, except where it penetrates from one cell to the other. (X6064)

PLATE 46 : Sub-cuticular runner hyphae (rh) and papilla (p) on Frensham leaf. Note the two parallel fungal strands beneath the distended cuticle (cu) and the altered fibrillar structure (arrowed) of the cell wall (cw). A papilla surrounds a portion of what is probably the haustorial neck (n). (X10499)



e
ia
cw



cu
rh
cw
p
n

PLATE 47 : Haustorium (hb) in epidermal cell of Frensham leaf. Note the large collar (c) and the broken extrahaustorial membrane (eh - vacuolar membrane and/or host plasmalemma). (X15632)

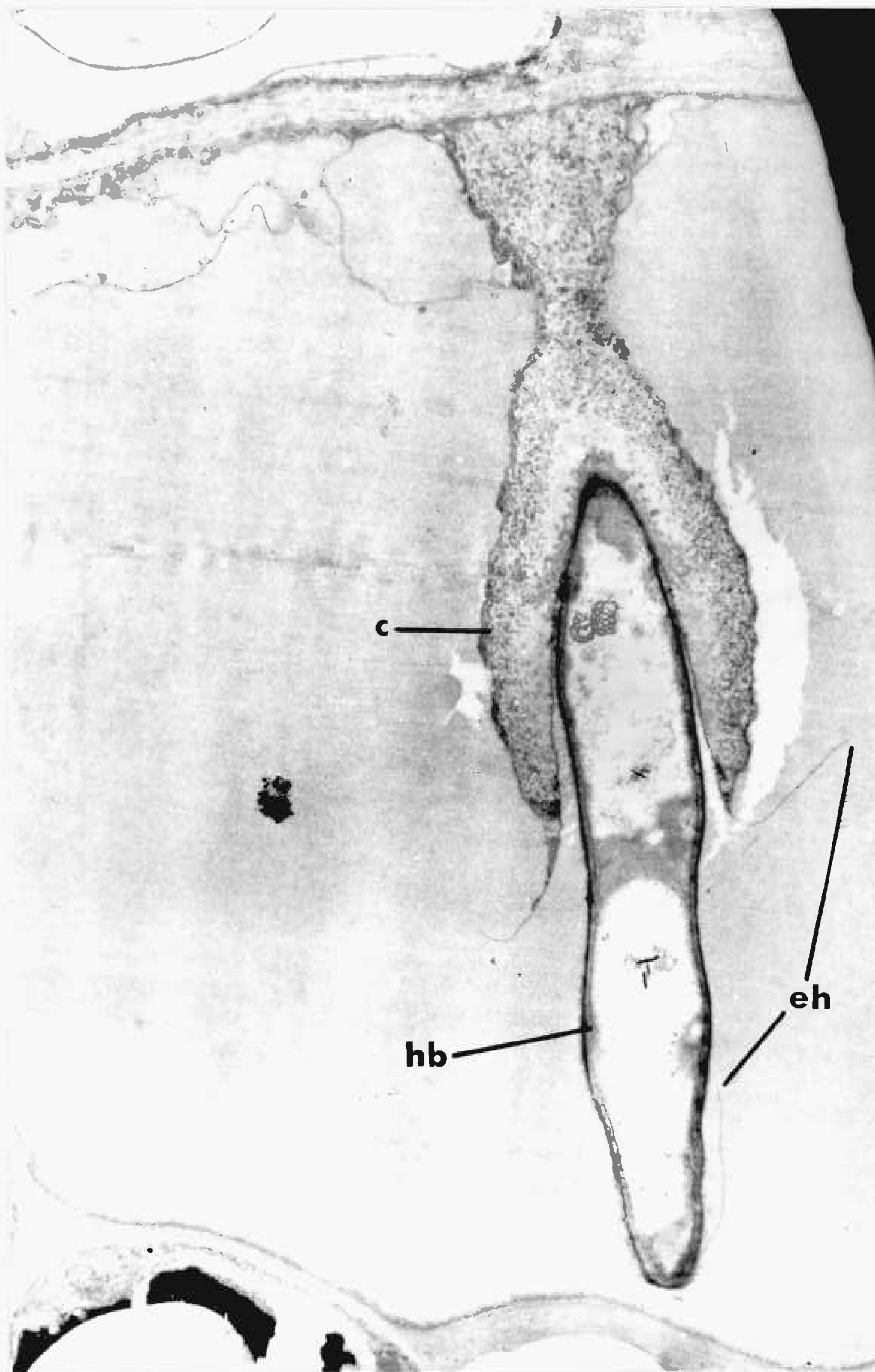

c
hb
eh

PLATE 48 : T.S. through epidermal cell of Frensham leaf, in which a haustorial neck (n) is seen embedded in collar (arrowed) material. Note the darkly staining particles (e) immediately around the haustorial neck. (X63600)

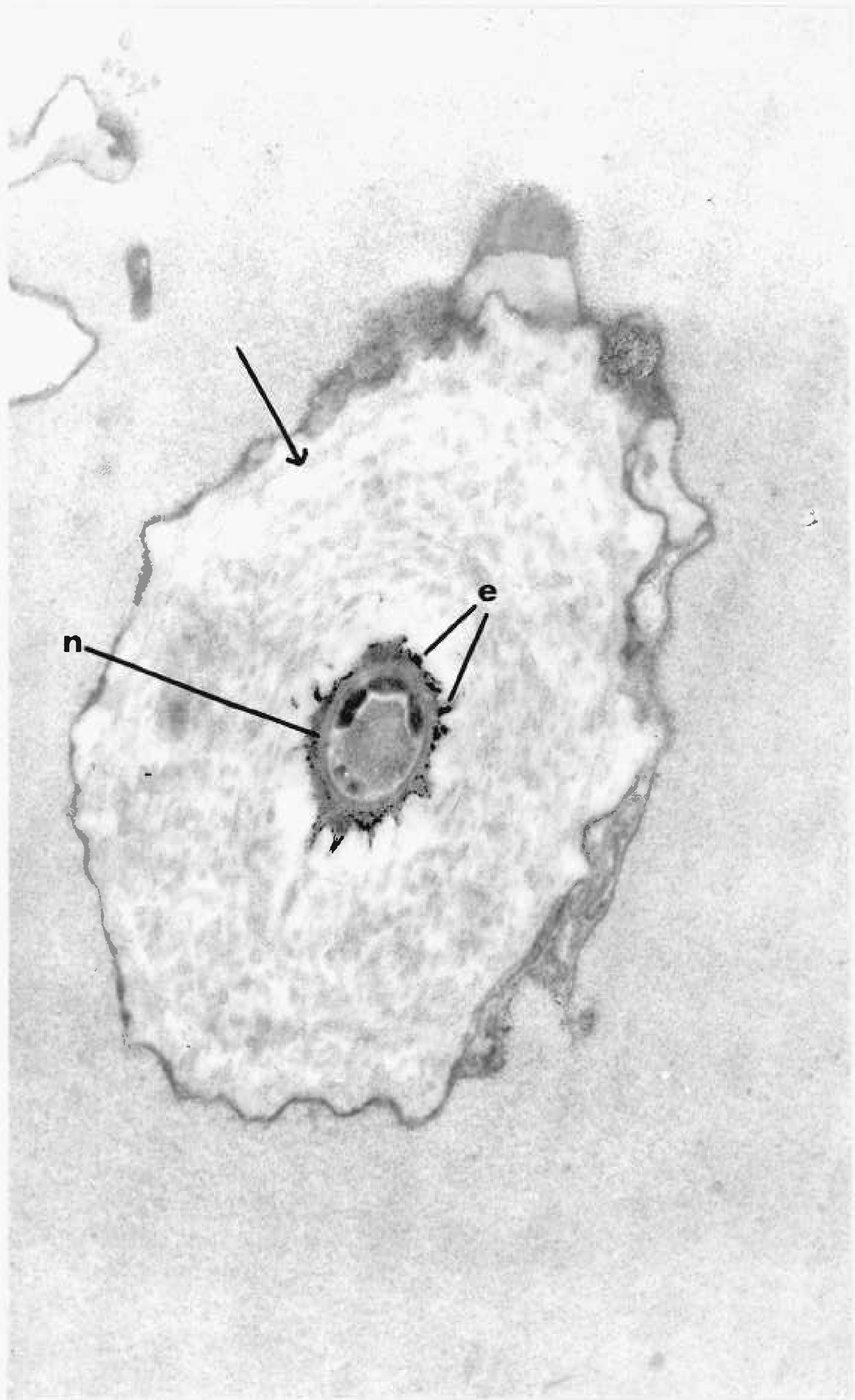

e
n

PLATE 49 : Intracellular hyphae (ia) in epidermal cell of Frensham leaf. Note the electron dense material (e) around the intracellular hyphae and the lack of constriction (arrowed) where it penetrates into the palisade cell (pc). (X24256)

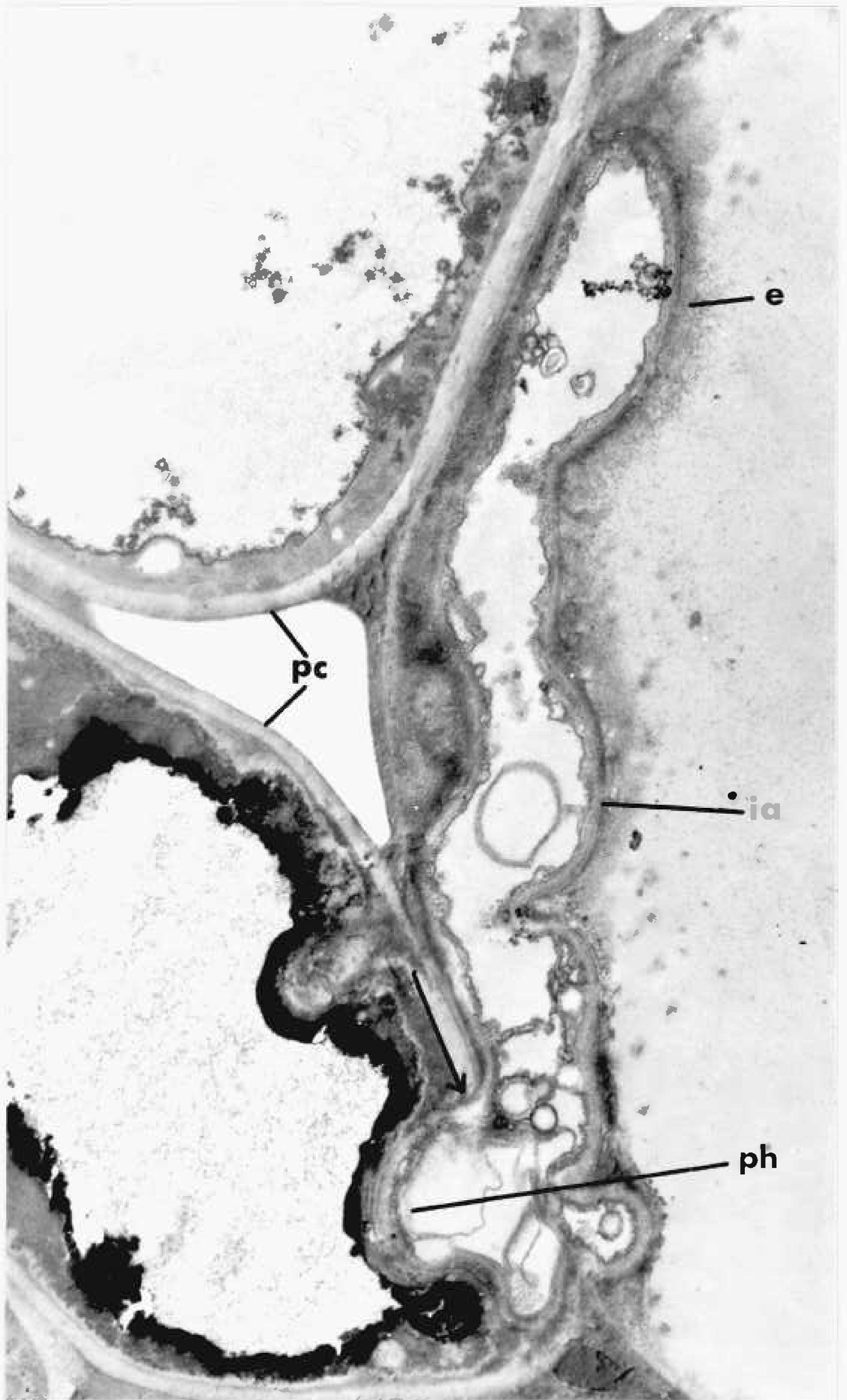

e
pc
ia
ph

PLATE 50 : Intracellular hypha (ia) passing from an epidermal to a palisade cell (pc) of Frensham leaf. Note the slight constriction at the site of penetration (arrowed) and the dark staining areas (sa). A papilla (p) surrounds the penetration hypha. (X28880)

PLATE 51 : Intracellular haustorial mother cell (hm) surrounded by electron dense material (e) in epidermal cell of Frensham leaf penetrating into the palisade cell (pc). There is no constriction of the penetration hypha (arrowed) at the site of penetration but the fungal cell wall (fw) is thinner at this point and the penetrated area stains darkly (sa). A haustorial neck septum (s) is seen close to the tip of the penetration hypha which is enclosed by a papilla (p). (X8604)

ia
sa
p
pc

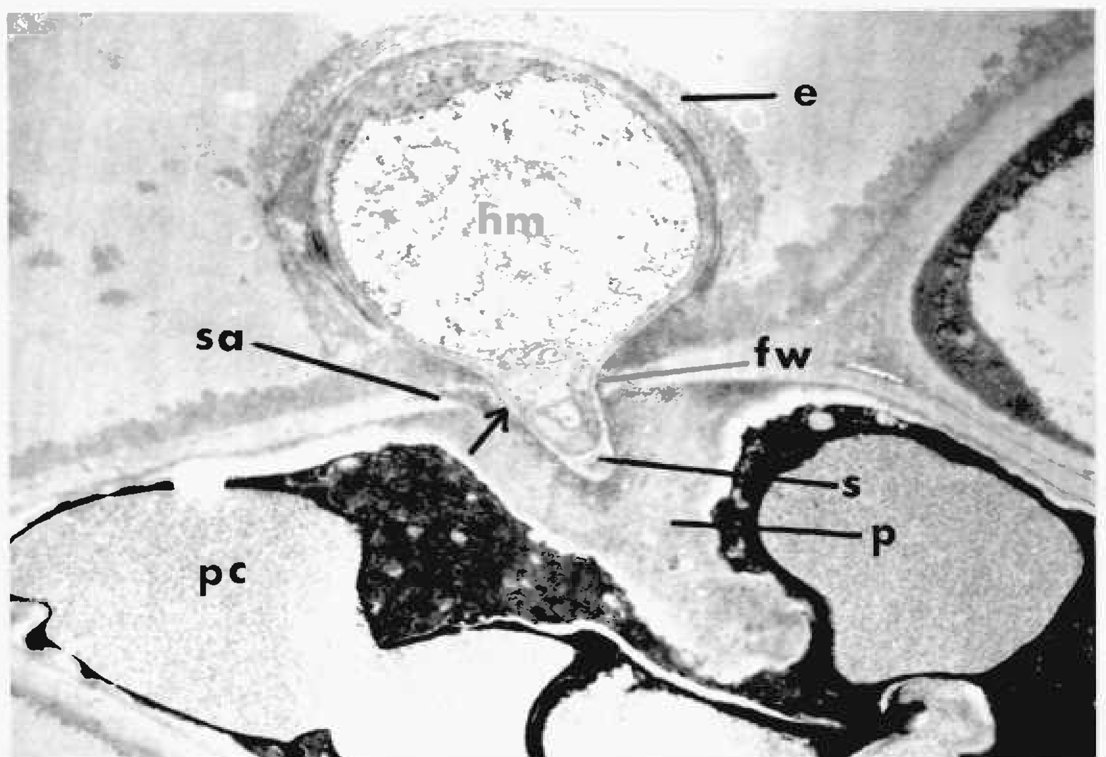
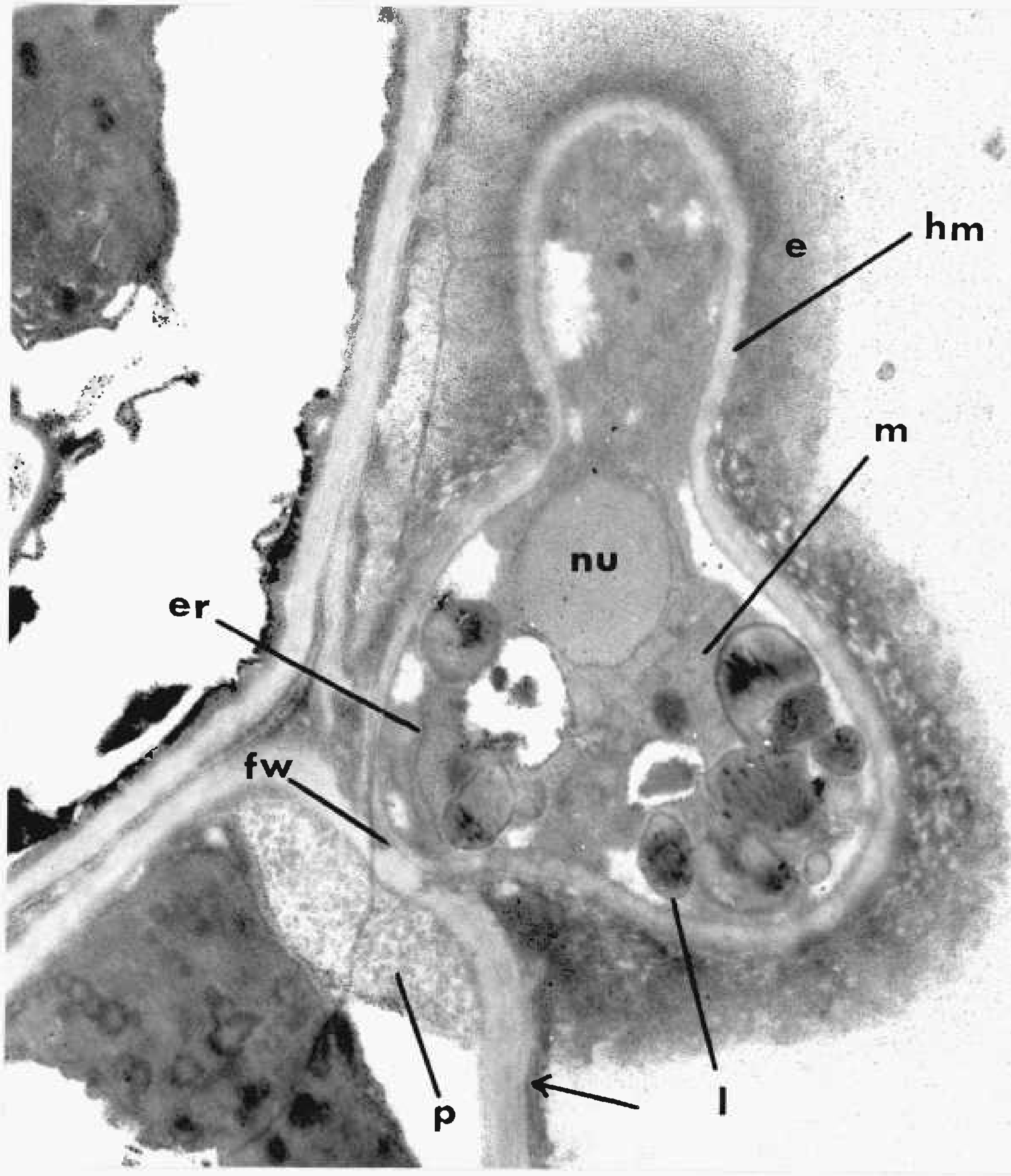


PLATE 52 : Intracellular haustorial mother cell (hm) in epidermal cell of Frensham leaf at early stage of penetration into palisade cell (arrowed). Note the papilla (p) on the palisade cell wall beneath the site of penetration. The haustorial mother cell is encased in an electron dense material (e) and contains a nucleus (nu), mitochondria (m), lipid bodies (l), endoplasmic reticulum (er), and the fungal wall (fw) is thinner at the penetration site. (X36416)



hm
e
m
nu
er
fw
p
l

<u>PLATE 53</u> : Intracellular haustorial mother cell (hm) in an epidermal cell of Frensham leaf giving rise to a haustorium (hb) inside a palisade cell. Note the haustorial neck septum (s) and the collar (c). The haustorium is enclosed in an extrahaustorial matrix (em) bounded by a membrane possibly of vacuolar and/or host plasmalemma origin (arrowed). The adjacent cell is in the process of being penetrated. (X20860)

