Scanning Microscopy

Volume 9 | Number 3 Article 23

9-8-1995

Low Temperature Techniques as a Tool in Plant Pathology

Sigrun Hippe-Sanwald
University of Kiel

Scanning Microscopy, Vol. 9, No. 3, 1995 (Pages 881-899) 0891-7035/95. 5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

LOW TEMPERATURE TECHNIQUES AS A TOOL IN PLANT PATHOLOGY

Sigrun Hippe-Sanwald*

University of Kiel, Institute of Phytopathology, D-24098 Kiel; Germany

(Received for publication December 19, 1994, and in revised form September 8, 1995)

Abstract

In plant pathology, low temperature preparation techniques now appear to be feasible methods to stabilize the dynamic ultrastructure of the host-(plant)-pathogen (fungi) interaction for an analysis by transmission electron microscopy. A well defined ultrastructure of small organisms (fungi) and large biological samples such as plant material and as well as the plant-pathogen (fungus) infection sites are presented. The mesophyll tissue of *Arabidopsis thaliana* is characterized by homogeneously structured cytoplasm closely attached to the cell wall. Infection sites of stem rust (*Puccinia graminis* f. sp. *tritici*) on primary leaves of wheat (*Triticum aestivum*) and powdery mildew (*Erysiphe graminis* f.sp. *hordei*) on coleoptiles of barley (*Hordeum vulgare*) are analyzed with regard to the fine structural preservation of the haustoria, the extrahaustorial matrix and the extrahaustorial membrane. Recent data on the immunocytochemical characterization of freeze substituted rust and powdery mildew infected plant tissue are described with special emphasis on the localization of elicitor glycoproteins involved in the cellular host-parasite interaction. There is clear evidence for the release of the elicitor glycoprotein into the extrahaustorial matrix of the haustorial complex. No elicitor molecules are seen in the plant host cytoplasm.

Key Words: Cryofixation, freeze substitution, low temperature embedding, fine structural preservation, extracellular material, fungi, wheat, barley, conidia, hyphae, haustoria, plant pathogen interface, extrahaustorial matrix, extrahaustorial membrane, immunocytochemistry, protein antigenicity, elicitor.

*Address for correspondence and present address:
Sigrun Hippe-Sanwald
Department of Agricultural Biology,
Korea University, College of Natural Resources,
#1 Anam-Dong, Sungbuk-ku,
Seoul 136-011, Korea
Telephone / FAX number: 82-2-7939326

Introduction

The introduction of low temperature (LT-) preparation techniques can be regarded as a major advance in biological electron microscopy in recent years. Biological specimens prepared by cryofixation (propane-jet freezing, plunge freezing, high pressure freezing, etc.) and freeze substitution in combination with LT-embedding may exhibit superior preservation to those prepared by conventional methods. This is because freeze-substituted specimens are rapidly immobilized by cryofixation. After preparation under low temperature conditions, they retain most of their water, forming the hydration shells of the cellular components.

High pressure freezing followed by freeze substitution and LT-embedding has been the method of choice for superior preservation of plant specimens (Gilkey and Staehelin, 1986; Kiss *et al.*, 1990; Lancelle *et al.*, 1986; Studer *et al.*, 1989). However, potential limitations of high pressure freezing should also be considered, following observations of significant disruption of large peripheral vesicles in sporangia of *Phytophthora cinnamomi* and *P. palmivora* (Hyde *et al.*, 1991c, 1993). The data published to date fall into two categories: those that explore the potential of freeze substitution (in combination with adequate freezing techniques) for the preservation of the structural integrity of various biological systems; and those that employ freeze substitution as a tool for stabilizing soluble components and sensitive antigenic sites. The majority of studies fall into the first category, i.e., improved visualization of the fine structure including the detection of novel features of cellular organization (Hippe-Sanwald, 1993).

In plant pathology as well as in mycology, LT-preparation techniques are regarded as important tools in stabilizing the dynamic ultrastructure of the host-(plant)-pathogen (fungi) interaction as it exists under physiological conditions (Hippe-Sanwald, 1993; Read, 1991). In particular, LT-preparation prevents major conformational changes of proteins, leading to better retention of protein antigenicity. Presumably, the hydration shells of proteins are better preserved at low temperatures; this

Abbreviations

b	blebbing
C	chloroplast
cw	cell wall
CY	cytoplasm
EC	epidermal cell
em	extrahaustorial membrane
ema	extrahaustorial matrix
er	endoplasmic reticulum
ERL	vinyl cyclohexene dioxide
FS	freeze substitution
g	glycogen
go	Golgi-body
h	Haustorium
hcw	haustorial cell wall
HP-	High Pressure-
IC	intercellular space
ihy	intercellular hyphae
LV-	Low Viscosity-
LT-	Low Temperature-
M	mitochondrion
MC	mesophyll cell
MT	microtubules
N	nucleus
V	vacuole

---

enhances the accessibility of antigenic sites (Bendayan, 1984; Kellenberger *et al.*, 1987; Monaghan and Robertson, 1990). A combined cell biological and molecular analysis of the mechanisms of host-pathogen relationship is now available. Consequently, an increasing number of investigations are dealing with the immunocytochemical characterization of fungal antigenic sites.

Two important criteria are prerequisites for optimum LT-preparation. First, the biological tissue has to be cryofixed to maintain the complex integrity of the cell, trapping all the solutes, macromolecules and membranes without segregation effects and damage caused by ice-crystallization. Second, the sensitive biological system has to be prepared for a fine structural and molecular analysis. This can be done in the frozen-hydrated state involving cryosectioning and freeze-fracture techniques or in the resin embedded state involving conventional thin sectioning. For the latter procedure, freeze substitution (FS) or freeze drying of the frozen material and embedding preferentially under low temperature conditions is necessary.

One of the main goals in modern plant pathology is to analyse the nature and dynamics of the host-parasite interface in the native state (*in situ*). The understanding of the recognition between host and pathogen, the nature of pathogenesis, and the genetics of host pathogen interactions are of particular interest. The introduction of "Low temperature techniques as a tool in cellular plant pathology" opened new perspectives for an answer of many fundamental questions. The integration of improved fine structural preservation with a molecular analysis of the infected host cell is achieved by the application of secondary probing techniques, i.e., immunocytochemistry.

In this short review on low temperature techniques in the study of fungi and fungus-plant interactions, a broad survey on ultrastructural aspects of freeze substituted plant pathogenic fungi and other pathogens like bacteria and viruses is not provided. The paper is focussed on certain foliar pathogens and a brief survey of the main literature is given. Examples of excellent visualization of the ultrastructure of extracellular material, small organisms (fungi) and large biological samples, such as, plant tissue as well as the plant-pathogen (fungus) interface are presented. Recent data on the molecular characterization of rust or powdery mildew infected plant tissue are exemplified with special emphasis on the subcellular detection of elicitor glycoproteins known to induce cell death. Even though it is not known how cell death is induced, the process is related to the hypersensitive resistance response against stem rust in wheat lines.