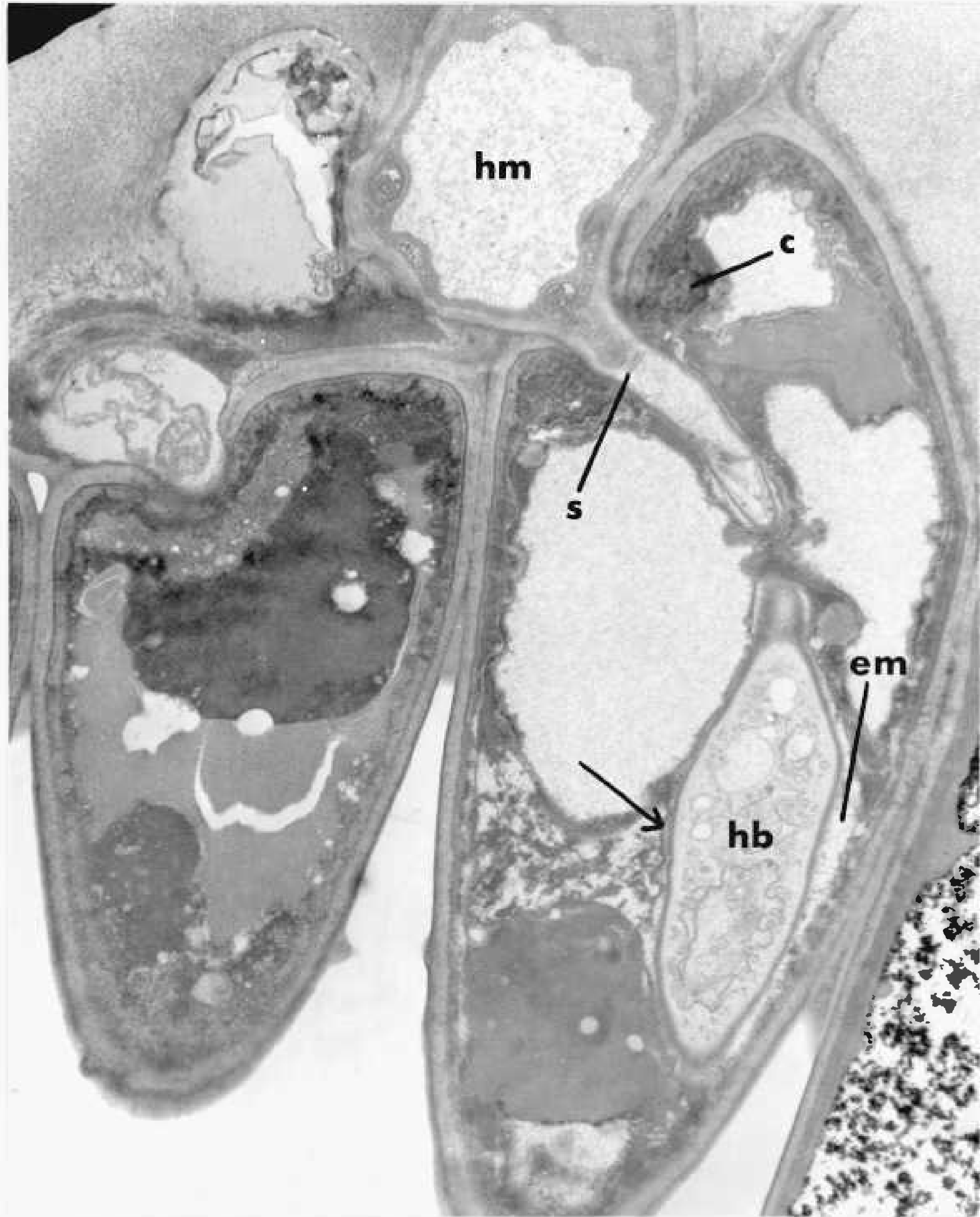

hm
c
s
em
hb

PLATE 54 : Intracellular haustorial mother cell (hm) giving rise to a haustorium (hb) in palisade cell of Frensham leaf. Note the haustorial neck septum (s) and collar (c), fungal plasmalemma (fp), and an extrahaustorial matrix (em) surrounded by membranes (arrowed) possibly of vacuolar and/or host plasmalemma origin. (X28880)

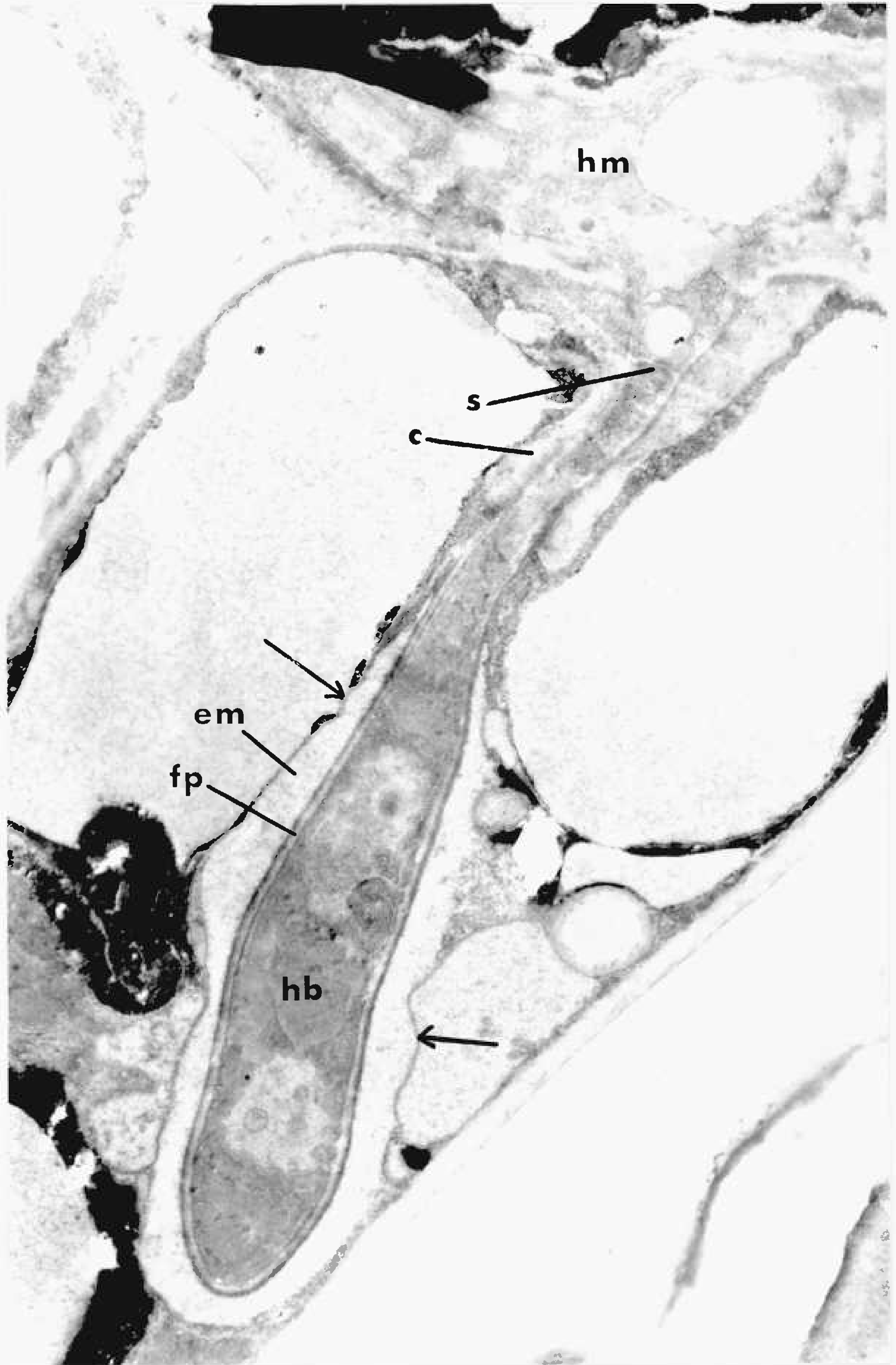

hm
s
c
em
fp
hb

PLATE 55 : Haustorium (hb) in palisade cell of Frensham leaf. Note fungal plasmalemma (fp), the extrahaustorial membrane (host plasmalemma and/or vacuolar membrane - arrowed) around the haustorium and the small collar (c) but the absence of any extrahaustorial matrix. Also note the papilla (p) in adjacent cell. (X28880)

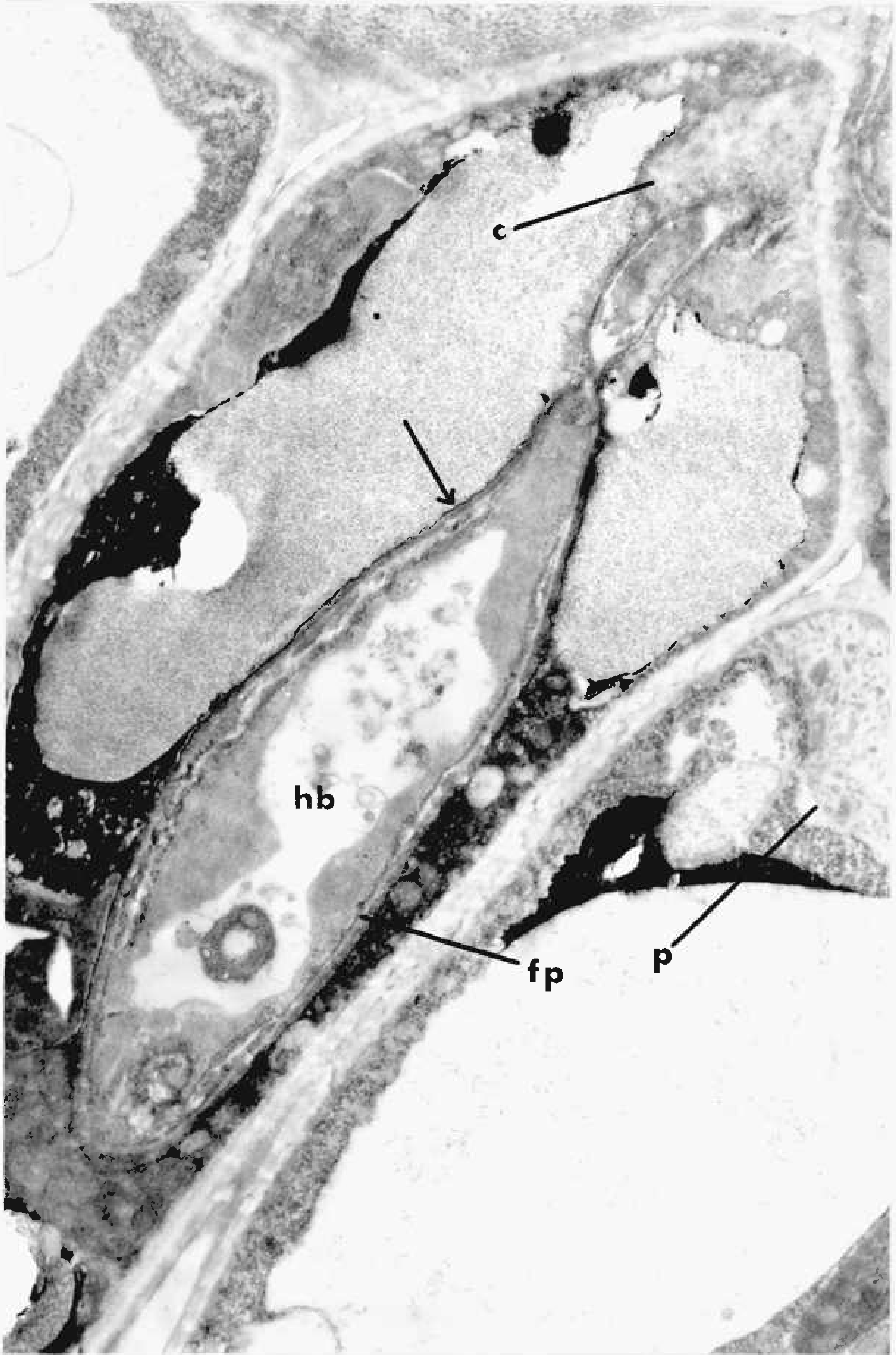
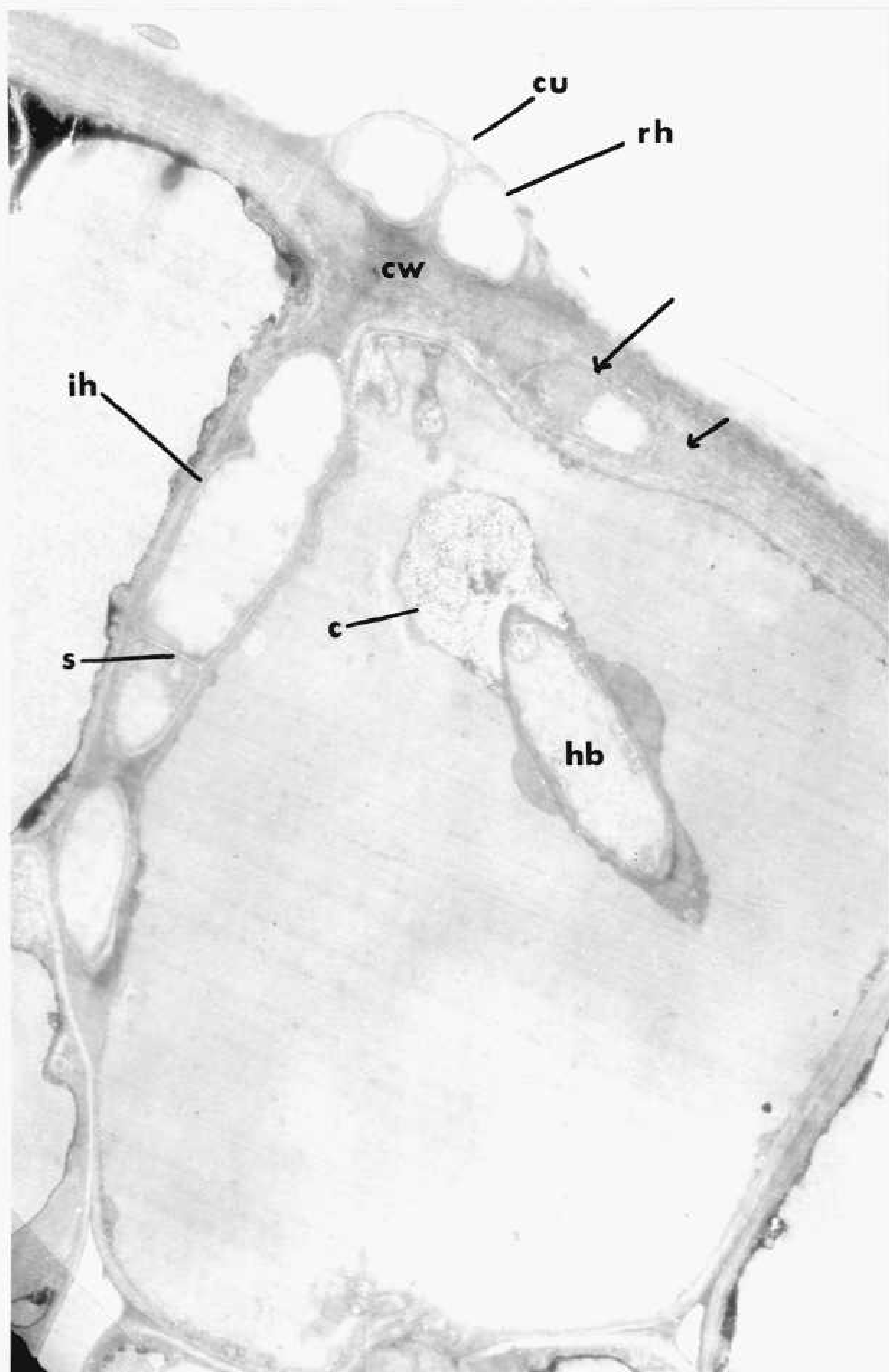
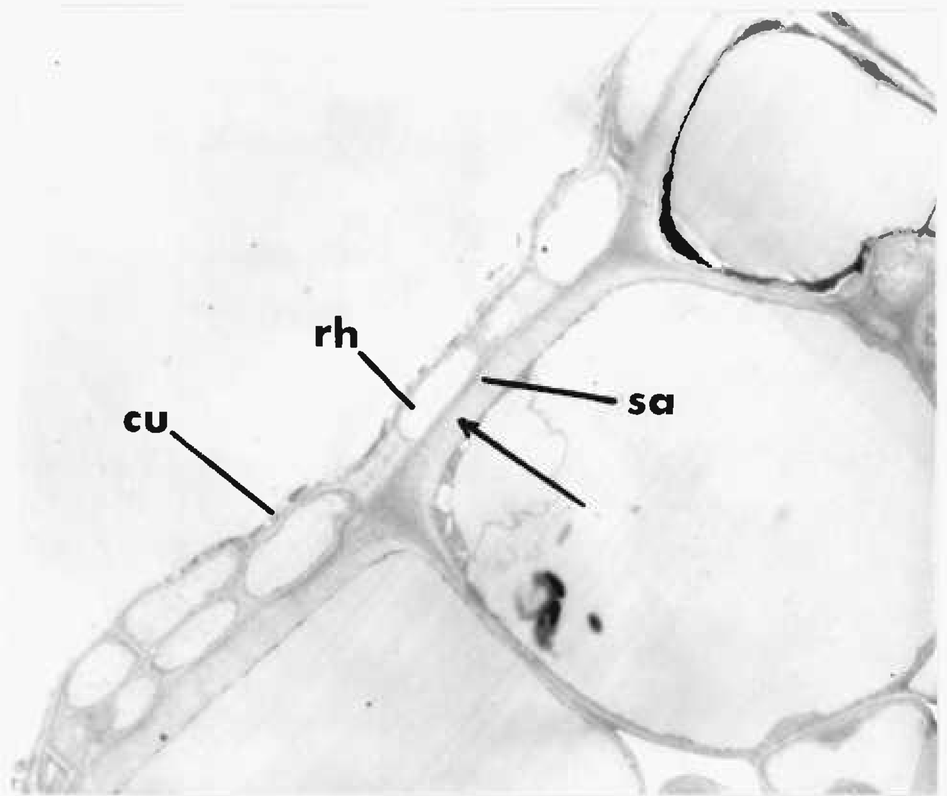

c
hb
fp
p

PLATE 56 : Intercellular hypha (ih) with a septum (s) between two epidermal cells of Allgold leaf (susceptible reaction). Note also the subcuticular hyphae (rh) and the distended cuticle (cu), hyphae (long arrow) within the epidermal cell wall (cw), haustorium (hb), and collar (c). Changes in the cell wall associated with the passage of hyphae are indicated by short arrows. (X9680)


cu
rh
cw
ih
c
s
hb

<u>PLATE 57</u> : Sub-cuticular hyphae (rh) in Allgold leaf (susceptible reaction). Note the displaced cuticle (cu), and the dark staining areas (sa) of cell wall (arrowed) near the sub-cuticular hyphae. (X1573)

<u>PLATE 58</u> : Sub-cuticular hyphae (rh) penetrating into epidermal cell of Allgold leaf (susceptible reaction). Note the side branch (arrowed) just above site of penetration and the crystal (k) at its base. The penetrating hypha (ph) is surrounded by a collar (c) and there is a haustorial neck septum (s) near its tip. Note also part of the haustorium (hb) with extrahaustorial matrix (em) bounded by a membrane (eh) possibly of vacuolar and/or host plasmalemma origin. (X20860)


rh
sa
cu

rh
k
c
s
ph
em
hb
eh

PLATE 59 : Sub-cuticular hyphae (rh) penetrating into an epidermal cell of Allgold leaf (susceptible reaction). Note the papilla (p) and the electron dense particles (e) adhering to the tip of the penetrating hypha (arrowed). Note also the haustorium (hb) in the same cell. (X15632)

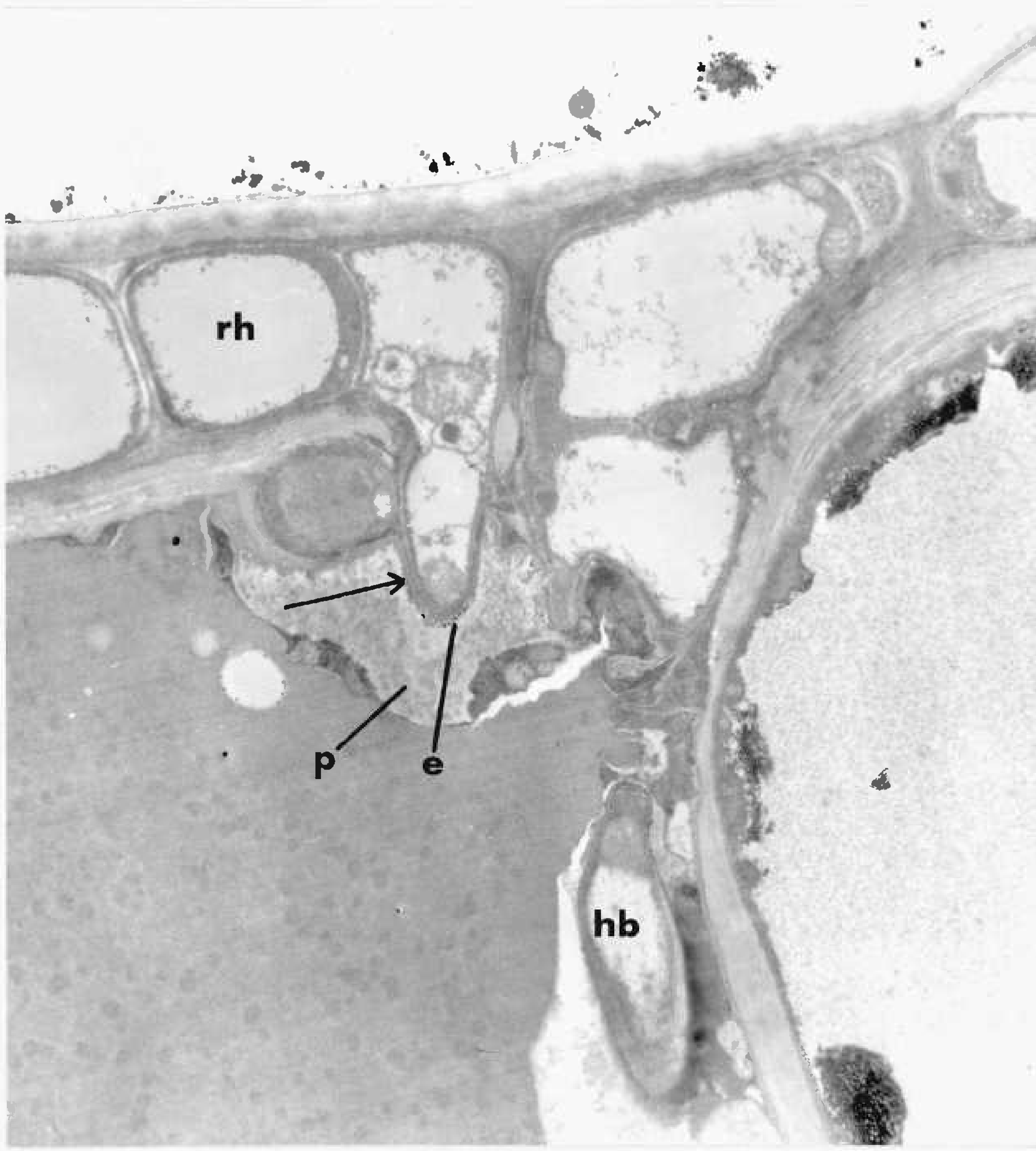

rh
p
e
hb

PLATE 60 : Papilla (p) formation in an epidermal cell of Allgold leaf (susceptible reaction). Note the electron dense particles (e) at base of papilla, sub-cuticular hyphae (rh) and hyphae (arrowed) in cell wall (cw) near papilla. (X15632)

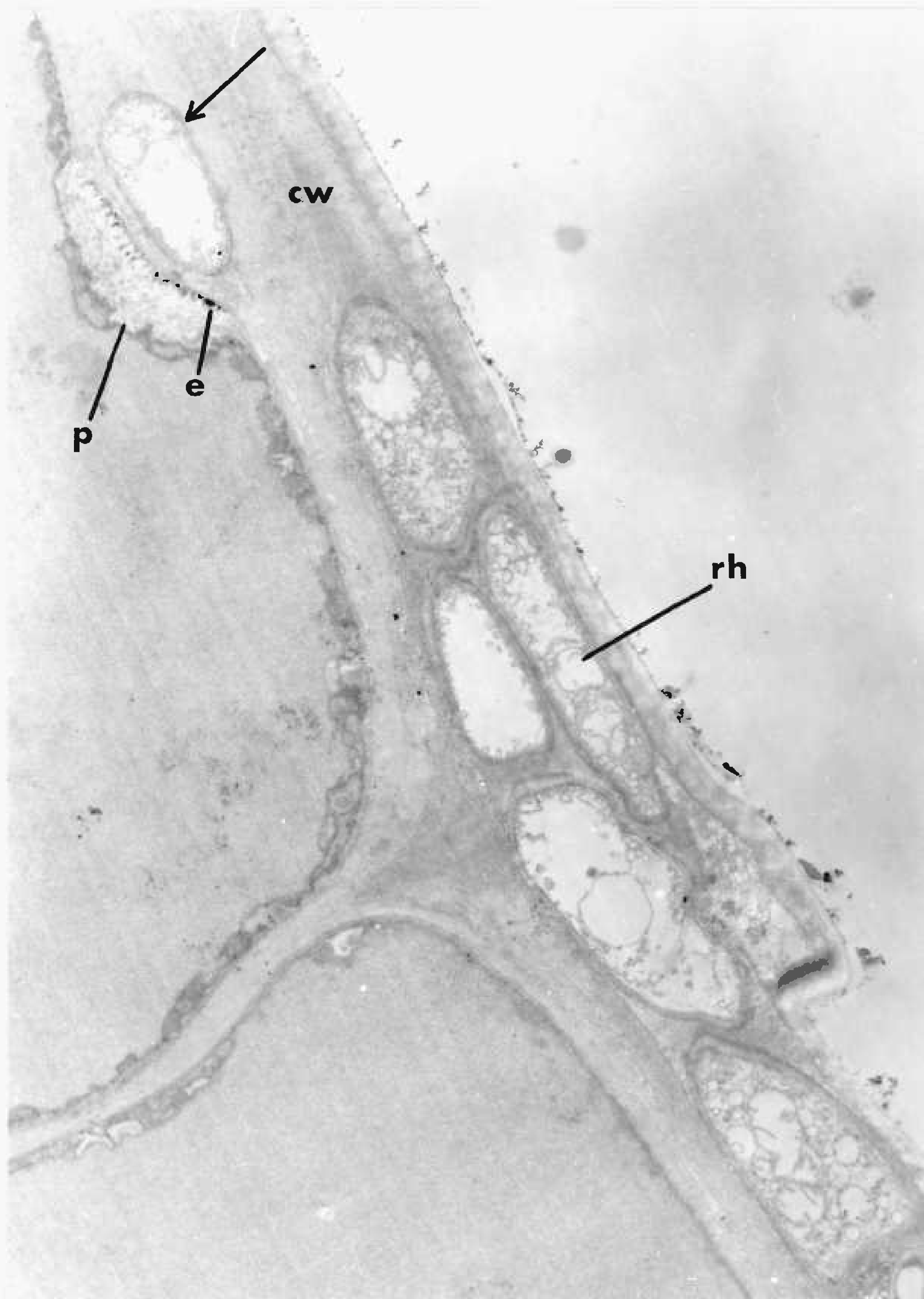
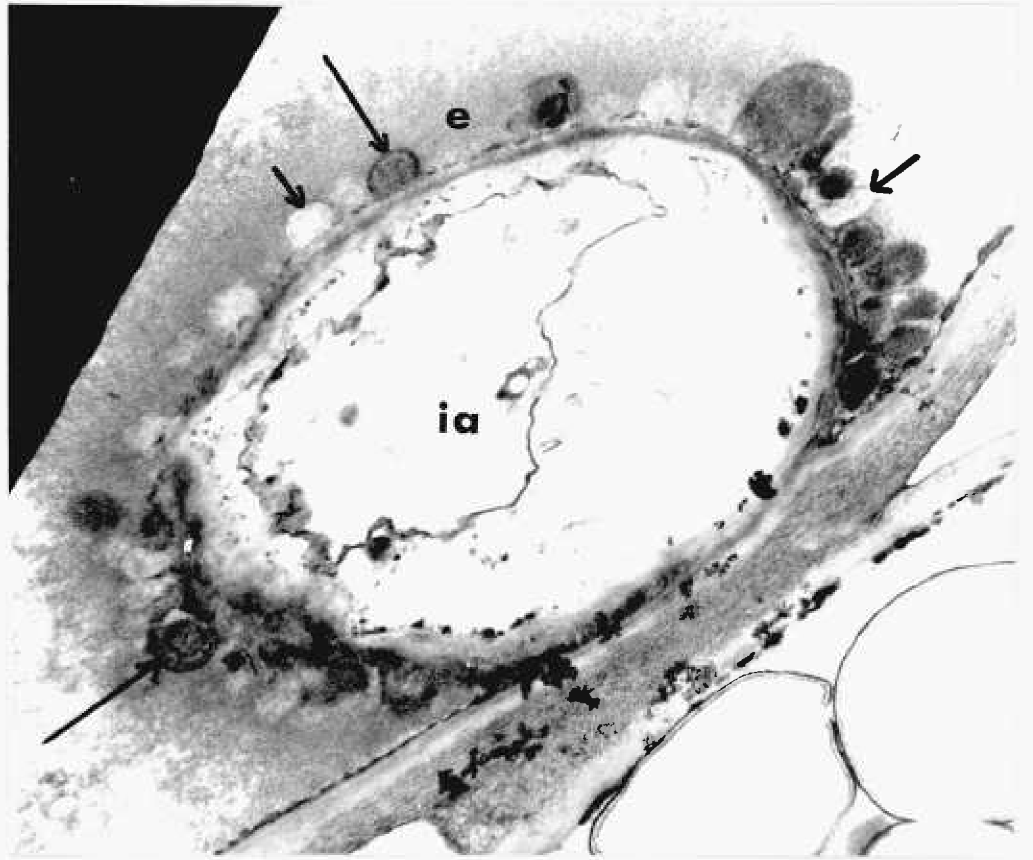

cw
e
p
rh

PLATE 61 : Intracellular hypha (ia) in epidermal cell of Allgold leaf (susceptible reaction). The hypha is surrounded by an amorphous electron dense material (e) in which are embedded electron dense (long arrows) and electron lucent particles (short arrows) which adhere closely to it. (X20860)

PLATE 62 : Haustorium (hb) with collar (c) in epidermal cell of Allgold leaf (susceptible reaction). Note the haustorial neck (n), haustorial neck septum (s), the electron dense material (e) in the lower half of the haustorial neck and the extrahaustorial matrix (arrowed) which is bounded by a membrane (eh) of vacuolar and/or host plasmalemma origin. (X15632)



s
c
n
e
eh
hb

PLATE 63 : Haustorium (hb) with collar (c) in an epidermal cell of Allgold leaf (susceptible reaction). The extrahaustorial membrane (vacuolar and/or host plasmalemma - arrowed) seems to have broken. Note the subcuticular hyphae (rh) and distended cuticle (cu). (X15632)

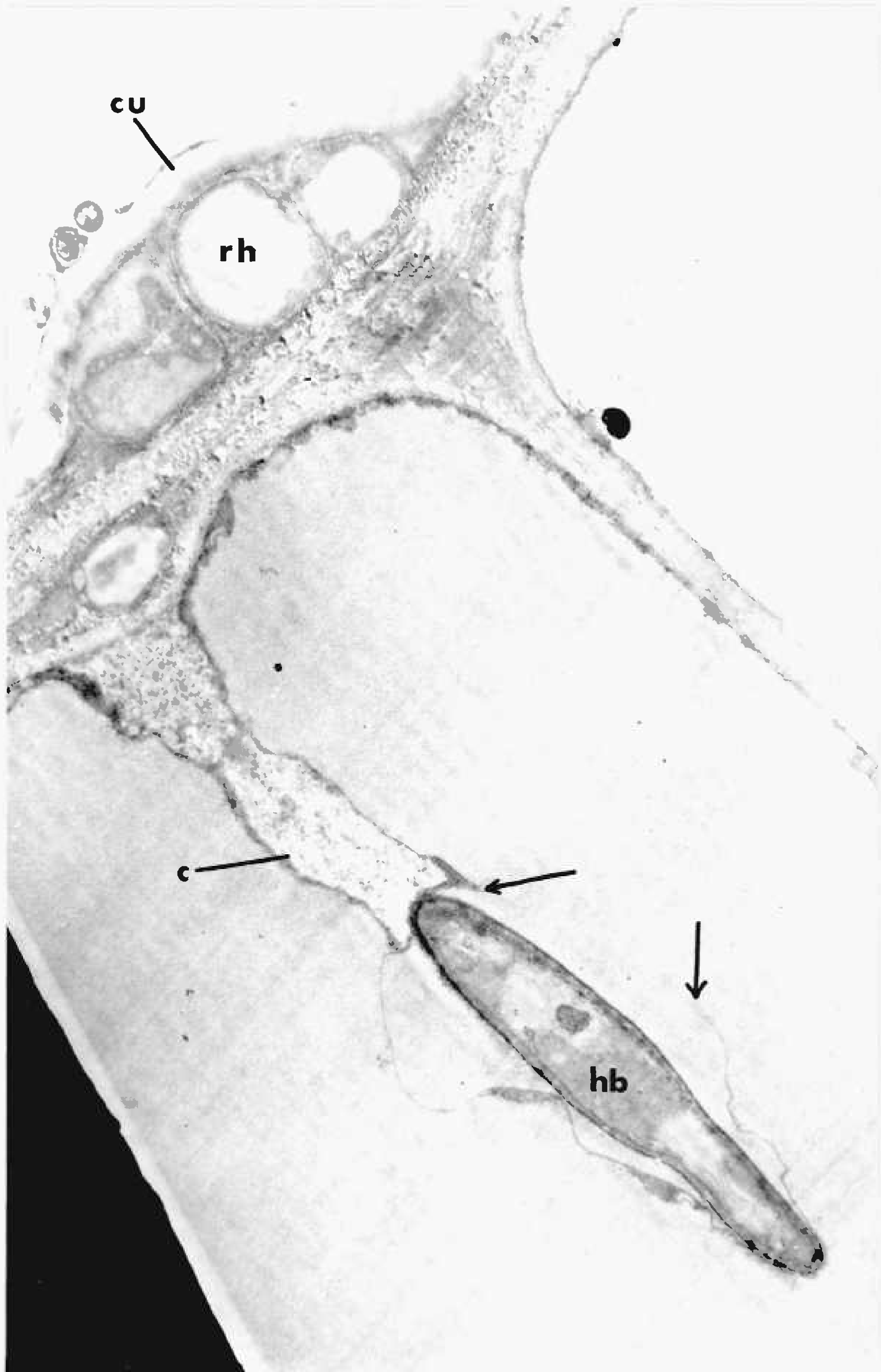

cu
rh
c
hb

PLATE 64 : Intracellular hypha (arrowed) forming a haustorium (hb) inside a palisade cell of Allgold leaf (susceptible reaction). Note the large collar (c) and electron dense material (e) around intracellular hypha. (X20860)

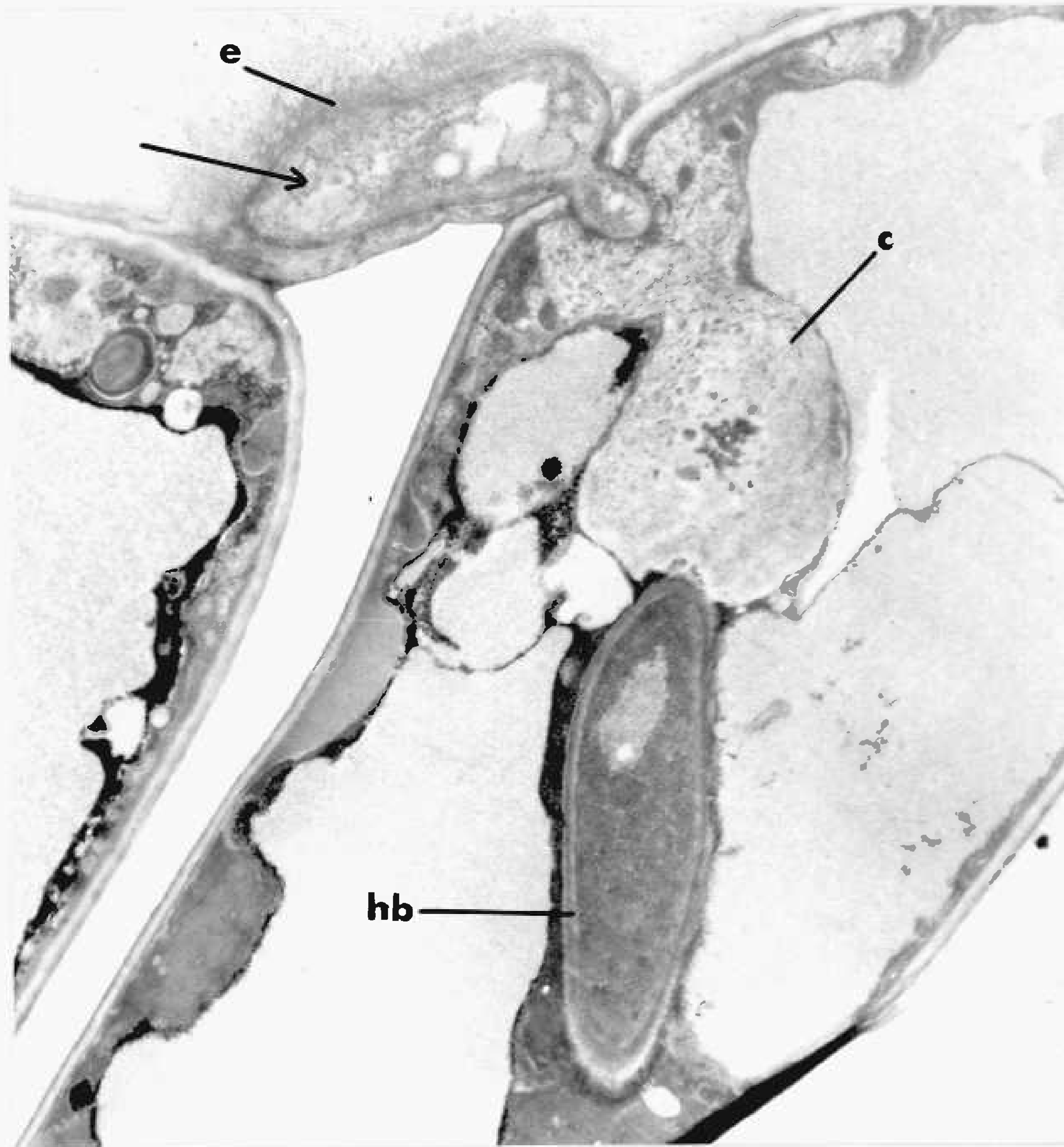
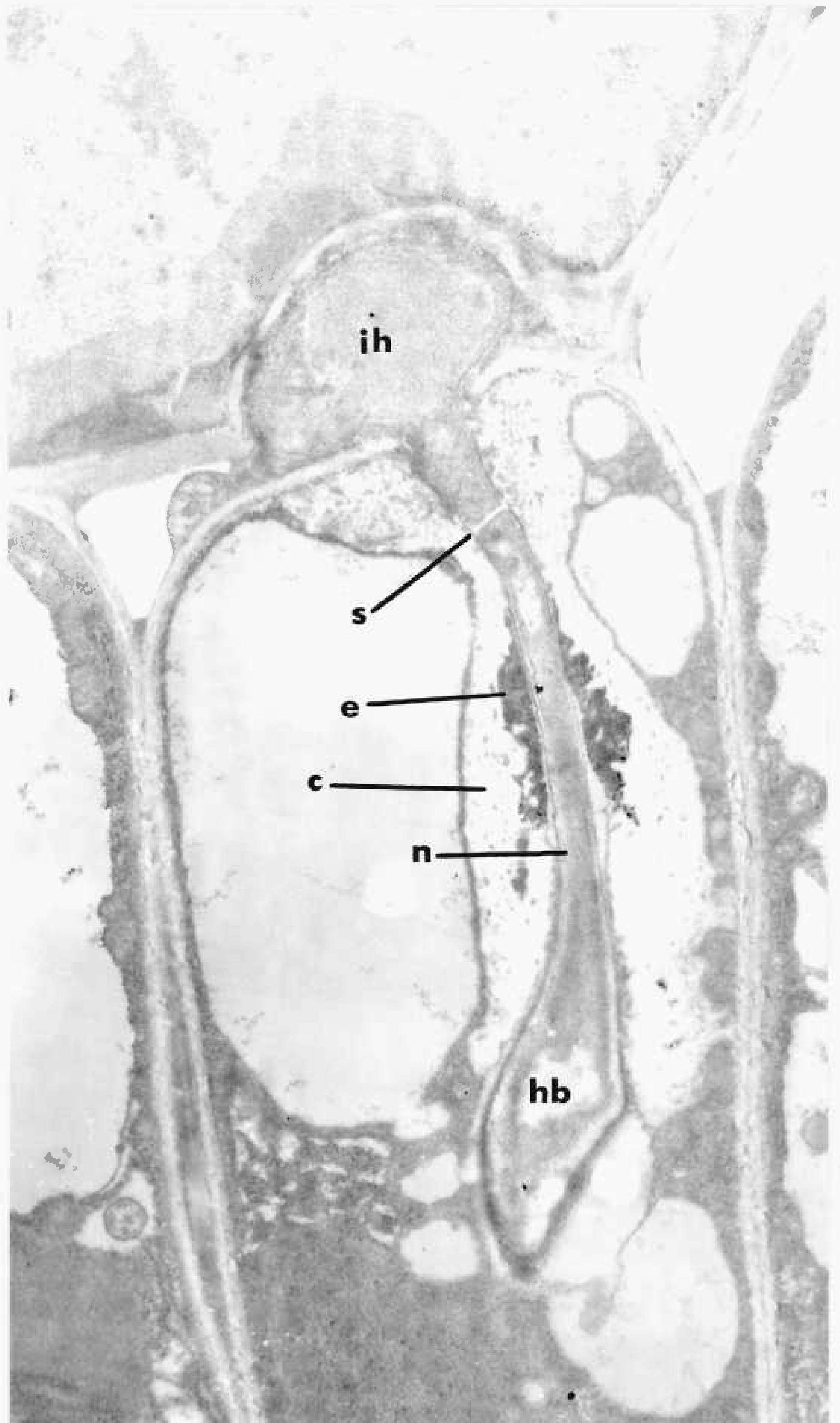

e
c
hb

PLATE 65 : Intercellular hypha (ih) forming a haustorium (hb) inside a palisade cell of Allgold leaf (susceptible reaction). A large collar (c) surrounds the haustorial neck (n). Note the haustorial neck septum (s) and electron dense material (e) around the lower part of the haustorial neck. (X24266)


ih
s
e
c
n
hb

<u>PLATE 66</u> : T.S. through a palisade cell of Allgold leaf (susceptible reaction) showing a part of the haustorial neck (n) containing a fungal plasmalemma (fp). Note the electron dense material (e) around the haustorial neck; collar material and host membranes (arrowed). A chloroplast (cl) adheres closely to the collar. (X51588)