Materials and Methods

Plant materials

The near-isogenic barley (*Hordeum vulgare* L.) lines, CI-16137 (AlgR), with the Ml-a1 powdery mildew resistance allele, and CI-16138 (AlgS), with the ml-a allele for susceptibility, were used. Barley seeds were surface disinfected with bleach and then allowed to germinate on Petri plates of potato dextrose agar (Klecan *et al.*, 1990). Seeds of near-isogenic wheat (*Triticum aestivum* L.) lines, Prelude-Sr5, carrying the Sr5 allele for the stem rust resistance, and Prelude-sr5, with the sr5 allele for susceptibility, were used. For comparison, healthy seedlings of *Arabidopsis thaliana* were grown using aseptic techniques (Fig. 1). The conditions for growing and maintaining the plant cultures are described in the literature (Hippe-Sanwald *et al.*, 1992, 1994; Klecan *et al.*, 1990).

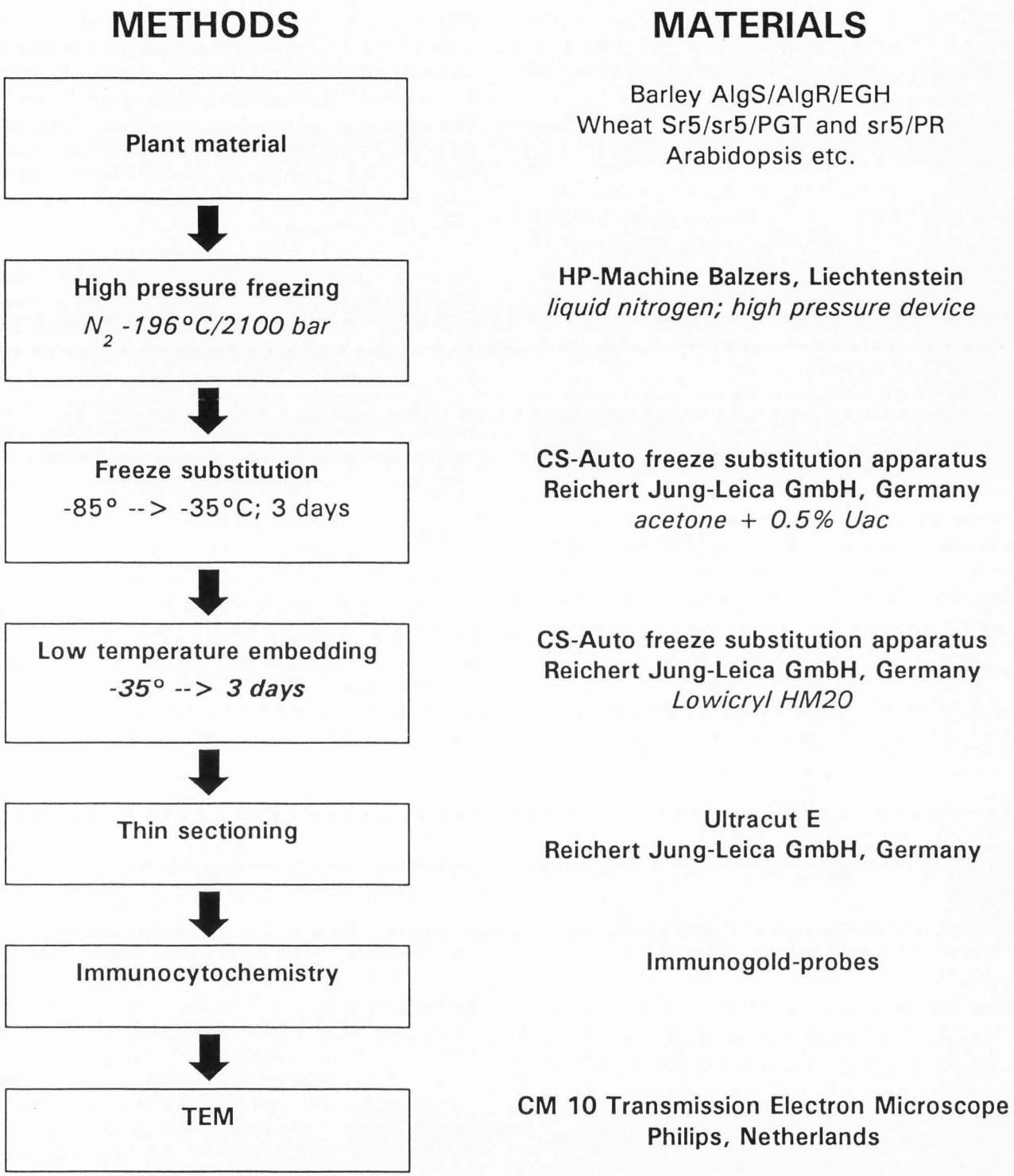


Figure 1. Schematic overview of the low temperature methods and the materials used.

Fungal material and inoculation

Uredospores of the wheat stem rust pathogen, *Puccinia graminis* f.sp. *tritici* Eriks. and E. Henn., race 32, which contain, among other genes for avirulence, the P5 avirulence allele, were collected from adult, greenhouse-grown plants of the wheat cultivar, Little Club. *Erysiphe graminis* DC.: Fr. f.sp. *hordei* Em Marchal [= *Blumeria graminis* (DC.) E.O. Speer f.sp. *hordei* Marchal], race CR3, was maintained under sterile conditions on leaf segments of AlgS or Manchuria (Cl-2330) in Petri

dishes (Klecan *et al.*, 1990). The inoculation of the wheat and barley plants with uredospore suspensions or conidia powder using a settling tower were identical to methods already documented (Hippe-Sanwald *et al.*, 1992, 1994; Klecan *et al.*, 1990).

Cryofixation

The most crucial step of any cryotechnique is a good cryofixation by rapid freezing. High pressure freezing offers the option of freezing even large plant samples of 50 to 200 μm near vitrification. Stem rust- and leaf rust-infected wheat leaves and healthy *Arabidopsis* leaf material were frozen under high pressure using an HPM 010 device [(Fig. 1) (BAL-TEC GmbH, Balzers, Liechtenstein)].

For the cryofixation procedure, infection sites on wheat leaves were screened under a stereo microscope at 5-6 days after inoculation. Small plant tissue pieces, each containing a rust colony, were removed with a hole punch (1.5 mm in diameter), transferred into 1-hexadecane and evacuated for 5-10 minutes to fill intercellular gas spaces. The shock freezing procedure of the specimens including *Arabidopsis* leaf material under liquid nitrogen at -196°C at a hydrostatic high pressure of 2.5 x 108 Pa (2500 bar) was according to Hippe-Sanwald (1993) and Hippe-Sanwald *et al.* (1994). After freezing, the sample was immediately transferred to liquid nitrogen for storage.