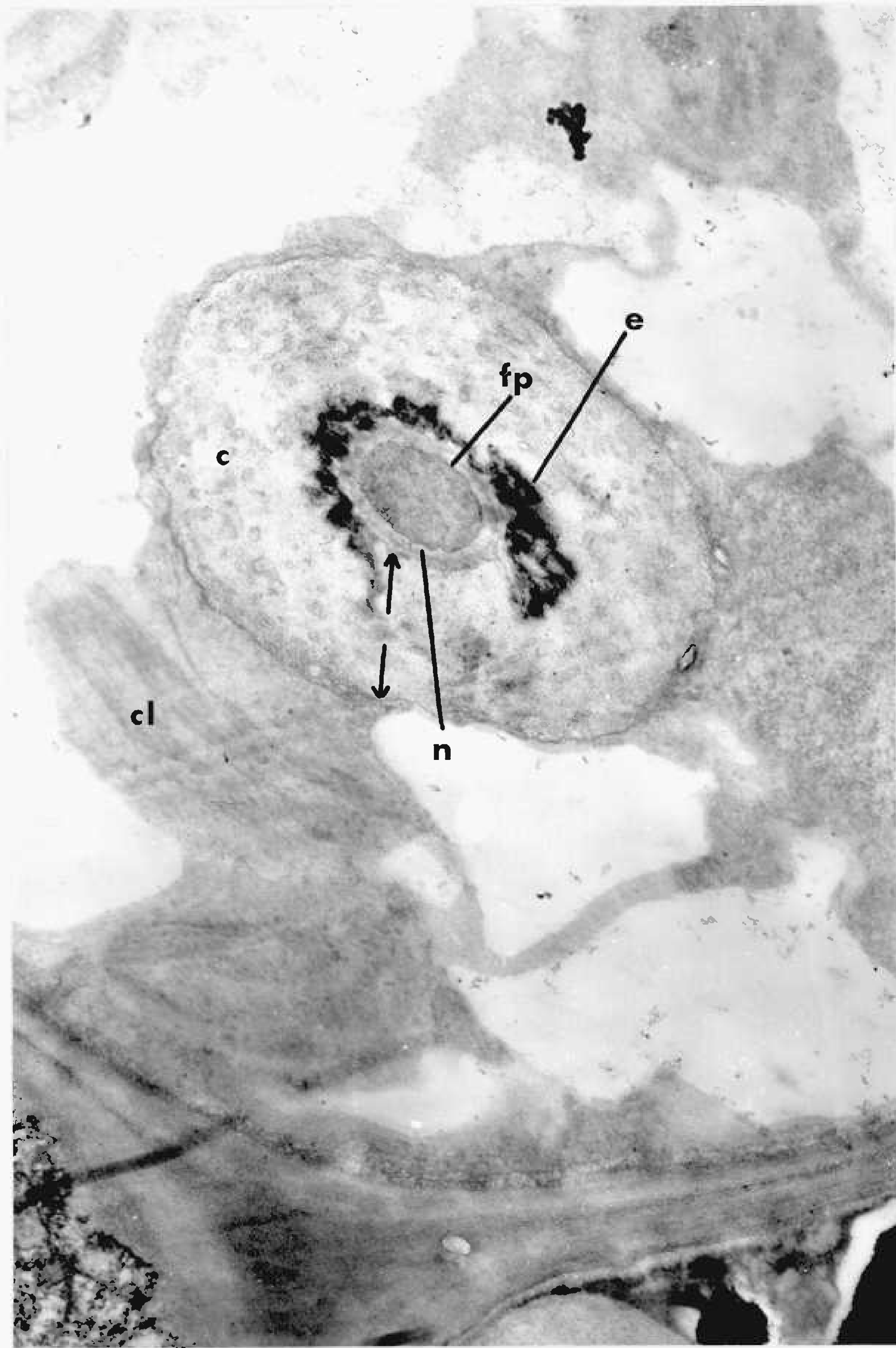

e
fp
c
cl
n

PLATE 67 : A haustorium (hb) with a collar (c) inside a palisade cell of Allgold leaf (susceptible reaction). Note the membrane (eh - of vacuolar and/or host plasmalemma origin) surrounding and adhering closely to the haustorium. Note also a section through an intercellular hypha (arrowed). (X18016)

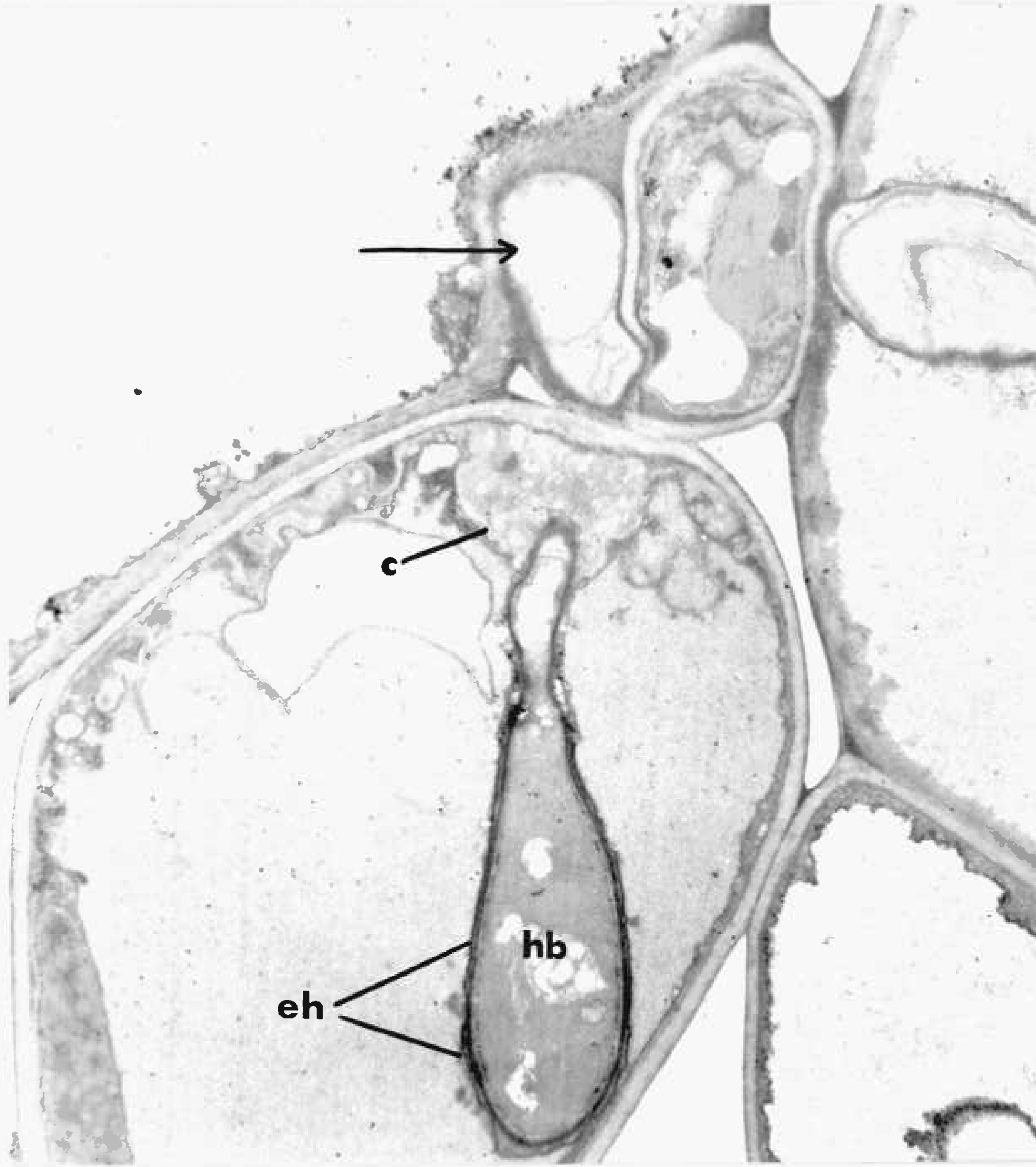
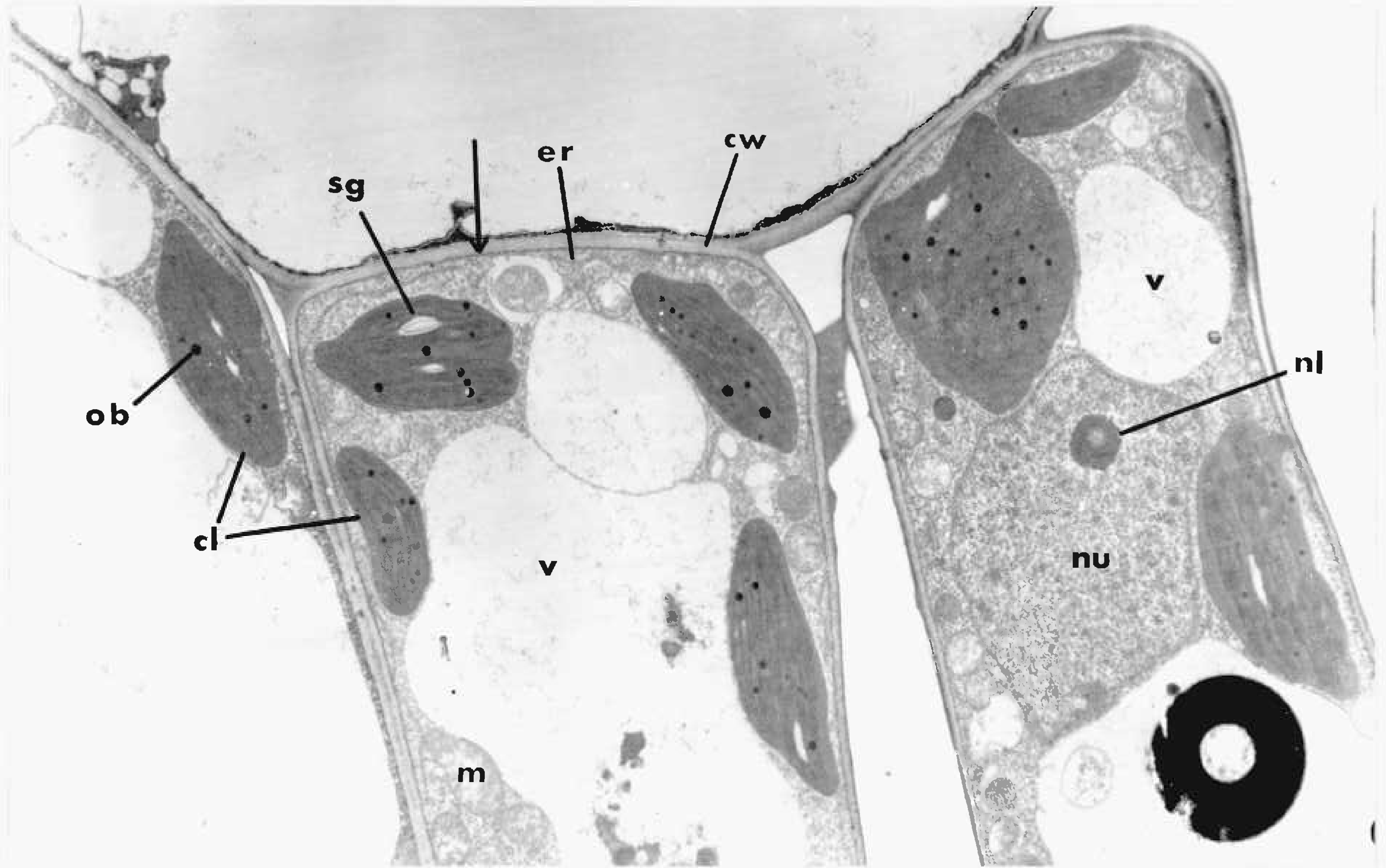

c
hb
eh

PLATE 68 : Allgold leaf - uninfected palisade cells. Note the cell wall (cw), chloroplasts (cl) containing starch grains (sg) and osmiophillic bodies (ob), plasmalemma (arrowed), nucleus (nu) with a nucleolus (nl), endoplasmic reticulum (er), vacuoles (v) and mitochondria (m). (X15632)



sg
er
cw
v
nl
ob
cl
v
nu
m

PLATE 69 : Sub-cuticular hyphae (rh) initiating the development of an acervulus on Allgold leaf (susceptible reaction). Note the well marked, unbroken but distended cuticle (cu) and unaltered epidermal cell wall (cw). Note also the expanding sub-cuticular hyphal cell and the junction (arrowed) between the vacuoles (v). (X15632)

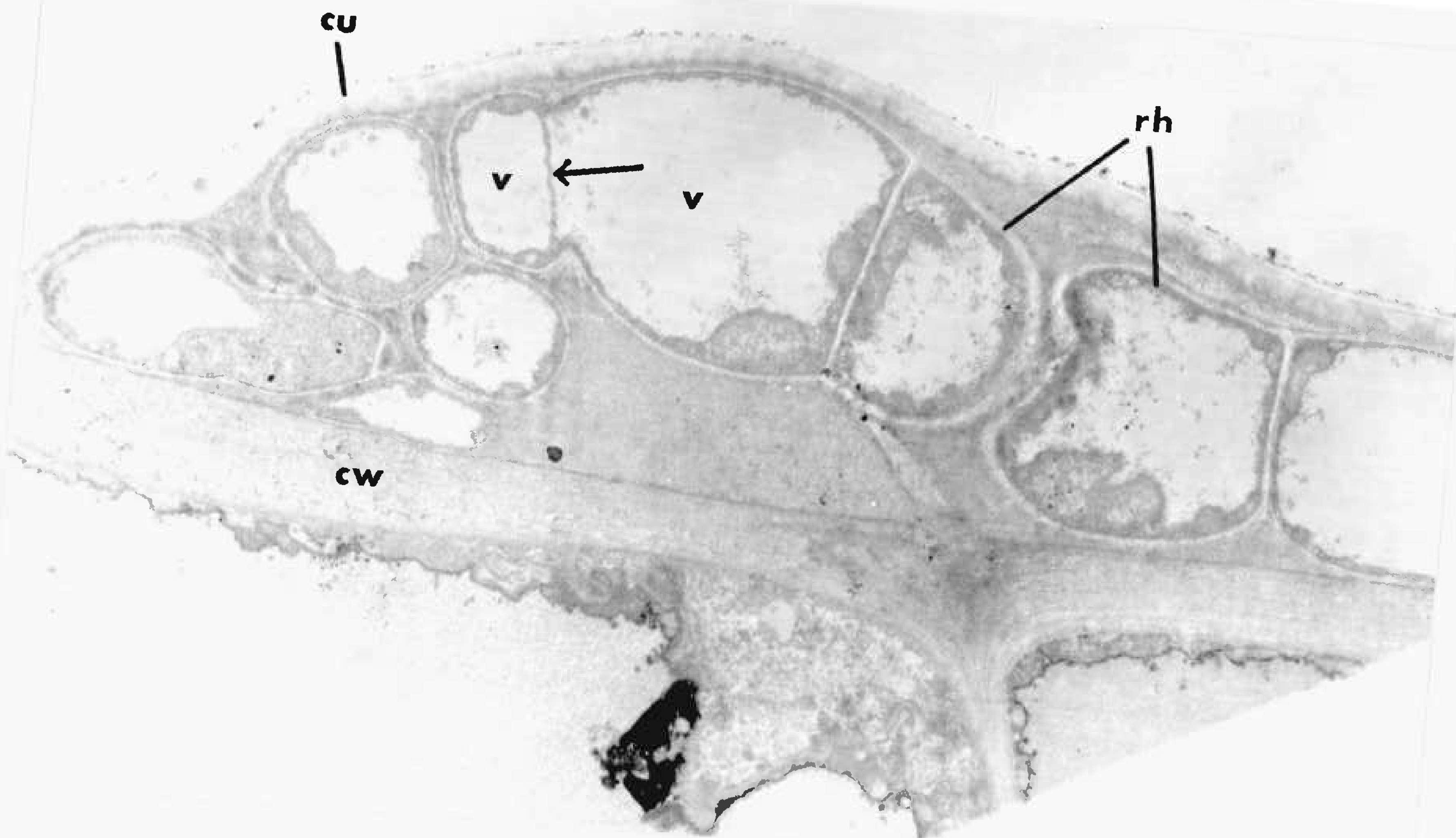

cu
rh
v
v
cw

PLATE 70 : Intercellular hypha (ih) penetrating a palisade cell of Allgold leaf (susceptible reaction). Note the haustorial neck septum (s) and the papilla (p). (X28884)

PLATE 71 : Section through acervulus on Allgold leaf (susceptible reaction). Note the spore mother cell (mo) on which two spores (arrowed) delimited by cross walls (w) are forming. The acervulus contains a structured matrix (st) and a clear structureless material surrounds the developing spores. (X6064)

ih

s

p

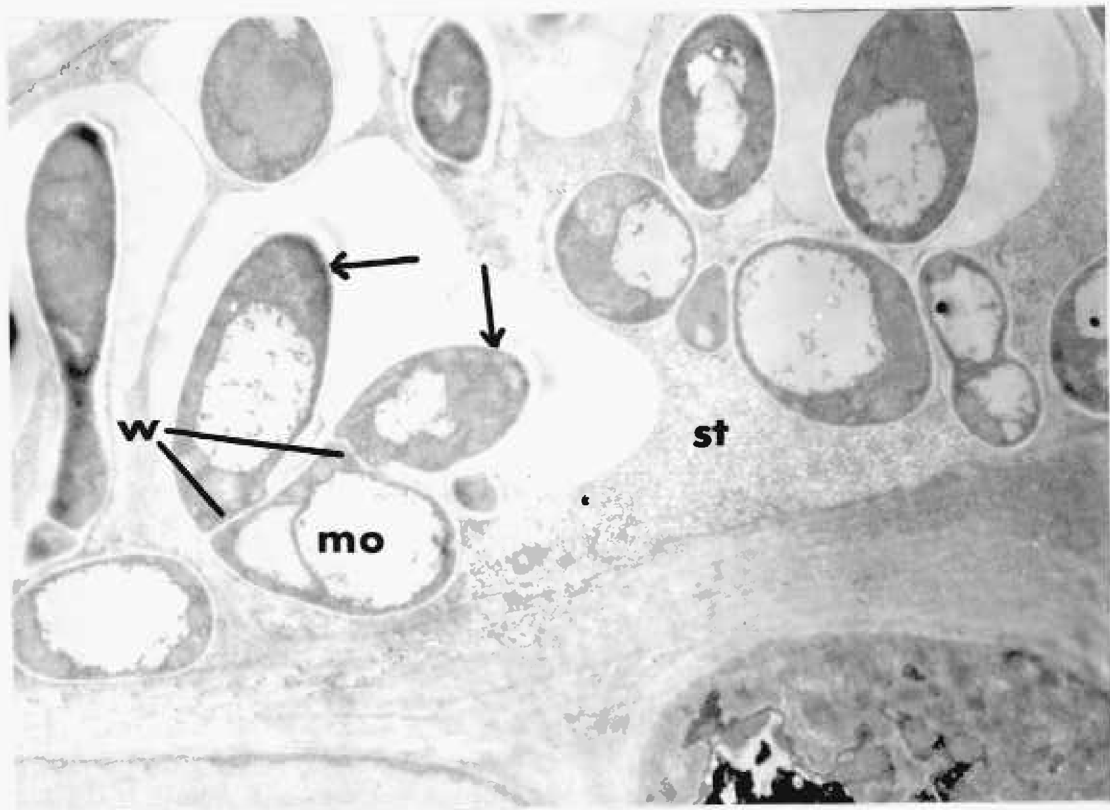


PLATE 72 : Section through part of an acervulus on an epidermal cell of Allgold leaf (susceptible reaction). Note the sub-cuticular hyphae (rh) and the developing spores (arrowed) both with unstained cell walls. The epidermal cell contains two haustoria (hb) : one seen in L.S. and with a well defined extrahaustorial membrane (eh) of vacuolar and/or host plasmalemma origin; the second seen in T.S. and showing part of the haustorial neck (n) surrounded by an electron dense material (e) and collar (c). (X15632)

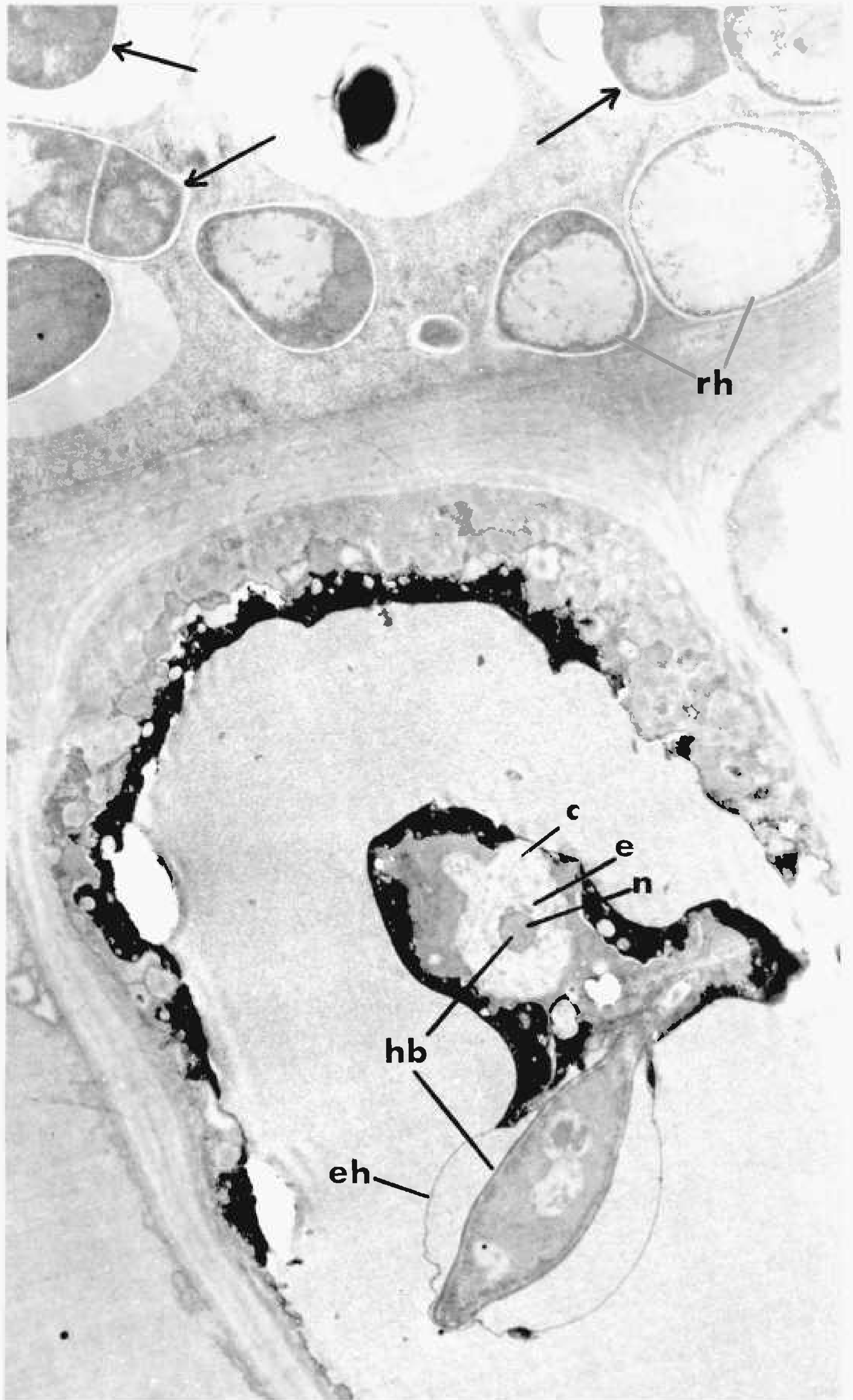

rh
c
e
n
hb
eh

PLATE 73 : Section through acervulus on Allgold leaf (susceptible reaction). The cuticle (cu) is distended but not broken and the epidermal cell wall (cw) is unaltered. Note the spore mother cells (mo) with developing spores (sp) attached but separated by a cross wall (arrowed). The acervulus contains a structured matrix (st) and a clear structureless material surrounds the developing spores. (X15632)

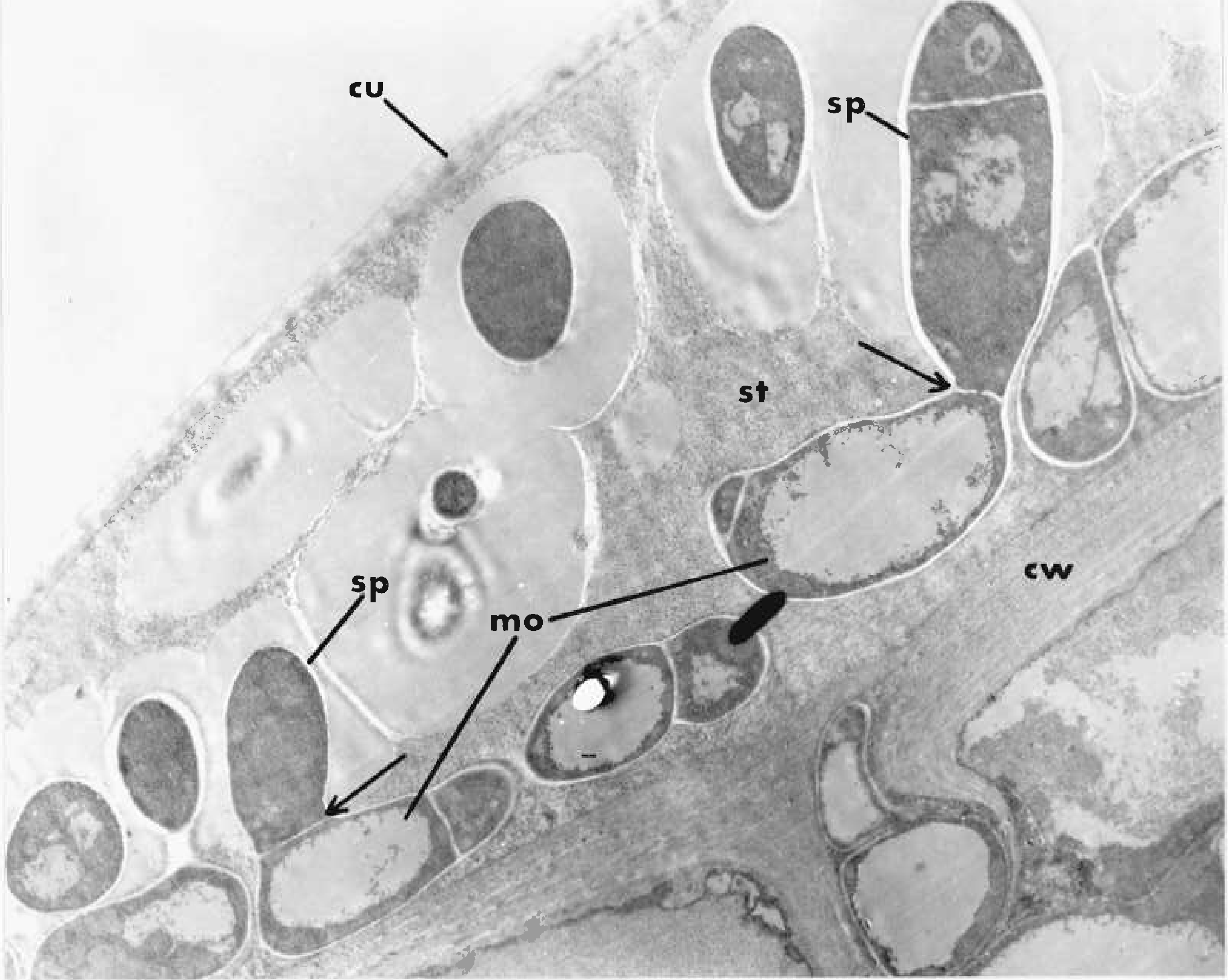
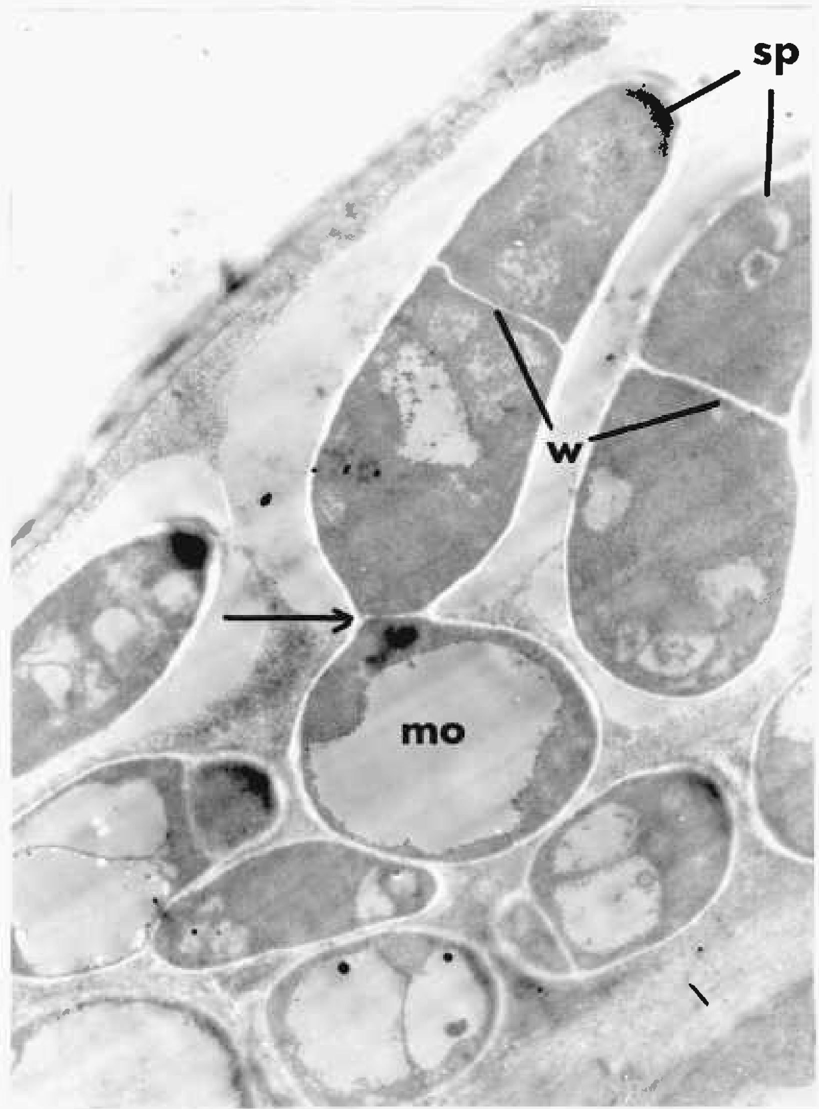

cu
sp
st
sp
mo
cw

PLATE 74 : Section through acervulus on Allgold leaf (susceptible reaction). Note the two mature spores (sp) one of which still adheres to its mother cell (mo), though there is a marked constriction (arrowed) at the point of attachment. A cross wall (w) delimits the two cells of the *D. rosae* spores. (X7220)

PLATE 75 : Section through acervulus on Allgold leaf (susceptible reaction). Only one of the three mature spores (arrowed) has a cross wall (w). Note the developing spore (sp) and its mother cell (mo). (X3908)


sp
w
mo

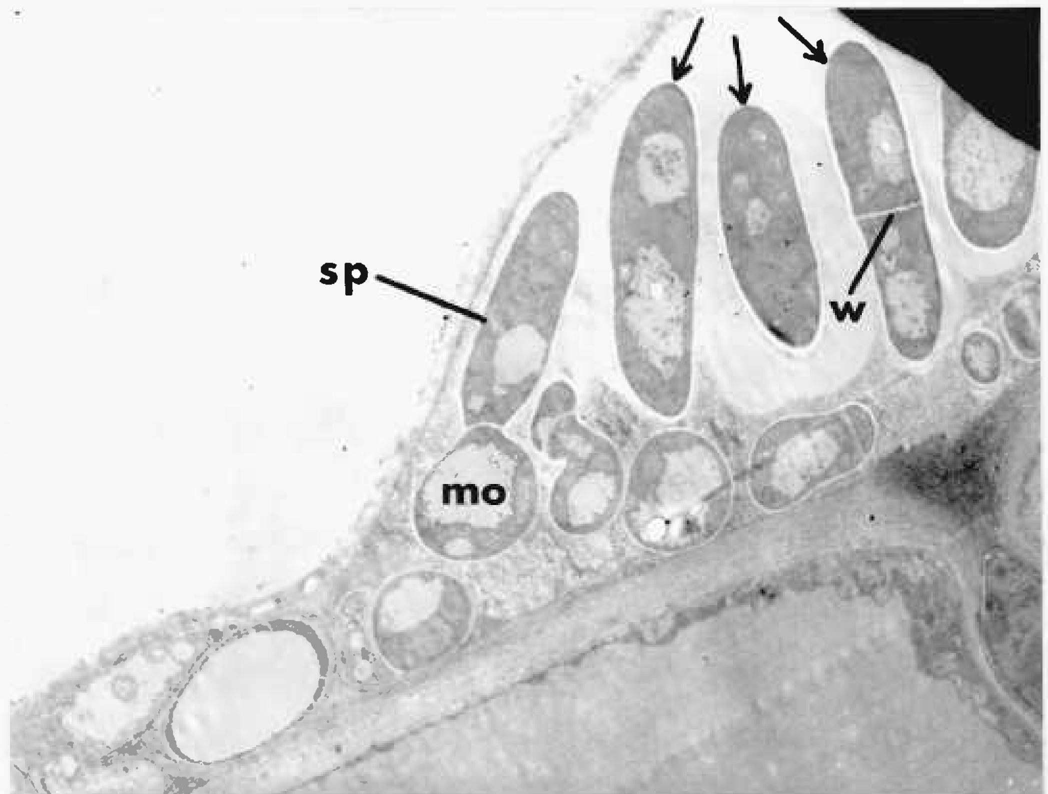

sp
w
mo

PLATE 76 : Section through epidermis and palisade of Allgold leaf (resistant reaction). Note the intact cuticle (cu) which is displaced by the runner hyphae (rh) growing beneath it and the intercellular hyphae (ih) separating the two epidermal cells and attempting to penetrate (arrowed) into a palisade cell (pc). (X2188)

PLATE 77 : Sub-cuticular hyphae (rh) in Allgold leaf (resistant reaction). Note the distended cuticle (cu), fibrillar changes (arrowed) in the cell wall (cw) and the dark staining areas (sa). (X6064)

cu
rh
ih
pc

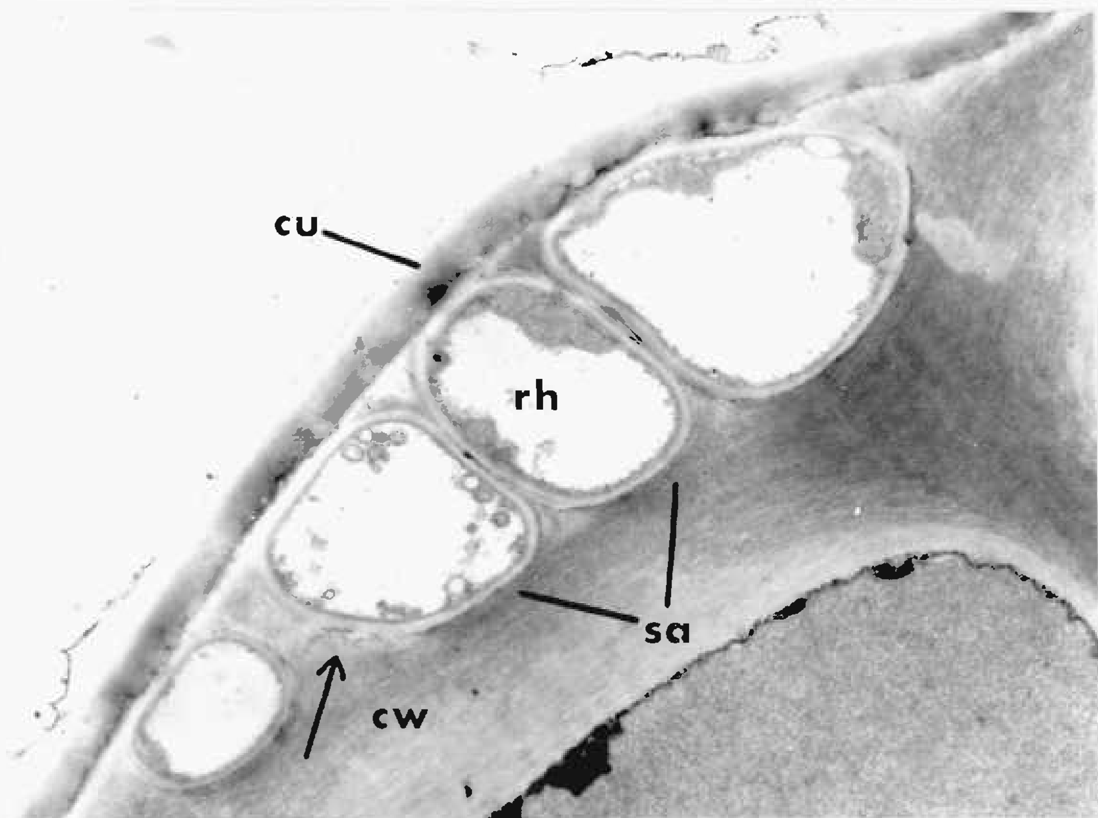
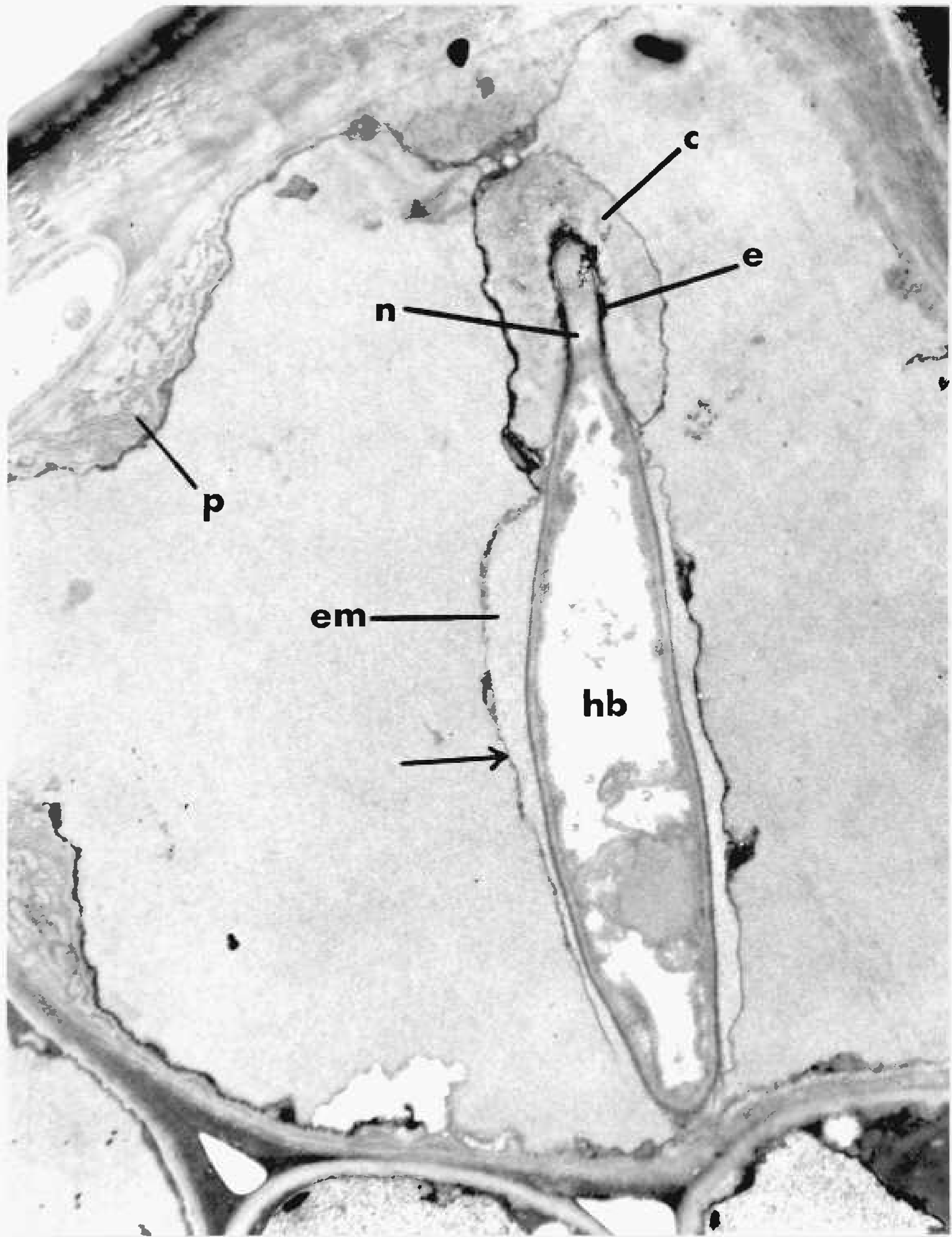
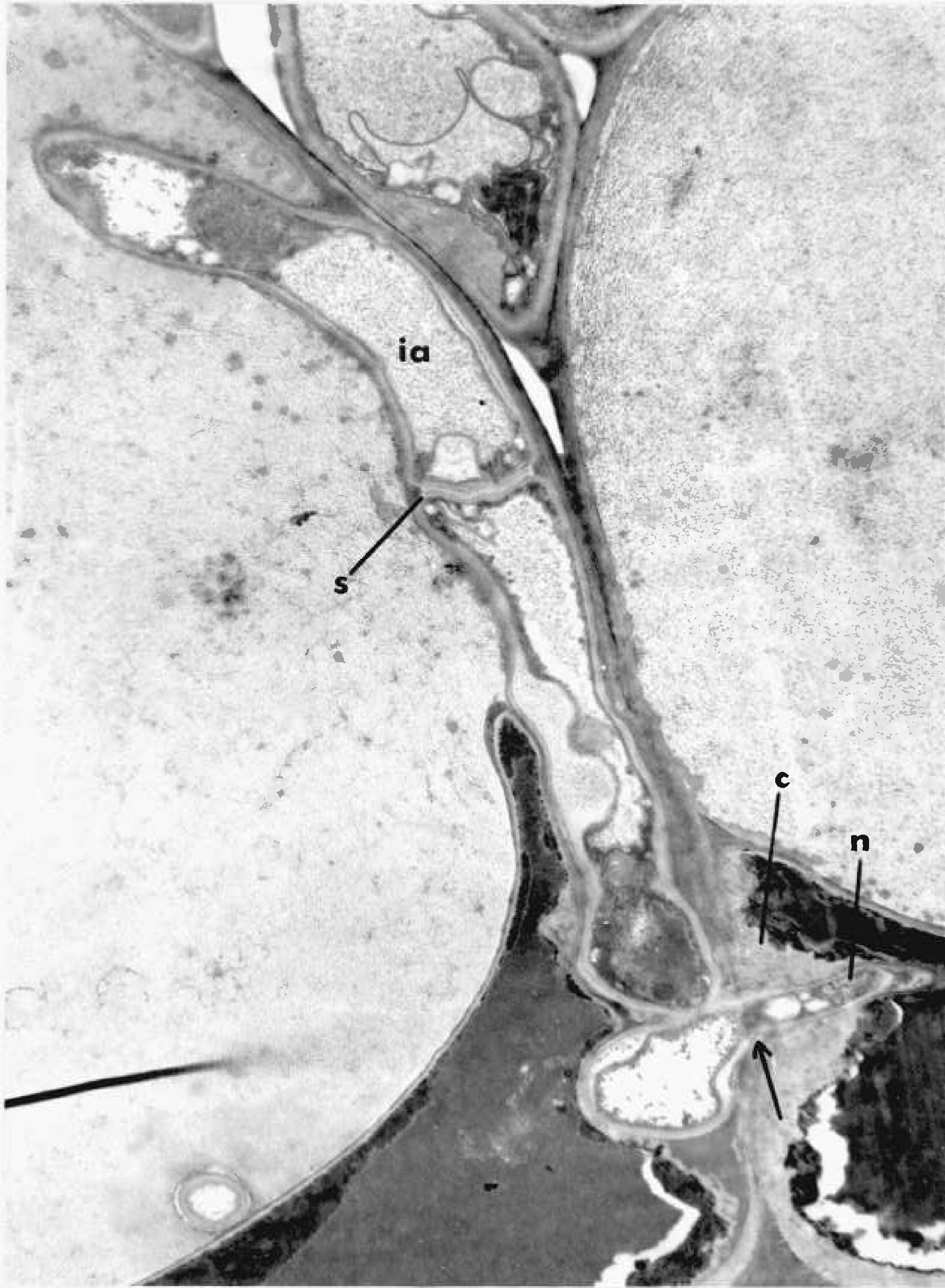