The powdery mildew disease is particularly suited to low temperature methods because the infection sites are confined to one cell layer. For rapid freezing of infection areas of powdery mildew on barley coleoptiles with the Gilkey-Staehelin Propane-Jet Ultra-Rapid Freezer (Research & Manufacturing Co., RMC, Tucson, AZ, USA; Gilkey and Staehelin, 1986), the fungus was identified with a stereo microscope at 3 days post-inoculation and excised to give an epidermal sample that was one cell layer thick (Hippe, 1985; Hippe-Sanwald, *et al.*, 1992).

Freeze substitution

Biological molecules (nucleic acids, proteins etc.) are surrounded by hydration shells of pure water on the surface of the molecules. During freeze substitution, a preservation of the surface-bound water on the molecules occurs (Hippe-Sanwald, 1993). Freeze substitution and LT-embedding of the HP-frozen samples were carried out in a commercial automatic CS Auto apparatus (Reichert Jung, CS Auto; Leica GmbH, Hamburg, Germany). The frozen specimen sandwiches were opened under liquid nitrogen, transferred to the CS Auto while attached to one of the platelets, and substituted with 0.5% uranyl acetate and 0.5% glutaraldehyde in anhydrous acetone over 3 days (Fig. 1). Freeze substitution was performed as described previously (Hippe-Sanwald *et al.*, 1994). Freeze substitution of the powdery mildew infected barley samples was done according to Hippe-Sanwald *et al.*, (1992).

Figure 2. Cross-sectioned germ tube of *Puccinia graminis* f.sp. *tritici* processed according to low temperature preparation using HP-freezing, freeze substitution and Lowicryl HM20 embedding (Hippe-Sanwald, 1993). The germ tube contains dense cytoplasm. Numerous ribosomes, small mitochondria and vacuoles are visualized. Superior retention of a non-collapsed coat of extracellular matrix (arrows) attached to the cell wall (cw). Bar = 0.5 μm.

Figure 3. Overview of the fine structure of freeze substituted hypha of powdery mildew, *Erysiphe graminis* f.sp. *hordei*, grown on the surface of barley leaf; low temperature preparation according to Hippe (1985). Homogeneously preserved cytoplasm composed of smooth cytoplasmic matrix, the nucleus (n), a high amount of ribosomes, peripherally arranged mitochondria (m), endoplasmic reticulum (er), and vacuoles (v). Small ring-like or rod-like Golgi-bodies (arrow) and mitochondria (m) are smoothly contoured. Bar = 0.5 μm.

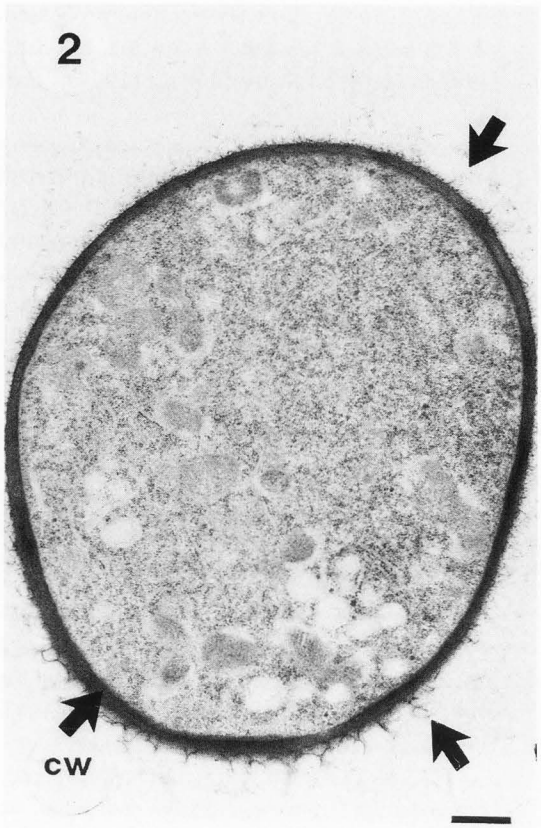
Figure 4. Conidium of powdery mildew, *Erysiphe graminis* f.sp. *hordei*, processed according to Hippe (1985). Numerous glycogen particles (g) a few vacuoles (v) are seen. The periphery of the conidium exhibits a small area of cytoplasm. Bar = 1 μm.