PLATE 78 : Haustorium (hb) in epidermal cell of Allgold leaf (resistant reaction). Note the collar (c), haustorial neck (n) with electron dense material (e), extrahaustorial membrane (vacuolar and/or host plasmalemma - arrowed) and extrahaustorial matrix (em). A papilla (p) is seen in the same cell. (X15632)



c
e
n
p
em
hb

PLATE 79 : Intracellular hypha (ia) in epidermal cell of Allgold leaf (resistant reaction). Note the septum (s) and the absense of electron dense material around the intracellular hypha. Note also the penetration (arrowed) into palisade cell and the collar (c) around haustorial neck (n). (X15632)



ia
s
c
n

PLATE 80 : Intracellular hypha (ia) surrounded by an electron dense material (e) in an epidermal cell of Allgold leaf (resistant reaction). An intracellular haustorial mother cell (hm) gives rise to a haustorium (arrowed) from which it is delimited by a septum (s). (X15632)

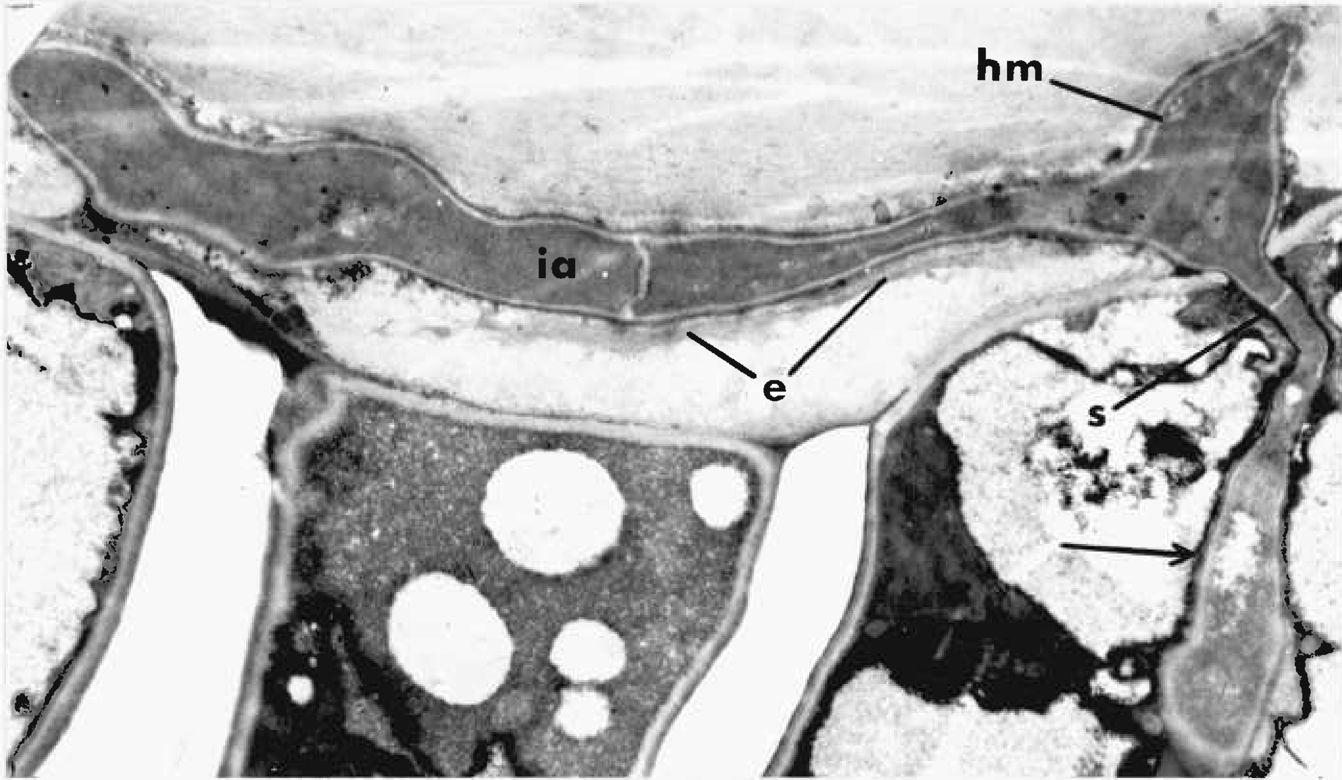
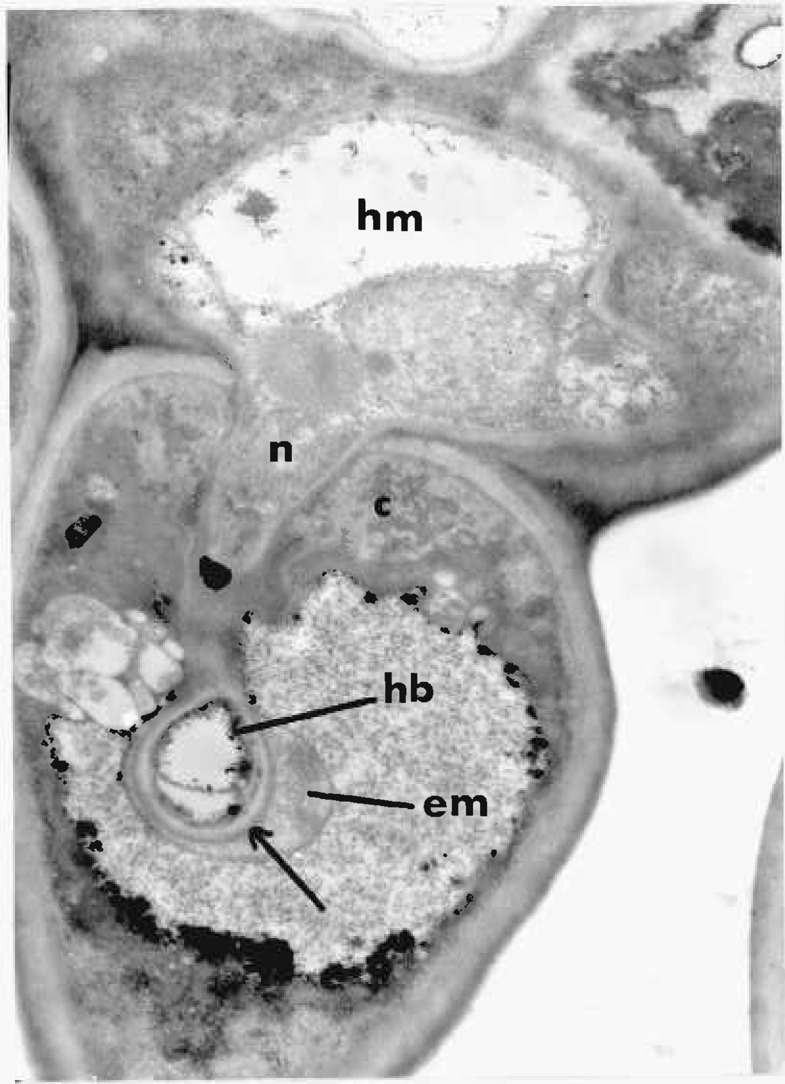

hm
ia
e
s

PLATE 81 : Intracellular haustorial mother cell (hm) in epidermal cell of Allgold leaf (resistant reaction) giving rise to a haustorium (hb) in a palisade cell. Note the small collar (c), the absence of a cross wall in the haustorial neck (n) and the poor development of the haustorial body which has a bulbous tip (arrowed) and is surrounded by an unidentified extrahaustorial matrix (em). (X10499)

PLATE 82 : Haustorium (hb) in palisade cell of Allgold leaf (resistant reaction). Note the large collar (c), haustorial neck with septum (s) and electron dense material (e), and the convoluted extrahaustorial membrane (vacuolar and/or host plasmalemma - arrowed). (X7220)


hm
n
c
hb
em

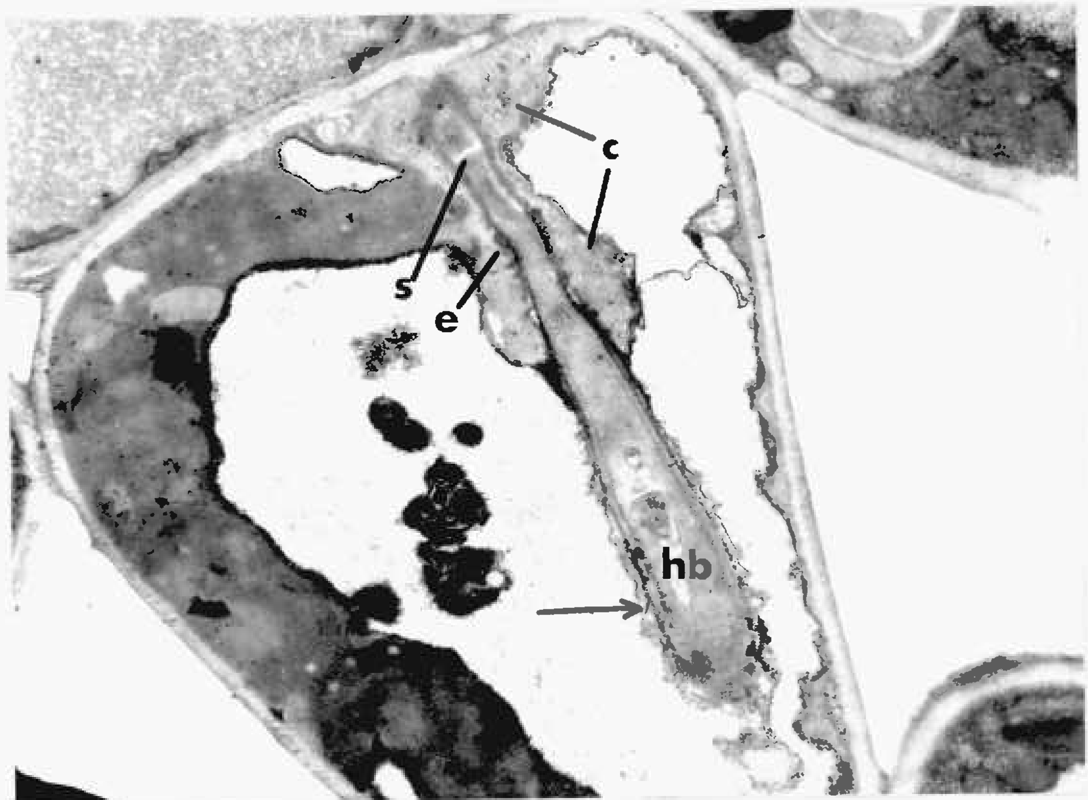

c
s
e
hb

PLATE 83 : Intracellular hypha (ia) penetrating a palisade cell of Allgold leaf (resistant reaction). Note the small collar (c), long haustorial neck (n) with no cross wall and poor development of haustorial body (hb) which has a bulbous tip (arrowed) and is surrounded by an extrahaustorial matrix (em). (X2880)

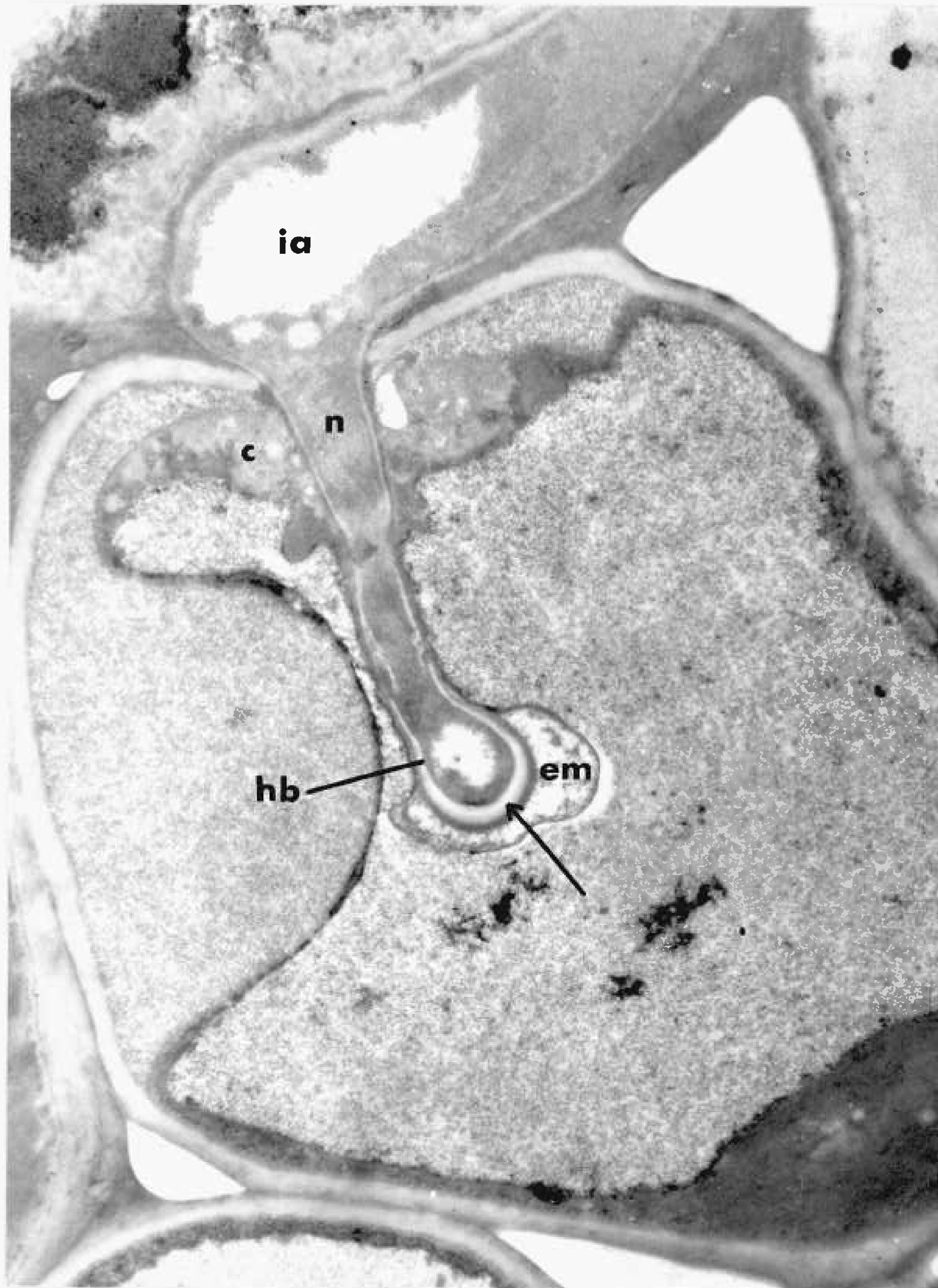

ia
n
c
hb
em

PLATE 84 : Intracellular haustorial mother cell (hm) in epidermal cell of Allgold leaf (resistant reaction) penetrating a palisade cell. Note the large collar (c), long haustorial neck (n) with areas of electron dense material (e) but no cross wall and poor development of haustorial body (arrowed). (X20860)

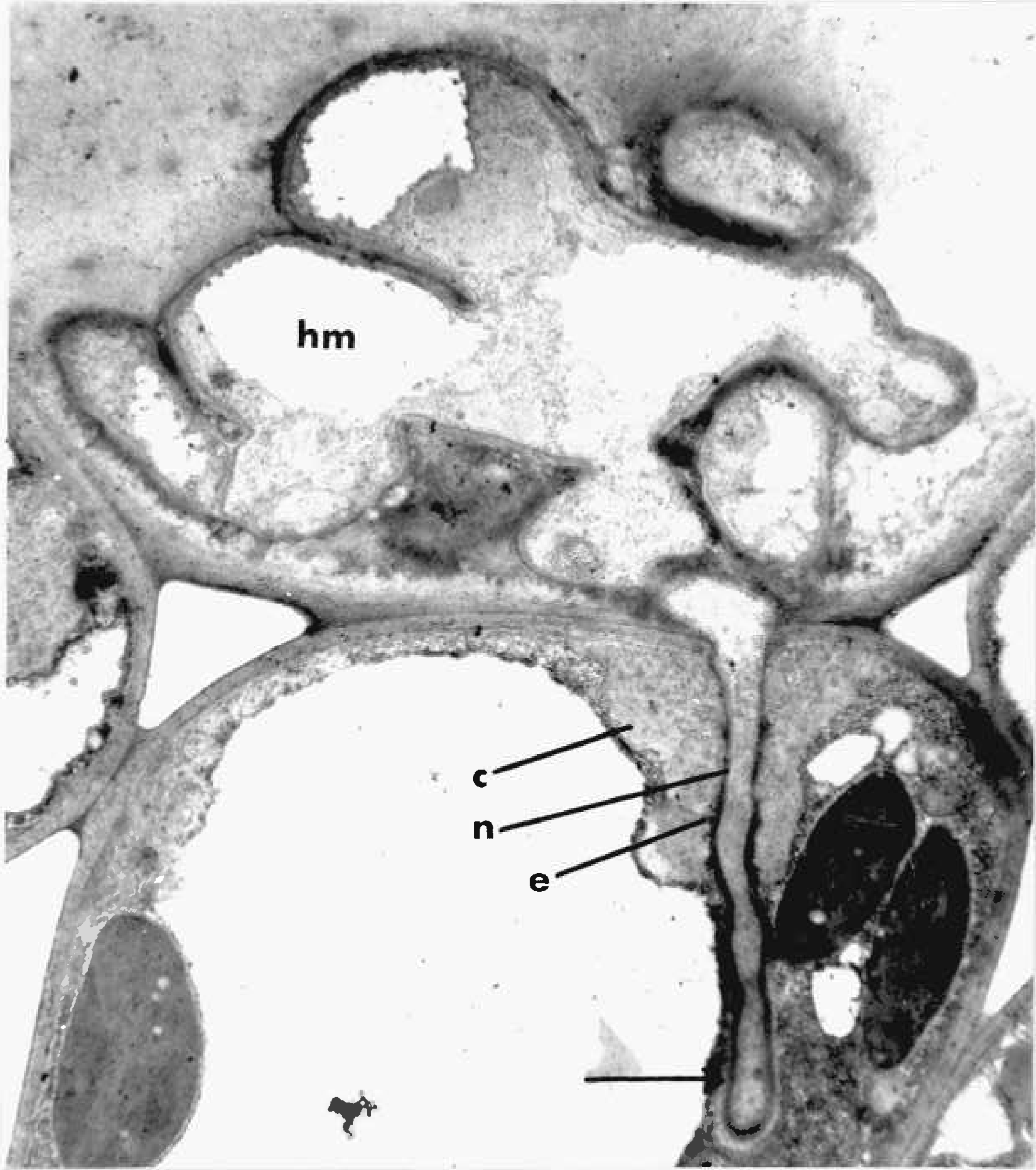

hm
c
n
e

PLATE 85 : Intracellular hyphae (ia) penetrating into palisade cells of Allgold leaf (resistant reaction). Note the collar (c) and well developed haustorium (hb) with a neck septum (s) and convoluted extrahaustorial membrane (eh - vacuolar and/or host plasmalemma). (X15632)