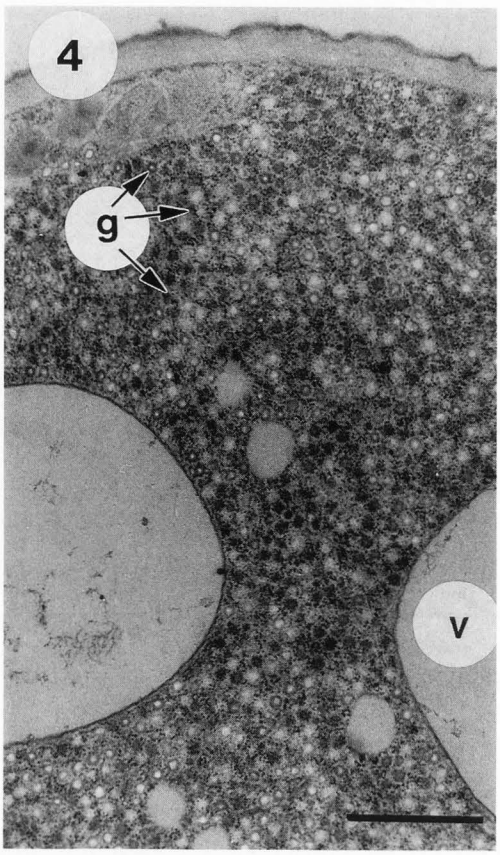
Embedding

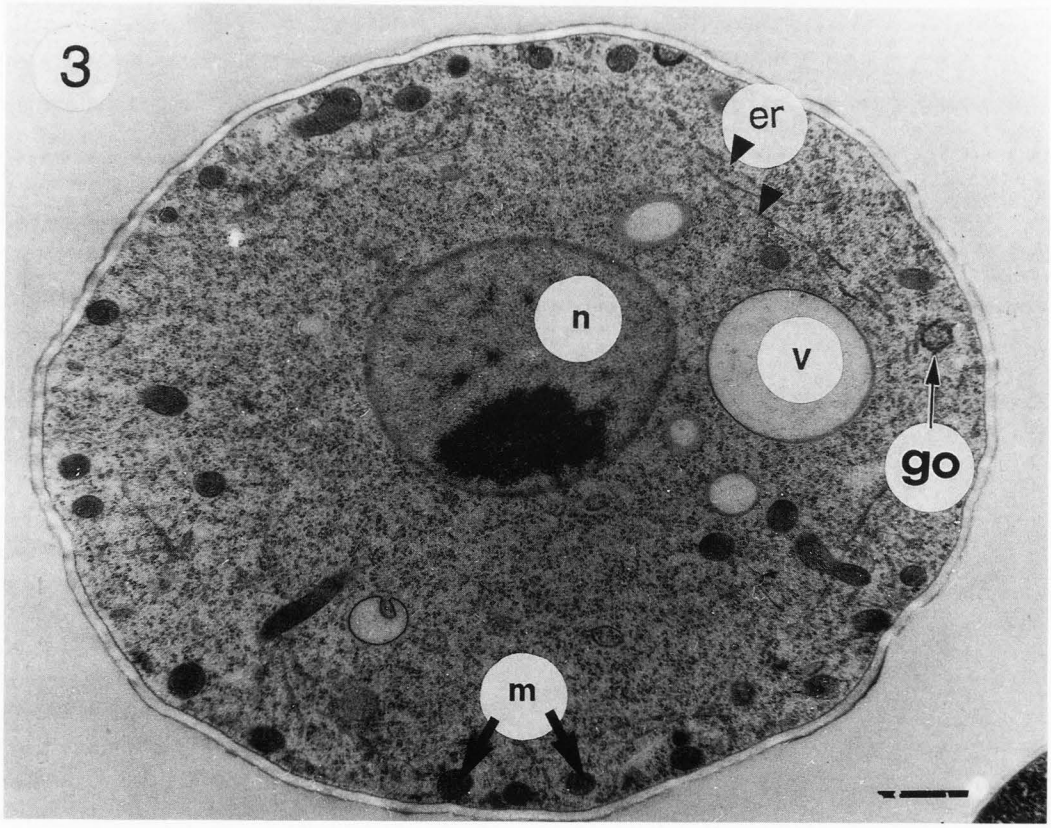
Following the freeze substitution, the objects were embedded at low temperatures using Lowicryl resin embedding kits. Lowicryl embedding permits lowering the temperatures considerably during the infiltration and polymerization processes (Fig. 1). Since Lowicryl resins are compatible with water the hydration shells on the surface on the molecules are preserved (Hippe-Sanwald, 1993). The low temperature-embedding was carried out in three stages, with a progressively increasing ratio of resin to acetone. At the end of the infiltration process the specimens were transferred into flat embedding molds and polymerized by diffuse UV irradiation (Hippe-Sanwald *et al.*, 1992, 1994).

Thin sectioning

Thin sections from various blocks were cut with a DIATOME diamond knife at a thickness of about 60 nm on a Reichert-Jung Ultracut E Ultramicrotome (Fig. 1). The sections were mounted on pioloform and carbon-coated (Auto 306 Turbo Coating Device, Edwards High


2
cw


4
g
v


3
er
n
v
go
m

Vacuum, Marburg, Germany) nickel grids, stained with 8% aqueous uranium acetate and alkaline lead citrate for 10 minutes each and examined either with an EM10 electron microscope (Zeiss, Oberkochen, Germany) operating at 60 kV (Hippe-Sanwald *et al.*, 1992) or with a Philips CM10 Electron Microscope (Philips, Eindhoven, The Netherlands) operating at 80 kV (Hippe-Sanwald *et al.*, 1994).

Immunocytochemistry

Because low temperature preparation achieves a superior localization of fixation-sensitive antigenic sites and minimizes the molecular distortion and movement of molecules, the biological tissue can be evaluated by immunocytochemistry. For the immunocytochemical detection of the PGT (*Puccinia graminis* f.sp. *tritici)* elicitor in the haustorial complexes of the stem rust and powdery mildew diseases, a monoclonal mouse anti-elicitor (MAB-E14e; MAB-E10e) antibody was applied (Tiburzy *et al.*, 1991). Immunostaining was performed according to previously described methods (Hippe *et al.*, 1989; Hippe-Sanwald *et al.*, 1994).

Results and Discussion

Theoretical considerations and experimental results obtained with low temperature preparation techniques in biological electron microscopy support the view that these techniques are a prerequisite for maintaining native cell and tissue properties (Gilkey and Staehelin, 1986; Hippe-Sanwald, 1993; Humbel and Müller, 1986; Robards and Sleytr, 1985). Ultrastructural features have become increasingly important for the analysis of host-pathogen relationships. In this context, freeze substitution techniques may provide new insight into ultrastructural characteristics.

Macromolecules and extracellular material in plant pathogenic fungi

Studies on the preservation of the fine structure of freeze substituted macromolecules, such as heterogeneous chromatin, bundles of microfilaments and microtubules as well as extracellular material of plant and fungal cells are available in the literature (Aist and Bayles, 1991a, 1991b; Bourett and Howard, 1992; Carlemalm *et al.*, 1986; Ding *et al.*, 1991; Hippe and Hermanns, 1986; Horowitz *et al.*, 1990; Jensen *et al.*, 1991; O'Donnell, 1992; Roberson, 1992; Studer *et al.*, 1989).

The fungal chromatin of the sporidia of the smut fungi *Ustilago avenae* is coarse when prepared conventionally at room temperature (Hippe, 1984) but less coarse when ultrarapidly frozen and cryosubstituted at 193K (Hippe and Hermanns, 1986). In the latter case, the DNA of *Ustilago avenae* sporidia and the nuclear

Figures 5 and 6. Low temperature processed leaf tissue of *Arabidopsis thaliana* according to Hippe-Sanwald (1993) and Hippe-Sanwald *et al.* (1994). Bars = 5 μm.

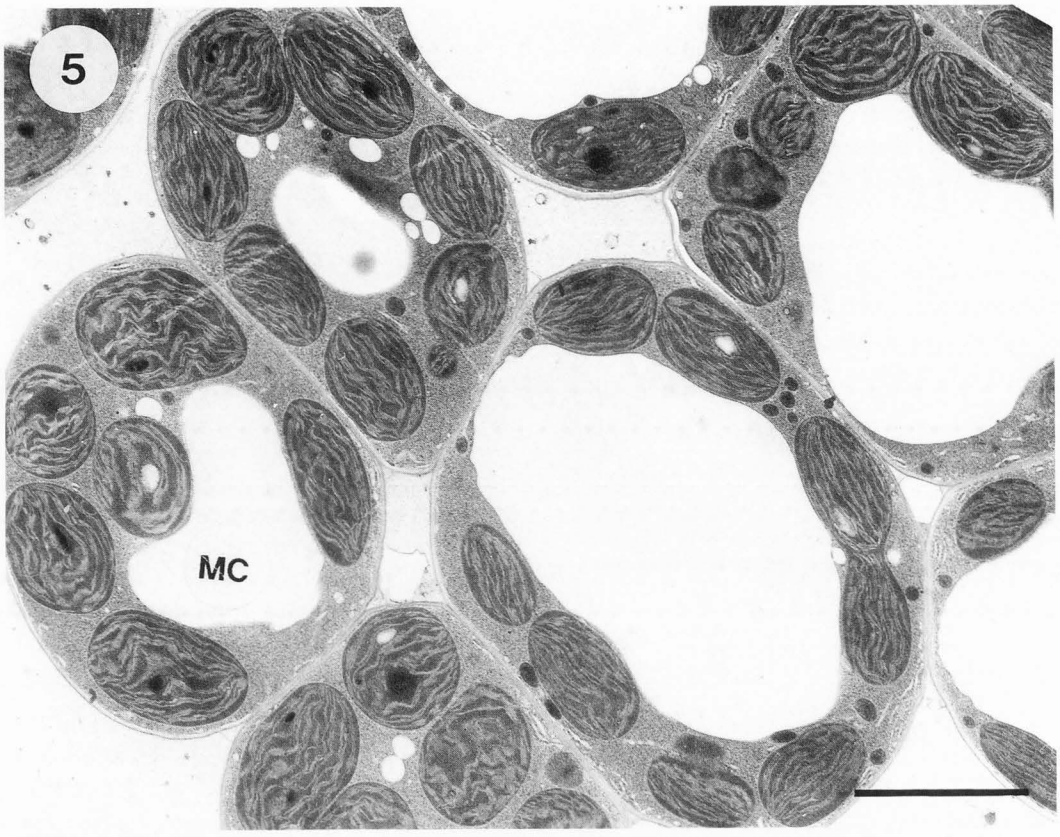
Figure 5. Homogenously structured mesophyll tissue of *Arabidopsis thaliana.* Large areas of intercellular space are seen between the mesophyll cells (MC). The distinctly structured mesophyll cytoplasm containing large chloroplasts and small, darkly stained mitochondria are arranged at the periphery of the cell. A large electron transparent vacuole is situated in the centre of each cell.

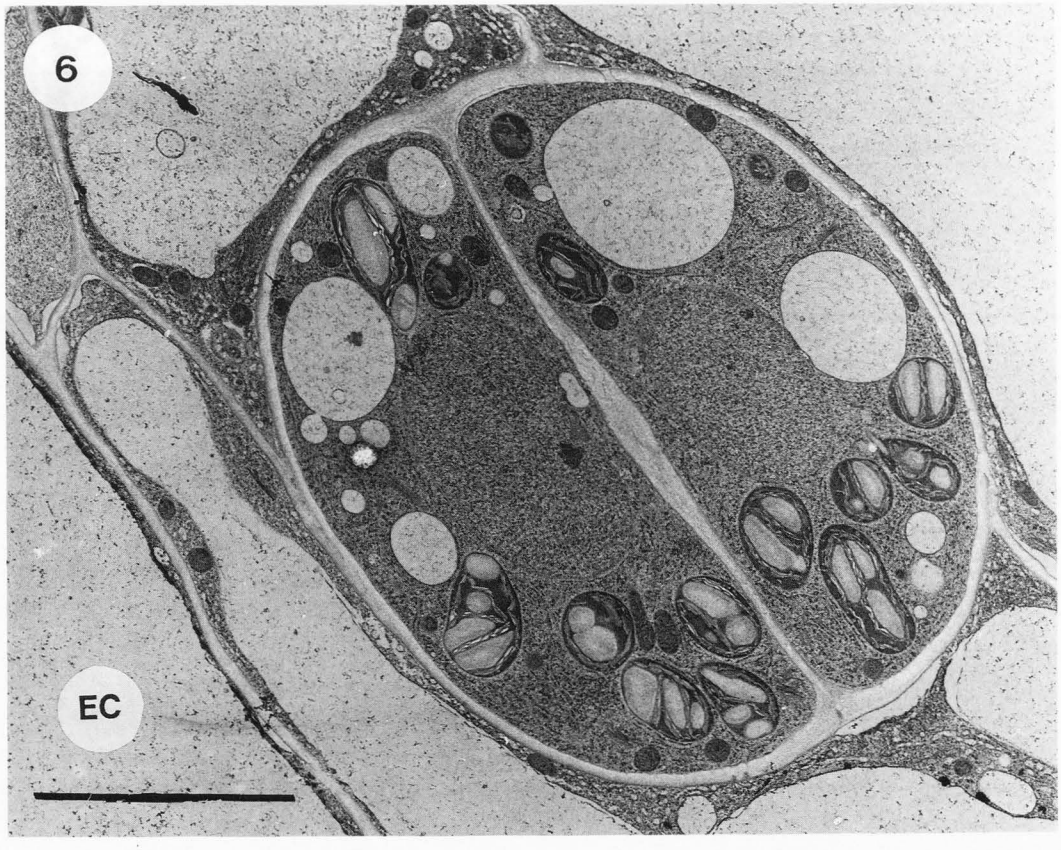
Figure 6. Section of epidermal tissue of *Arabidopsis thaliana* exhibiting an excellently preserved stomata surrounded by epidermal cells (EC). Chloroplasts, nuclei and vacuoles are seen in the cytoplasm of the stomata cells. A thin layer of cytoplasm is closely attached to the cell walls of the epidermal cells.

---

structures of basidia of *Ustilago maydis* are homogeneously distributed in a distinctly structured fungal cytoplasm containing numerous ribosomes (Hippe and Hermanns, 1986; O'Donnell, 1992).

Complex fungal extracellular material including characteristic fimbriae, fibrillar coating of appressoria, as well as the fungal extrahaustorial matrix that are usually collapsed and washed out after conventional preparation are well preserved by the LT method (Clement *et al.*, 1993b; Hippe *et al.*, 1989; Knauf *et al.*, 1989; Kwon *et al.*, 1991; van Dyke and Mims, 1991). Evidence has been given for an involvement of the extracellular matrix of various plant pathogenic fungi in non-specific attachment to the host surface, which is regarded as a prerequisite for successful infection (Clement *et al.*, 1993b; Hamer *et al.*, 1988). It is suggested that the extracellular matrix associated with rust germlings is also involved in signal transduction (Epstein *et al.*, 1985, 1987). However, the composition of extracellular matrix from only a few fungi has so far been examined (Bergstrom and Nicholson, 1981; Epstein *et al.*, 1985; Kunoh *et al.*, 1990; Pringle, 1981; Tunlid *et al.*, 1991). The release of extracellular matrix during the pre-invasion phase on the host plant has been observed during the germination of urediospores of *Uromyces appendiculatus* and *Uromyces viciae-fabae* clearly visualized by LT-preparation (Clement *et al.*, 1993a; Kwon *et al.*, 1991). The outer cell wall of cross-sectioned germ tubes of *Puccinia graminis* f.sp. *tritici* is also characterized by a distinctly resolved extracellular matrix (Fig. 2). Novel features of the immunogold labeling of the fungal haustorial cell wall and extrahaustorial matrix of biotrophic rust parasites and powdery mildew are discussed below.