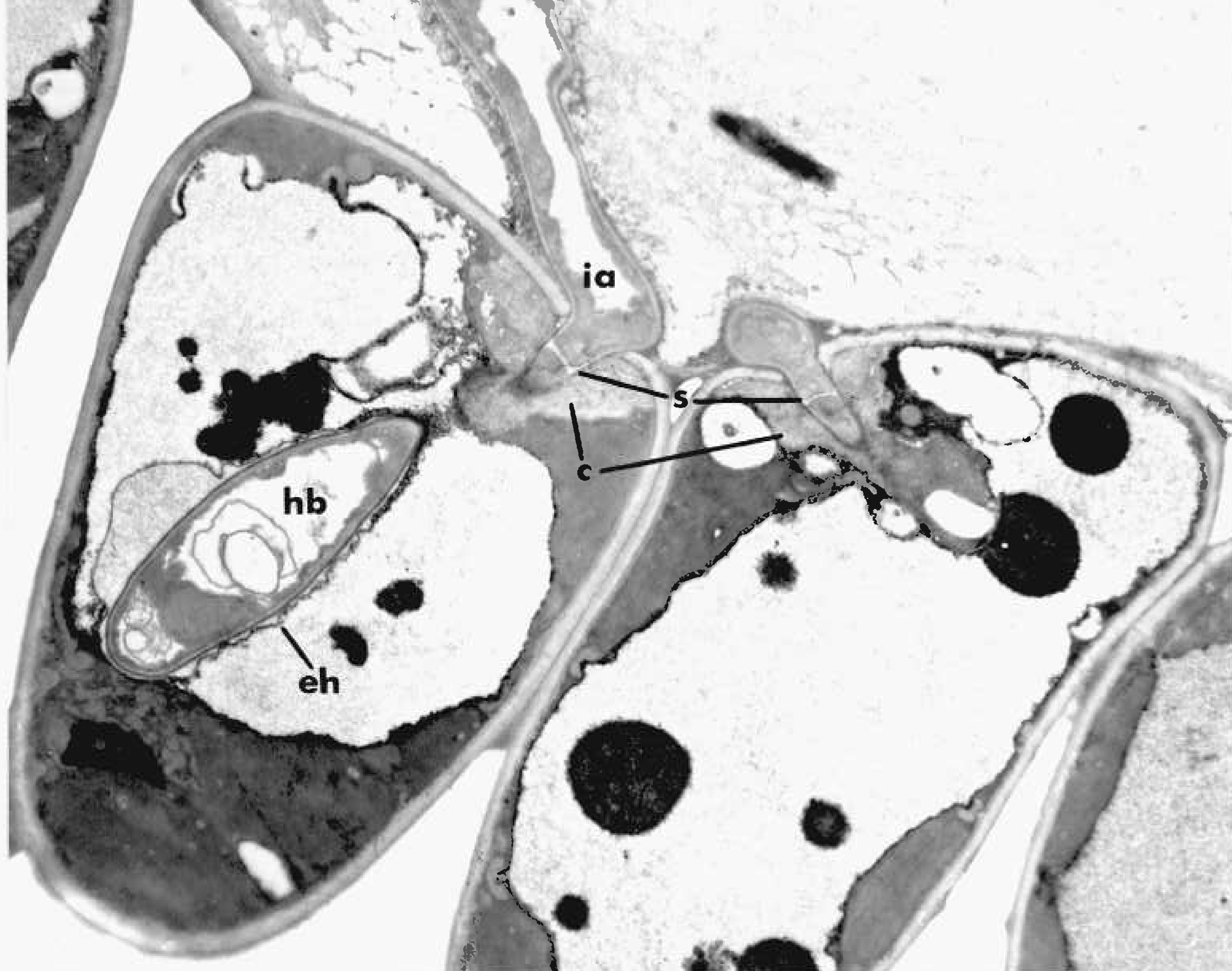
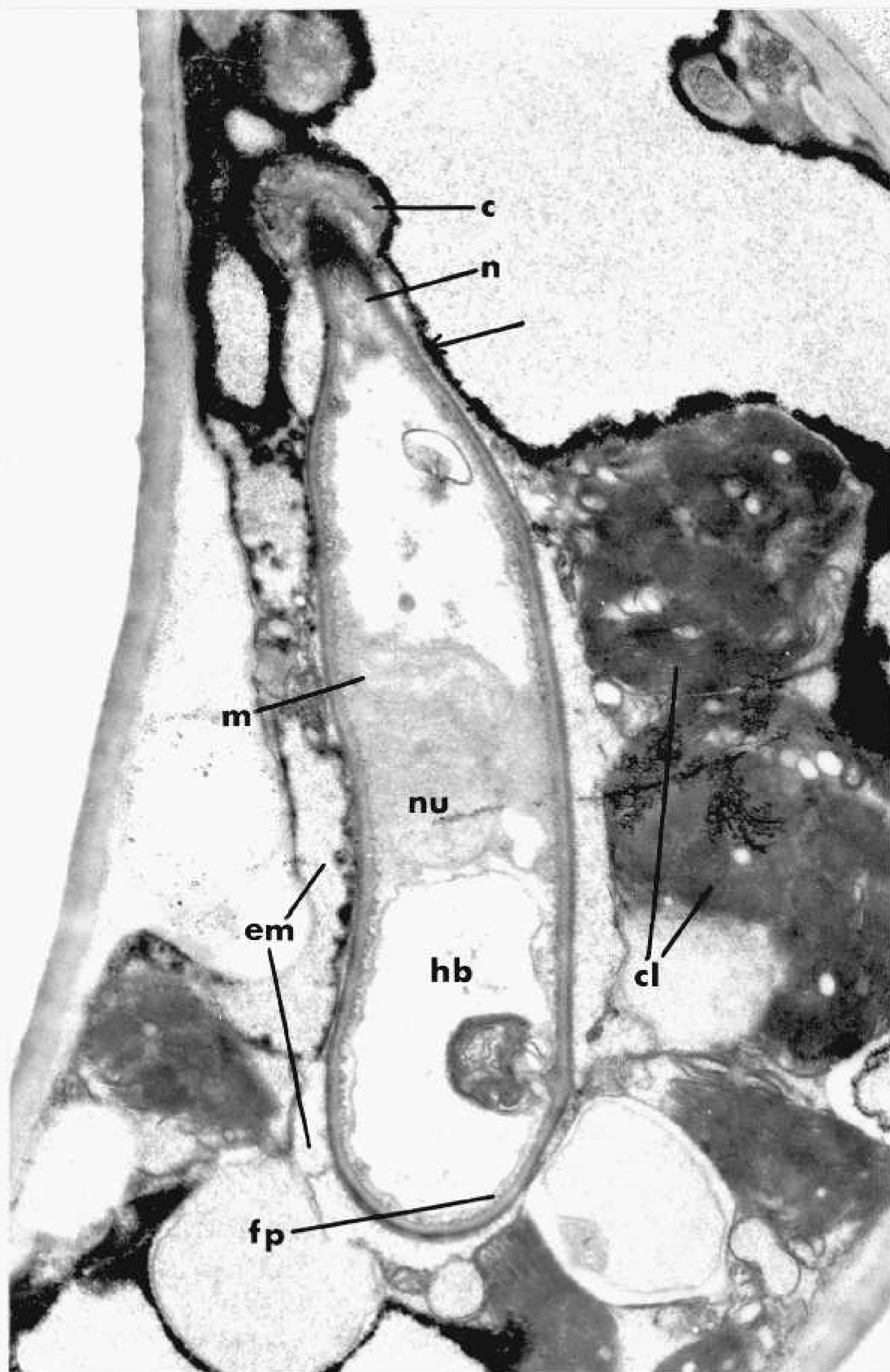

ia
s
c
hb
eh

PLATE 86 : Fully expanded haustorium (hb) in palisade cell of Allgold leaf (resistant reaction). Note part of the collar (c), haustorial neck (n), convoluted fungal plasmalemma (fp) nucleus (nu), mitochondria (m), a section of extrahaustorial membrane (arrowed - vacuolar and/or host plasmalemma) and extrahaustorial matrix (em) with closely adhering chloroplasts (cl). (X36416)



c
n
m
nu
em
hb
cl
fp

APPENDIX

Key to abbreviations:

(1) D.F. - Degrees of freedom

S.S. - Sums of squares

M.S. - Mean square

F. - F test

(2) Significance levels

n.s. - not significant

* - $\underline{P} < 0.05$

** - $\underline{P} < 0.01$

*** - $\underline{P} < 0.001$

APPENDIX TABLE 1 : Germination of *D. rosae* (F-76-Ash) on Frensham and Allgold leaf disks.

Replicate	% germination					
	24 h		48 h		72 h	
	F	A	F	A	F	A
1	11	6	19	17	17	15
2	7	3	17	6	21	17
3	10	4	17	11	19	12
4	7	4	11	17	14	18
5	8	3	19	22	18	18
6	7	2	14	15	17	17
7	5	4	21	5	12	18
8	6	4	12	16	20	8
9	8	6	17	18	16	16
10	7	4	20	14	17	17
Mean	7.6	4	16.7	14.1	17.1	15.6

Analysis*

	24 h	48 h	72 h
S.D. =	1.86	3.90	2.60
df =	18	18	18
t =	5.40	2.24	0.74
<u>P</u> =	<0.001	<0.05	n.s.

* Analysis based on angular transformation of % germination data.

APPENDIX TABLE 2 : Lesion development (diameter in mm) on 5 rose cultivars inoculated with *D. rosae* (F-76-Ash).

Replicate	Frensham	Allgold	Masquerade	Iceberg	Orange Sensation
1	9	4	6	7	6
2	8	4	0	5	5
3	6	0	0	4.5	6
4	3	0	0	5	3.5
5	8	0	0	8	3.5
6	8	0	0	6	6
7	5	0	0	8	5
8	5.5	0	0	9	5
9	0	0	0	3	4.5
10	0	0	0	7	5
Mean	5.25	0.8	0.6	6.25	4.75

Analysis

Analysis of variance

Factor	D.F.	S.S.	M.S.	F.
Treatments	4	253.53	63.38	13.991***
Error	45	203.88	4.53	
Total	49	457.41		

S.E. = ± 0.673

Difference between treatments (Duncan's new multiple range test - 1% level)

M. A. O.S. F. I.
(M. and A. underscored by one line; O.S. and F. underscored by one line; F. and I. underscored by one line)

Cultivars not underscored by the same line differed significantly (P <0.01)

APPENDIX TABLE 3 : Germination of *D. rosae* (F-77-Ash) on Frensham (F) and Allgold (A) leaf disks.

Replicates	% germination							
	24 h				48 h			
	F_1	A_1	C_1*	$W.A._1$*	F_2	A_2	C_2	$W.A._2$
1	18	8	35	74	20	12	45	75
2	12	12	36	63	17	13	46	73
3	13	14	28	86	11	13	36	76
4	10	9	33	81	12	10	37	74
5	13	12	30	81	21	11	38	76
Mean	13.2	11	33	76.7	16.2	11.8	40.4	74.8

Analysis (based on angular transformation of % germination values)

Analysis of variance

Factor	D.F.	S.S.	N.S.	F.
Treatments	7	10706.34	1529.48	161.34***
Error	32	303.48	9.48	
Total	39	11009.82		

S.E. = ± 1.377

Difference between treatments (Duncan's new multiple range test - 1% and 5% levels)

	A_1	A_2	F_1	F_2	C_1	C_2	$W.A._1$	$W.A._2$
5% level	———	———	———	———			———	———
1% level	———	———	———	———	———	———	———	———

(Underlined groups: 5% level — A_1 to F_2; $W.A._1$ to $W.A._2$. 1% level — A_1 to F_2; C_1 to C_2; $W.A._1$ to $W.A._2$.)

* Key: C - germination of conidia on slide

W.A. - germination of conidia on 2% water agar.

APPENDIX TABLE 4 : Lesion development (mm) by *D. rosae* (F-77-Ash) on Frensham and Allgold leaf disks.

Replicate	Frensham	Allgold
1	8.5	7
2	5.5	8
3	6	8
4	3.5	4.5
5	5	6
6	6	0
7	6	0
8	4.5	0
Mean	5.6	4.2

Analysis

S.D. = 2.73

df = 14

t = 1.08

P = N.S.

APPENDIX TABLE 5 : Lesion development (mm) by *D. rosae* (F-77-Ash) on Frensham and Allgold leaf disks.

Replicate	Frensham	Allgold
1	5.0	3.5
2	4.5	4.5
3	2.5	6.0
4	5.0	7.0
5	4.5	4.5
6	3.5	3.5
7	5.5	6.0
8	5.0	0
9	5.5	0
10	6.0	0
11	2.0	0
12	3.0	0
13	0	0
14	0	0
Mean	3.7	2.5

Analysis

S.D. = 3.29

df = 26

t = 2.61

P = 0.05

APPENDIX TABLE 6 : Lesion development (mm) by *D. rosae* (F-77-Ash) on Frensham and Allgold leaf disks.

Replicate	Frensham	Allgold
1	6.0	6.0
2	8.0	6.5
3	8.0	6.5
4	5.0	8.5
5	4.5	7.5
6	4.0	7.5
7	5.5	5.0
8	6.5	8.0
9	6.0	7.5
10	7.5	9.0
Mean	6.1	7.2

Analysis

S.D. = 1.33

df = 18

t = 1.85

P = N.S.

APPENDIX TABLE 7 : Development of *D. rosae* (F-78-G.H.) on Frensham (F) and Allgold (A) leaf disks.

Replicate	% germination* 24 h		% germination* 48 h		Lesion diameter (mm)	
	F	A	F	A	F	A
1	55	58	60	56	7.0	5.5
2	54	66	62	63	8.5	7.5
3	63	61	66	49	5.5	8.0
4	52	69	73	75	6.0	7.5
5	53	66	70	69	7.5	9.0
6	63	67	71	47	6.0	7.5
7	55	54	61	70	5.5	5.5
8	57	67	68	52	6.0	6.5
9	59	62	64	62	7.5	7.5
10	64	55	59	62	6.0	8
Mean	57.5	62.5	65.4	60.4	6.5	7.2
Analysis						
S.D. =	12.65		4.52		1.08	
df =	18		18		18	
t =	0.52		1.44		1.46	
$\underline{P}$ =	n.s.		n.s.		n.s.	

* Analysis based on angular transformation of % germination data.

APPENDIX TABLE 8 : Lesion development of *D. rosae* isolate (F-78-G.H.) on Frensham leaf disks incubated with (L) or without light at different temperatures.

Replicate	Lesion diameter (mm)							
	$10^{o}C$	$15^{o}C$	$15^{o}C$ (L)	$20^{o}C$	$20^{o}C$ (L)	$25^{o}C$	$25^{o}C$ (L)	$30^{o}C$
1	2	4.5	5.5	6.5	7.5	5.5	6.75	9.5
2	1	3.5	6.5	6.5	10.5	7.75	7.5	10.0
3	1	5.5	4.75	5.5	8.0	6.5	4.5	11.0
4	1	6.0	6.5	5.5	10.5	7.0	6.5	7.75
5	1.5	5.5	6.5	4.5	8.0	6.0	7.5	10.0
6	2	5.0	5.5	4.5	9.0	8.5	7.5	8.0
7	1.5	5.0	5.5	6.5	8.0	5.5	7.5	11.0
8	1	6.5	6.0	5.0	7.75	8.0	7.0	11.0
9	1	5.25	5.5	5.5	6.0	8.5	6.5	9.5
10	1	5.0	4.0	4.5	7.5	6.5	6.0	11.0
Mean	1.3	5.18	5.63	5.45	8.28	6.98	6.73	9.88

Analysis

Analysis of variance

Factor	D.F.	S.S.	M.S.	F.
Treatments	7	446.92	63.85	70.01***
Error	72	65.67	0.91	
Total	79	512.59		

S.E. = ± 0.302

Differences between treatments (Duncan's new multiple range test)

	$10^{o}C$	$15^{o}C$	$20^{o}C$	$15^{o}C$ (L)	$25^{o}C$ (L)	$25^{o}C$	$20^{o}C$ (L)	$30^{o}C$
1% level		————	————	————				
				————	————			
					————	————		
5% level		————	————	————	————	————		

APPENDIX TABLE 9 : Lesion development of *D. rosae* isolate (F-78-G.H.) on Allgold leaf disks incubated with (L) or without light at different temperatures.

Replicate	Lesion diameter (mm)							
	10°C	15°C	15°C (L)	20°C	20°C (L)	25°C	25°C (L)	30°C
1	1	4.5	2	3	5.5	5.5	6.0	9.0
2	1	1.5	1	1.5	0	4.0	0	10.0
3	1.5	1	1.5	1	0	4.5	0	7.5
4	1	1	1	1.5	0	5.0	0	8.0
5	2	2	1	2	0	5.0	0	7.0
6	1.5	3.5	1.5	3.5	0	6.0	0	10.0
7	1	3.0	2	2	0*	7.0	0*	9.5
8	1	1	1	1	0*	5.0	0*	8.5
9	1	1.5	0	0	0*	0	0*	7.5
10	1	0	0*	0	0*	0	0*	0
Mean	1.2	1.9	1.1	1.55	.55	4.2	0.6	7.7

Analysis

Analysis of variance

Factor	D.F.	S.S.	M.S.	F.
Treatments	7	420.75	60.11	19.708***
Error	72	219.95	3.05	
Total	79	640.7		

S.E. = ± 0.552

Differences between treatments (Duncan's new multiple range test)

	20°C (L)	25°C (L)	15°C (L)	10°C	20°C	15°C	25°C	30°C
1% level	————	————	————	————	————	————		
5% level	————	————	————	————	————	————		

* 'Browning' characteristic of the resistant response on Allgold observed on these disks.

APPENDIX TABLE 10 : Lesion development of *D. rosae* isolate (F-78-G.H.) on Frensham (F) and Allgold (A) leaf disks from plants grown at different temperatures.

Replicates	10°C		15°C		20°C		25°C		30°C	
	Lesion diameter (mm)									
	F	A	F	A	F	A	F	A	F	A
1	7	7	6.5	5.5	7.5	5	8.5	5	9	8
2	7	0	7	6.5	5.5	0	9.5	7	9	7
3	4	0	5.5	5	6	0	6.5	0	8	6.5
4	4	0	5.5	6	4.5	0	8	0	7	7
5	0	0	0	6	4.5	0	8	0	8.5	0
6	0	*	0	0	7	0	7.5	0	8.5	0
7	0	*	0	0	5.5	*	0	0	9.5	0
8	0	*	0	0	5	*	0	0	8.5	0
9	0	*	0	0	7.5	*	*	0	10	*
10	0	*	0	0	5.5	*	*	0	9	*
11	0	*	0	0	4	*	*	0	*	*
12	*	*	0	0	0	*	*	0	*	*
13	*	*	0	0	0	*	*	*	*	*
14	*	*	*	0	0	*	*	*	*	*
15	*	*	*	0	*	*	*	*	*	*
16	*	*	*	0	*	*	*	*	*	*
17	*	*	*	0	*	*	*	*	*	*
18	*	*	*	0	*	*	*	*	*	*
Mean	2	1.4	1.88	1.61	4.46	0.83	6	1	8.7	3.56
t =	0.37		0.29		3.05		3.65		4.12	
df =	14		29		18		18		16	
P =	n.s.		n.s.		<0.01		<0.01		<0.001	

* No disks available for examination of lesion development.