5
MC


6
EC

Yeast-mycelium dimorphism

The yeast-mycelium dimorphism is exhibited by a variety of plant pathogenic fungal species. *Ustilago maydis* and *Ustilago avenae*, which cause the smut disease on maize and oat, respectively, represent two examples of dimorphic fungi extensively examined by electron microscopy (Hippe, 1984; Hippe and Hermanns, 1986; O'Donnell, 1992; Snetselaar and Mims, 1994). The transformation of the haploid, yeast-like sporidial phase to the dikaryotic, mycelial stage that is obligately parasitic on the host plant is basically a matter of morphogenesis which involves changes of cell shape, cell wall structure and fine structural features. Early infection processes of *Ustilago maydis* on maize have recently been examined using high pressure freezing followed by freeze substitution and conventional embedding (Snetselaar and Mims, 1994). Conventional electron microscopy on the smut fungi (*Ustilaginales*) has been a problem in the past because thick cell wall layers prevent penetration of fixatives into the cells. Low temperature preparation now has been proved as the method of choice for an improved elucidation of the fine structure of yeast cells including smut fungi (Baba and Osumi, 1987; Hippe and Hermanns, 1986; O'Donnell, 1992). Thus, on the basis of this methodological progress, a revised meiotic spindle pole body cycle for *Ustilago* basidia has been reported (O'Donnell, 1992).

Fungal hyphae

The process of hyphal elongation in filamentous fungi is of basic importance for vegetative cell growth that involves many cytoplasmic activities (Wessels, 1990, 1993). Thus, cytological research is particularly focussed on the cellular organization of the hyphal tip. During the past 15 years, insights into the fine structure of the hyphal apex advanced with the application of cryotechniques in specimen preparation (Heath *et al.*, 1985; Hoch and Howard, 1980; Howard, 1981; Howard and Aist, 1979; Hoch and Staples, 1983; Newhouse *et al.*, 1983; Roberson, 1992; Roberson and Fuller, 1988; Vargas *et al.*, 1993). So far, three types of cytoplasmic organizations of secretory vesicles have been identified by conventional fixation in the apical hyphal region (Grove and Bracker, 1970). In the *Oomycetes*, no particular apical organization of secretory vesicles has been found, whereas a crescent-shaped accumulation of vesicles near the periphery of the apical zone is typical in the *Zygomycetes*. Fungi with septated hyphae (*Ascomycetes*, *Basidiomycetes*, *Deuteromycetes*) contain an apical body called Spitzenkörper. Freeze substituted Spitzenkörper are now described as apical, cytoplasmic regions containing dense aggregation of apical vesicles, microvesicles and filasomes, surrounded by a vesicle-free zone in hyphal tip cells of various fungi (Howard, 1981). The process of apical growth in fungi involves the migration of cytoplasmic, secretory vesicles transporting materials for cell growth from Golgi-equivalents to the hyphal tip (Heath *et al.*, 1985). The actin cytoskeleton visualized by ultrastructural immunocytochemistry of freeze substituted hyphal tips of the basidiomycetous, plant pathogenic fungus *Sclerotium rolfsii*, is suggested to be involved in the coordinated movement of vesicles (Roberson, 1992). In this system, the coat of filasomes contains actin. The data indicate the filasomes represent the ultrastructural equivalent of actin plaques identified by light microscopy using immunofluorescence techniques and are principally localized in the first 10 to 12 μm of the hyphal tip (Roberson, 1992).

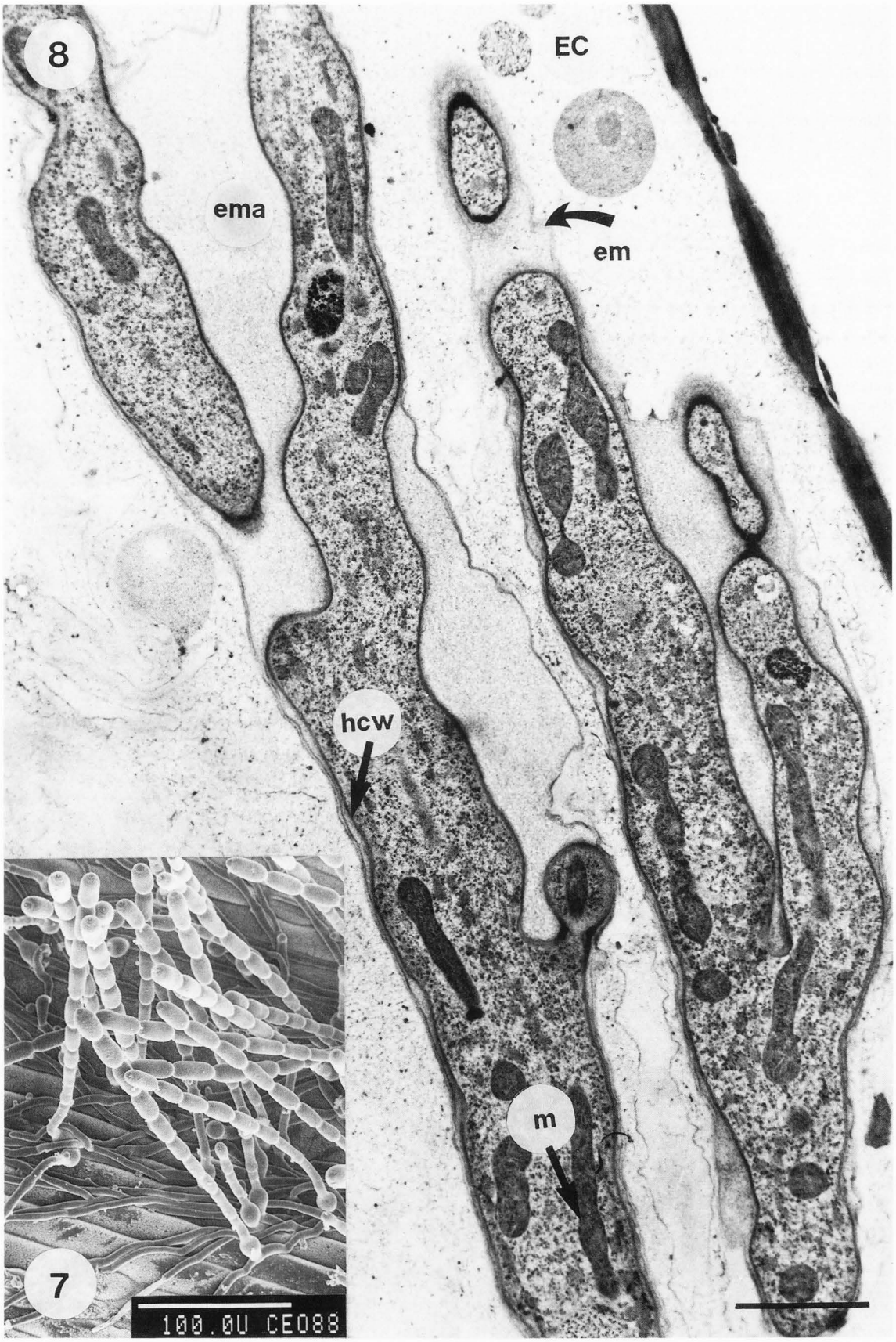
The superior visualization of labile cytoskeletal elements is particularly advantageous for freeze substitution in filamentous hyphae. It enables a better understanding of the functional role of microtubules in organelle positioning, of the mechanisms of mitosis and of other dynamic processes in the cell. Ultrastructural studies on mitosis in fungi like *Zygorhynchus moelleri* (Heath and Rethoret, 1982), various *Saprolegnia* isolates (Heath *et al.*, 1984), and *Basidiobolus haptosporus* (McKerracher and Heath, 1985) are focussed on the arrangement of spindle and kinetochore associated microtubules.