APPENDIX TABLE 11 : Lesion development of *D. rosae* isolates A and B* on Frensham and Allgold leaf disks.

Replicate	Lesion diameter (mm)			
	Isolate A		Isolate B	
	Frensham	Allgold	Frensham	Allgold
1	8.0	0	6.5	6.5
2	8.5	0	7.5	7.5
3	5.5	0	7.5	8.0
4	6.5	0	5.5	8.0
5	6.5	0	7.0	9.0
6	6.5	0	7.5	5.0
7	9.0	0	5.5	8.5
8	6.0	0	6.5	8.0
9	6.0	0	0	8.5
10	7.5	0	0	0
Mean	7.3	0	5.35	6.9

Analysis: Infected disks only

s = 1.07

t = 1.89

df = 15

P = n.s.

* Details in main text (pg. 24)

APPENDIX TABLE 12 : Lesion development of *D. rosae* isolates C and D* on Frensham and Allgold leaf disks.

Replicate	Lesion diameter (mm) Isolate C		Isolate D	
	Frensham	Allgold	Frensham	Allgold
1	7.0	8.5	8.0	0
2	8.0	6.0	8.5	0
3	7.0	10.0	8.5	0
4	8.5	8.0	8.5	0
5	7.0	9.5	6.0	0
6	6.0	8.0	7.0	0
7	6.5	8.0	7.0	0
8	7.5	7.0	7.5	0
9	6.0	8.0	11.0	0
10	8.5	7.0	7.0	0
Mean	7.20	8.0	7.93	0

s = 1.06

t = 1.69

df = 18

P = n.s.

* Details in main text (pg. 24)

APPENDIX TABLE 13 : Lesion development of *D. rosae* isolates E, F and G* on Frensham (F) and Allgold (A) leaf disks.

Replicate	Isolate E		Isolate F		Isolate G	
	Lesion diameter (mm)					
	F	A	F	A	F	A
1	5.5	7.5	5.0	6.0	3.5	6.0
2	6.0	7.5	5.0	5.0	5.0	5.0
3	4.5	6.5	5.0	7.0	6.0	6.5
4	5.5	4.5	5.0	4.0	5.0	8.0
5	5.0	8.0	4.0	6.0	6.5	4.0
6	3.0	5.0	4.0	0	6.5	5.5
7	0	6.5	0	0	6.5	4.0
8	0	0	0	0	5.0	0
9	0	0	0	0	5.0	0
10	0	0	0	0	3.0	0
Mean	2.95	4.56	2.8	2.8	5.2	3.9

Analysis†

	Isolate E	Isolate F	Isolate G
s =	1.21	0.85	4.29
t =	2.38	2.14	0.19
df =	11	9	15
P =	0.05	n.s.	n.s.

Frensham (E and G)

s = 4.39

t = 0.14

df = 14

P = n.s.

Frensham (F and G)

s = 4.35

t = 0.40

df = 14

P = n.s.

* Details in main text (pg. 24)

† Analysis: Infected disks only

APPENDIX TABLE 14 : Chemical methods examined for the removal of rose leaf cuticle.

Agent	Method	Comments
1. PECTIC ENZYMES (Preece, 1962; Johnston *et al.*, 1965).	Disks were placed in 90 ml- citric acid - phosphate buffer (pH 4) containing 2, 4 or 6 g. pectic enzyme (Sigma Ltd.) plus 10 ml. 0.001% sodium ethyl mercuri-thiosalicylate (Hopkin and Williams Ltd.) to reduce bacterial contamination. The disks were infiltrated with this mixture by subjecting them to a partial vacuum for 10-30 min. Then they were kept at 37°C and examined daily for 8 days.	a. Cuticles of uninfected rose disks could be peeled off after 5-8 days. It was more difficult to remove cuticles from infected rose disks. b. Cuticles of broad bean (*Vicia faba*) and apple could be gently removed after 24 h, but apple cuticles did not float free in the solution as reported by Preece (1962). c. Concentrations above 4% pectinase did not make any difference to the isolation of cuticles. d. Longer periods of impregnation and immersion of leaf disks in the solution slightly improved removal of cuticle.

APPENDIX TABLE 14 : Continued.

Agent	Method	Comments
2. AMMONIUM OXALATE AND OXALIC ACID (Huelin & Gallop, 1951).	Uninfected Allgold and Frensham disks were placed in an aqueous mixture (pH 4) of 1.6% ammonium oxalate and 0.4% oxalic acid at 37°C and examined daily for 8 days.	Even after a weeks incubation it was not possible to remove the cuticles.
3. CELLULASE	Uninfected Allgold and Frensham disks were immersed in 10 ml. distilled water containing 0.15 g cellulase (BDH Chemicals Ltd.). The disks were incubated at 37°C and examined daily for 8 days.	Even after a weeks incubation it was not possible to remove the cuticles.
4. CELLULASE & PECTINASE	Uninfected disks of Allgold and Frensham were immersed in a solution of cellulase (as prepared in 3.), and 2% pectinase (as prepared in 1.), in a 1:1 mixture. Following infiltration of the cellulase/pectinase solution disks were incubated at 37°C and examined daily for 8 days.	The addition of cellulase did not improve the removal of cuticles.

APPENDIX TABLE 14 : Continued.

Agent	Method	Comments
5. ZINC CHLORIDE/ HYDROCHLORIC ACID & CELLULASE	Infected disks of Allgold and Frensham were immersed in a solution containing 5 g zinc chloride, 8.5 ml hydrochloric acid and 1.5% cellulase.	After 24 h, cuticles could be readily stripped off, although epidermal cells still adhered to the cuticle.

APPENDIX TABLE 15 : Agents and methods used to clear rose leaf disks.

Agent	Method	Comments
1. ETHANOL	Disks were immersed in concentrations of ethanol ranging from 50% - 99.8% (absolute ethanol), and left at 18-20°C for periods to 48 h.	A 24 h immersion in absolute ethanol consistently gave good results as judged by the ability to view the internal tissues of the disks. Lower concentrations of ethanol proved less useful.
2. METHANOL	Procedure as for ethanol.	Clearing not as good as with ethanol.
3. LACTOPHENOL	Disks were boiled in lactophenol for 5 min., while others were left to stand in the solution at room temperature for periods to 48 h.	Only boiling the disks resulted in good clearing but this also caused disruption of the tissues.
4. CHLORAL HYDRATE SOLUTION	Disks were immersed in a solution of chloral hydrate (5 g) and distilled water (2 ml), and left at 18-20°C for periods to 48 h.	Although disks were cleared they were too opaque for further observation.
5. METHANOL/ CHLOROFORM/ LACTIC ACID	Disks were immersed in a 1:1:1 mixture of methanol, chloroform, and lactic acid and left at 18-20°C for periods to 48 h.	Clearing occurred as early as 6 h but the disks proved too opaque for observations.

APPENDIX TABLE 15 : Continued.

Agent	Method	Comments
6. SODIUM HYPOCHLORITE	Disks were immersed in 4% aq. sodium hypochlorite and left at 18-20°C for 24 h.	Disks cleared after 24 h, but the cuticle ruptured in some areas and the disk centres often remained green.
7. CHLORINE GAS	Disks were exposed to chlorine gas for 10-30 min.	The disks yellowed and became brittle.
8. SODIUM HYDROXIDE	Some disks were boiled in 0.1 M NaOH for 5-10 min; others were left to stand in this solution at 18-20°C for periods to 48 h.	Only boiling the disks resulted in good clearing but this also caused disruption of the tissues, (cf. Experiment 3).

APPENDIX TABLE 16 : Staining techniques examined to demonstrate *D. rosae* in leaf disks*.

Agent	Method	Comments
1. COTTON BLUE IN LACTO-PHENOL (Aronescu, 1934).	Disks were immersed in cotton blue-lactophenol for 6, 12, 24 and 48 h with the aim of staining penetration points and runner hyphae of the sub-cuticular fungus. They were examined after being washed and mounted in distilled water.	Host tissues were stained dark blue, but neither penetration points nor runner hyphae took up the stain.
2. TOLUIDINE BLUE O (O'Brien *et al.*, 1965; Ghemawat, 1977).	Disks were immersed in 0.005% aq. toluidine blue O (BDH Chemicals Ltd.), for 1, 5, 10 and 30 min with the aim of staining penetration points. They were examined after being washed and mounted in distilled water.	Host tissues were stained bluish-violet but penetration points were not stained.
3. AZURE BLUE (Sargent & Gay, 1977).	Disks were immersed either in 0.1% aq. azure blue or 0.1 g. azure blue in 100 ml of 5% phenol for 10-15 min with the aim of staining penetration points. They	Penetration points were not stained but the runner hyphae took on a light blue colour when phenolic azure blue was used.

APPENDIX TABLE 16 : Continued.

Agent	Method	Comments
	were examined after being washed and mounted in distilled water.	
4. THIONIN AND ORANGE G (Stoughton, 1930).	Disks were immersed in a solution of 0.1 g. thionin (Solmedia Ltd.), in 100 ml of 5% phenol for 5, 10 or 30 min. After washing in water disks were put into 95% ethanol for ½ min and then in a saturated solution of orange G (Gurr Ltd.) in absolute ethanol for 1-3 min. They were then washed in absolute ethanol, cleared in xylol and mounted in balsam.	After staining with thionin runner hyphae appeared light blue and penetration points dark blue. However, with the completion of the technique the colour was lost, but haustoria now clearly differentiated stained bright green.
5. CRYSTAL VIOLET AND ORSEILLIN BB (Corlett & Kokko, 1975)	Disks were immersed in a solution of 3% acetic acid saturated with Orseillin BB (Pfaltz & Bauer) for 10-30 min. They were then washed in distilled water and immersed in 1% aq. crystal violet for 5-10 secs and mounted in distilled water.	Haustoria stained violet but other fungal structures and penetration points remained unstained.

APPENDIX TABLE 16 : Continued.

Agent	Method	Comments
6. CRYSTAL VIOLET	Disks were immersed in 1, 3, 5 and 7% phenol for 10-30 min. These disks (plus some untreated ones) were placed (with and without washing) into 0.001, 0.01, 0.1 and 1% aq. crystal violet for 1, 5, 10 or 30 min, after which they were mounted in distilled water. Some disks were infiltrated with the dye by subjecting them to a partial vacuum for 10-30 min.	Infected cells stained more deeply than non-infected cells. Impregnation of tissues with dye did not improve the staining of fungal structures. Other details are given in the main text (pg. 33).
7. CRYSTAL VIOLET AND THIONIN	Disks were immersed in 0.1 g. thionin in 100 ml. of 5% phenol for 5 min and then transferred without washing to 0.1% aq. crystal violet, after which they were mounted in water and examined.	The runner-hyphae stained reddish violet, but were poorly differentiated from the deeply stained host tissue.

* Infected disks (1 cm^2) were first cleared in absolute ethanol (24 h). Runner hyphae and penetration points on disks cleared in 1:1:1 methanol, chloroform and lactic acid failed to take up the dyes listed.

APPENDIX TABLE 17 : Dyes* examined to demonstrate *D. rosae* in 0.5 μm and 2.5 μm sections**.

Dye	Observation
1. 0.1% AQUEOUS TOLUIDINE BLUE	Fungal and host tissues stained dark blue in 2.5 μm section and there was little differentiation. However, for 0.5 μm sections this proved to be the most suitable dye, (details in main text, pg. 34).
2. 0.1% AQUEOUS AZURE BLUE	In contrast to toluidine blue, azure blue stained 2.5 μm sections extremely well, (details in main text, pg. 34), but 0.5 μm sections were very poorly stained.
3. COTTON BLUE IN LACTOPHENOL	Acervuli in 2.5 μm sections were stained particularly well. The young conidia developing in the acervulus were stained blue and the surrounding matrix a light brown colour.
4. 0.1% AQUEOUS THIONIN	Intracellular hyphae in 2.5 μm sections stained dark blue, whereas surrounding cells were stained light blue.
5. ORANGE G (saturated in absolute ethanol)	Although differentiation between fungus (which stained black) and host was very good in 2.5 μm sections the host cells were distorted by the treatment.
6. 0.03% AQUEOUS ROSE BENGAL	Fungal tissues stained brown in 2.5 μm sections, but differentiation between parasite and host was poor.

APPENDIX TABLE 17 : Continued.

Dye	Observation
7. ORSEILLIN BB (saturated in 3% acetic acid)	Fungal tissues stained brown-black in 2.5 μm sections, but many cells were disrupted.
8. 0.1% AQUEOUS CRYSTAL VIOLET	Both fungal and host tissues stained dark violet and differentiation was poor particularly in 2.5 μm sections.
9. IODINE (in KI solution)	Fungal tissues were darkly stained in 2.5 μm sections but the treatment damaged host cells.

* The following dyes were also tried but although fungal tissues were often stained there was no clear differentiation: lactofushsin, Giemsa stain, trypan blue and 1% aq. safranin.

** Preparation of sections and methods of staining are outlined in the main text (pg. 34).

APPENDIX TABLE 18 : Analysis of the mean distribution of browned groups* on Allgold after 48 and 72 hours, and 14 days : Experiment 2.

Comparing:

136 groups at 48 h 161 groups at 72 h	161 groups at 72 h 167 groups at 14 days
S = 17.2	S = 18.1
Ratio = 1.45	Ratio = 0.33
P = n.s.[†]	P = n.s.[†]

* Details in Table 14a.

[†] n.s. = means do not differ significantly.

APPENDIX TABLE 19 : Analysis (using a χ^2 test for homogeneity) of the distribution of group types* : Experiment 2.

Period	χ^2	df	P †
48 - 72 h	19.5	4	***
72 h - 14 days	17.6	4	**

* Details in Table 14b.

† Probability that distributions differ is indicated thus: ** $P < 0.01$ and *** $P < 0.001$.

APPENDIX TABLE 20 : Lesion development on Babylon, Red Rum and Astral inoculated with an isolate of *D. rosae* (F-77-Ash) : Experiment 1.

Replicate	Lesion diameter (mm)		
	Babylon	Red Rum	Astral
1	3.0	7.5	1.5
2	4.5	6.5	2.0
3	4.0	5.5	3.5
4	3.0	6.0	4.5
5	2.5	5.5	4.5
6	2.5	6.5	1.5
7	2.0	6.5	1.5
8	4.0	5.0	7.0
9	1.0	6.0	6.5
10	3.5	6.0	0
Mean	3.0	6.1	3.25

Analysis

Analysis of variance

Factor	D.F.	S.S.	M.S.	F.
Treatments	2	59.32	29.66	12.62***
Error	27	63.52	3.35	
Total	29	122.84		

S.E. = ± 0.48

Differences between cultivars (Duncan's new multiple range test)

	Babylon	Astral	Red Rum
1% level	————————	————————	
5% level	————————	————————	

APPENDIX TABLE 21 : Lesion development on Babylon, Frensham and Allgold inoculated with an isolate of *D. rosae* (F-77-Ash) : Experiment 2.

Replicate	Lesion diameter (mm)		
	Frensham	Babylon	Allgold
1	8.5	5.0	0
2	10.0	2.5	0
3	10.5	2.5	0
4	11.0	3.5	0
5	10.5	8.5	0
6	9.0	2.5	0
7	10.5	2.0	0
8	10.0	2.0	0
9	6.5	2.5	0
10	10.5	2.5	0
11	10.5	2.0	0
12	8.5	2.5	0
13	10.5	2.5	0
14	8.0	2.5	0
15	5.5	2.5	0
16	2.0	2.5	0
17	5.0	2.0	0
18	0	2.0	0
19	0	3.5	0
20	0	2.5	0
Mean	7.35	2.9	

S = 4.10

df = 38

t = 3.44

P < 0.01

APPENDIX TABLE 22 : Lesion development on Babylon, Red Rum and Astral inoculated with an isolate of *D. rosae* (F-77-Ash) : Experiment 3.

Replicate	Lesion diameter (mm)		
	Babylon	Red Rum	Astral
1	2	5.5	0
2	1	5.0	0
3	0	5.5	0
4	0	5.0	0
5	0	0	0
6	0	0	0
7	0	0	0
8	0	0	0
Mean	0.38	2.63	0

Analysis

Analysis of variance

Factor	D.F.	S.S.	M.S.	F.
Treatments	2	37.05	18.53	8.46**
Error	27	59.25	2.19	
Total	29	96.30		

S.E. = ± 0.52

Differences between cultivars (Duncan's new multiple range test)

	Astral	Babylon	Red Rum
1% level	————————	————————	
5% level	————————	————————	

APPENDIX TABLE 23 : Lesion development on 7 'un-named' cultivars, Frensham (F) and Allgold (A) with an isolate of *D. rosae* (F-77-Ash) : Experiment 1.

Lesion diameter (mm)

Replicate	J5	K3	K4	K8	K9	L1	L4	Controls F	Controls A
1	7.5	6.0	7.0	8.0	2.5	5.0	3.5	7.5	6.0
2	4.5	6.5	7.5	0	0	2.0	5.5	3.0	0
3	0	6.0	8.0	0	0	4.5	6.5	8.0	0
4	0	5.5	7.0	0	0	4.0	5.0	3.0	0
5	0	3.0	0	0	0	9.0	5.0	0	0
6	0	7.5	0	0	0	7.0	3.5	0	0
7	0	8.0	0	0	0	0	7.5	0	0
8	0	4.5	0	0	0	0	0	0	0
9	0	6.5	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
Mean	1.2	5.35	2.95	0.8	0.25	3.15	3.65	2.15	0.6

Analysis

Analysis of variance

Factor	D.F.	S.S.	M.S.	F.
Treatments	8	219.07	27.38	3.72**
Error	81	597.05	7.37	
Total	89	816.12		

S.E. = ± 0.86

Differences between cultivars (Duncan's new multiple range test)

	K9	A	K8	J5	F	K4	L1	L4	K3
1% level	—	—	—	—	—	—	—	—	
					—	—	—	—	—
5% level	—	—	—	—	—	—			
		—	—	—	—	—	—		
				—	—	—	—	—	
						—	—	—	—

APPENDIX TABLE 24 : Lesion development on 7 'un-named' cultivars, Frensham (F) and Allgold (A) with an isolate of *D. rosae* (F-77-Ash) : Experiment 2.

Lesion diameter (mm)

Replicate	J5	K3	K4	K8	K9	L1	L4	Controls F	A
1	2.0	6.5	3.5	10.0	5.0	5.5	2.0	8.5	7.0
2	0	7.5	2.5	9.5	0	8.5	0	5.5	7.5
3	0	7.0	2.0	9.5	0	7.0	0	6.0	7.5
4	0	8.0	2.0	7.5	0	9.0	0	3.5	4.5
5	0	6.0	2.0	9.0	0	6.0	0	5.0	6.0
6	0	7.5	0	9.0	0	6.0	0	6.0	0
7	0	6.0	0	9.0	0	7.5	0	6.0	0
8	0	9.5	0	8.0	0	7.0	0	4.5	0
Mean	0.25	7.25	1.5	8.94	0.63	7.06	0.25	5.63	4.06

Analysis

Analysis of variance

Factor	D.F.	S.S.	M.S.	F.
Treatments	8	741.67	92.71	33.84***
Error	61	166.91	2.74	
Total	69	908.58		

S.E. = ± 0.58

Differences between cultivars (Duncan's new multiple range test)

J5 L4 K9 K4 A F L1 K3 K8

1% level

5% level

APPENDIX TABLE 25 : Lesion development on 7 'un-named' cultivars, Frensham (F) and Allgold (A) with an isolate of *D. rosae* (F-78-G.H.) : Experiment 3.

Lesion diameter (mm)

Replicate	J5	K3	K4	K8	K9	L1	L4	Controls F	Controls A
1	5.5	6.0	7.5	8.0	2.5	6.0	7.0	7.0	5.5
2	5.0	6.0	6.5	7.0	3.0	8.0	4.5	8.0	7.5
3	5.0	5.5	7.0	6.0	0	6.5	6.5	5.5	8.0
4	6.0	4.5	6.5	7.5	0	8.5	5.5	6.0	7.5
5	7.0	4.5	7.5	7.0	0	8.5	6.5	7.5	9.0
6	6.5	5.0	9.5	7.0	0	6.5	5.5	6.0	7.5
7	6.0	6.5	7.0	9.0	0	4.5	7.5	5.5	5.0
8	6.0	6.5	5.0	6.0	0	5.5	6.0	6.0	6.5
9	4.0	5.0	0	7.5	0	5.5	5.5	7.5	7.5
10	7.5	0	0	0	0	7.5	5.5	6.0	8.0
Mean	5.85	4.95	6.0	6.5	0.55	6.7	6.35	6.5	7.2

Analysis

Analysis of variance

Factor	D.F.	S.S.	M.S.	F.
Treatments	8	320.96	40.12	15.55***
Error	81	208.7	2.58	
Total	89	529.66		

S.E. = ± 0.51

Differences between cultivars (Duncan's new multiple range test)

	K9	K3	J5	K4	L4	F	K8	L1	A
1% level		———	———	———	———	———	———	———	
			———	———	———	———	———	———	———
5% level		———	———	———					
			———	———	———	———	———	———	———