Hyphae of powdery mildew

Transmission electron microscopy (TEM) was used to examine the fine structure of freeze substituted hyphae of powdery mildew, *Erysiphe graminis* f.sp. *hordei*, grown on the leaf surface of barley plants (Fig. 3) (Hippe-Sanwald, 1993). The cross-section exhibits

Figure 7. Scanning electron micrograph demonstrating hyphal growth and conidia formation of powdery mildew, *Erysiphe graminis* f.sp. *hordei*, on the surface barley primary leaves. The preparation for low temperature scanning electron microscopy is according to Klecan *et al.* (1990). Bar = 100 μm.

Figure 8. Ultrastructure of a longitudinally sectioned, finger-like haustorium of *Erysiphe graminis* f.sp. *hordei* preserved by freeze substitution (Hippe, 1985). The haustorium is surrounded by extrahaustorial matrix (ema) and an extrahaustorial membrane (em). The darkly stained haustorium is located in a barley epidermal cell (EC). The sharply contoured extrahaustorial membrane (em) separates the slightly stained host cytoplasm from the fungal extrahaustorial matrix (ema). The material forming the extrahaustorial matrix appears homogeneously structured, closely attached to the haustorial cell wall (hcw). According to the low magnification, only mitochondria (m), ribosomes and the darkly stained haustorial cell wall (hcw) are visible. Bar = 1 μm.


8
EC
ema
em
hcw
m
7
100.0U CE088

typical compartments of a fungal hypha: in a smoothly contoured cytoplasm, the well-preserved nucleus, mitochondria, vacuoles, ribosomes and Golgi-equivalents are seen. Golgi bodies only occur in the Oomycota whereas single cisternae of Golgi-equivalents are found in other fungal divisions. The sensitive detection of Golgi bodies as well as Golgi-equivalents in freeze substituted fungal cells is regarded as a special advantage of LT-preparation since Golgi bodies are usually poorly preserved with conventional procedures (Hoch, 1986).

Fungal reproductive structures and spores

As mentioned above, examples of pre-penetration events often include adherence of spores and germ tubes to leaf surfaces by extracellular matrix and/or fibrillar coating (Clement *et al.*, 1993a; Hamer *et al.*, 1988). Because spores, germ tubes and appressoria are exposed on leaf surfaces, TEM studies are more complicated: specimens on leaves are difficult to find and orient for sectioning. Recently, promising fine structural results have been published on a variety of freeze substituted fungal reproductive structures and spores of *Phytophthora cinnamomi*, *Ascodemis nigricans*, *Colletotrichum truncatum*, *Gymnosporangium juniperi-virginianae*, *Uromyces viciae-fabae* and *Cryphonectria parasitica* (Clement *et al.*, 1993a; Hyde *et al.*, 1991a, 1991b, 1993; Mims, 1991; Mims and Richardson, 1990; Mims *et al.*, 1988, 1990; Newhouse *et al.*, 1990; van Dyke and Mims, 1991). In all cases, freeze substituted specimens yielded information not available in conventionally fixed materials regarding extracellular matrix, and nuclear and cytoplasmic events occurring during early stages of sporulation. This includes modifications of the plasma membrane and the endomembrane system during cell division, as well as multivesicular bodies and cytoplasmic and nuclear microtubules and the Golgi cisternae.

Freeze substituted conidia of powdery mildew *Erysiphe graminis* f.sp. *hordei* appear to be organized differently in comparison to the fine structure of spores of other fungi (Mims *et al.*, 1990; Clement *et al.*, 1993a). Conidia shortly separated from the fungal mycelium on the plant leaf surface contain a large amount of storage material (glycogen) and ribosomes (Fig. 4). Only small amounts of cytoplasm and a few mitochondria are found in the periphery of the conidia. Bundles of microfilaments are commonly preserved just beneath the plasma membrane and the cell wall. The thick cell wall is characterized by an electron-dense outer layer and a translucent inner layer (Fig. 2).

Plant-Fungi Interactions

The sequence of events occurring after spore germination depends strongly upon the type of nutritional relationship the plant-pathogenic fungus exhibits. Many

Figures 9 and 10. Details of the fine structure of a young haustorium of *Erysiphe graminis* f.sp. *hordei* in a barley epidermal cell. The low temperature preparation is according to Hippe-Sanwald *et al.* (1992). Bars = 0.5 μm

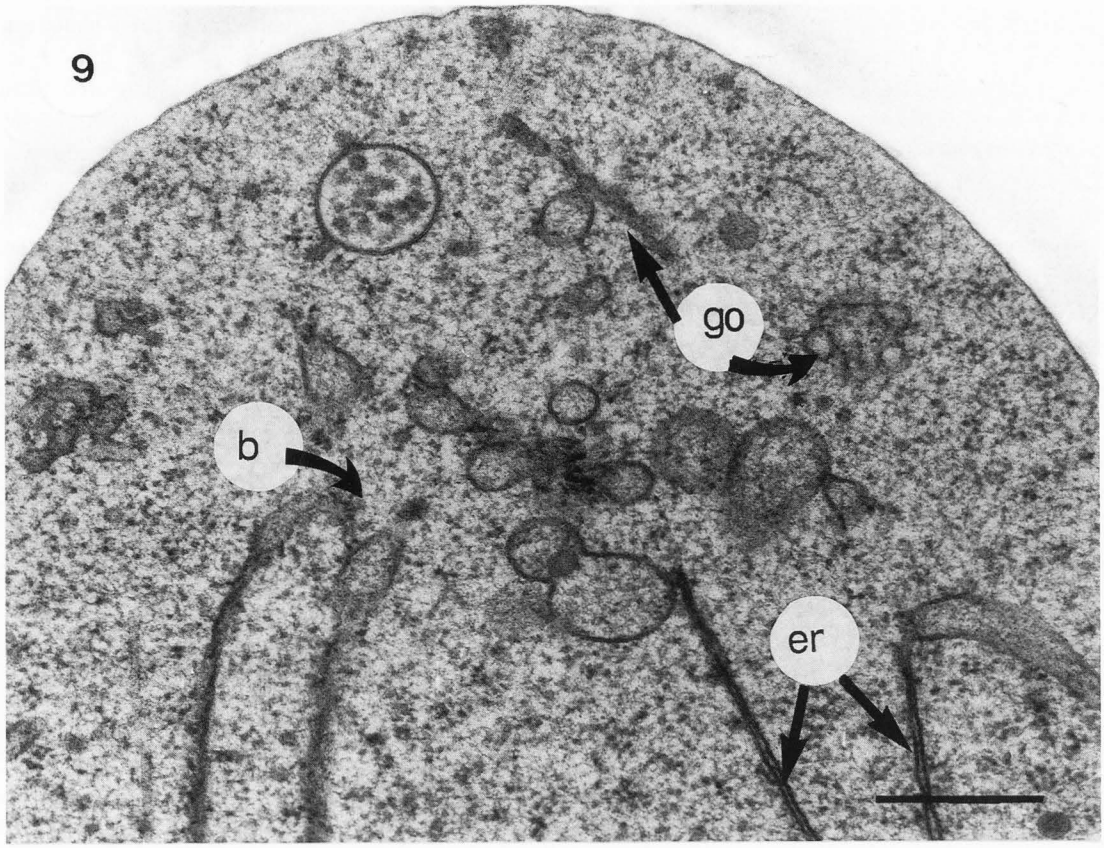
Figure 9. The tip of a haustorial finger illustrating blebbing (b) of the endoplasmic reticulum (er). Golgi-like structures (go) and a superior preservation of the cytoplasm, including microtubules and a fibrillar matrix around the ribosomes, is also seen in this area.

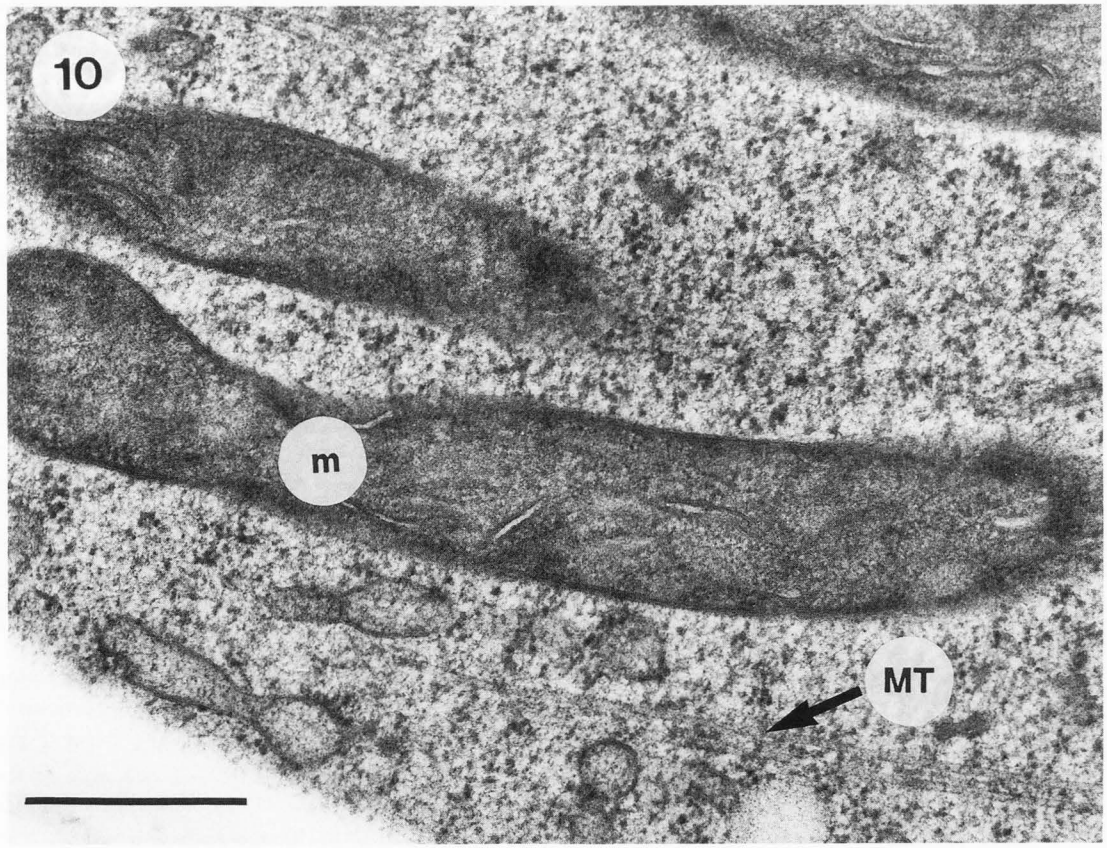
Figure 10. Another example of a freeze substituted fine structure of a young haustorium of powdery mildew under higher magnification. Mitochondria (m) are characterized by smooth cristae in a dense matrix. Extended areas of the endoplasmic reticulum and microtubules (MT) are seen at the periphery of the haustorium.

---

plant-pathogenic fungi produce specialized infection structures termed appressoria. Appressoria are examples of a pre-penetration process on the leaf surface and have been the subject of a considerable amount of research based on conventional as well as on LT-preparation (Bourett *et al.*, 1987; Bourett and Howard, 1990; Clay *et al.*, 1994; Hoch, 1986; Hoch and Staples, 1991; Mims and Richardson, 1989; Swann and Mims, 1991; van Dyke and Mims, 1991). After penetration into the host tissue, biotrophic fungi develop a sequence of specialized infection structures. Haustoria are specialized feeding structures that occur only in living host cells. These structures are surrounded by an extrahaustorial matrix forming the host-parasite interface and enclosed by a specialized extension of the host plasma membrane, the extrahaustorial membrane.

Freeze substitution data so far available in the literature are mostly focussed on the compatible host-pathogen interaction. An ultrastructural comparison of incompatible and compatible interactions in the barley powdery mildew disease based on low temperature preparation has been published recently (Hippe-Sanwald *et al.*, 1992). Fine structural studies have been performed on fungal haustoria of various species: of the apple scab fungus *Venturia inaequalis* on apple leaves (Dahmen and Hobot, 1986; Studer *et al.*, 1988, 1989), of the bean rust *Uromyces appendiculatus* on beans (Knauf *et al.*, 1989; Mendgen *et al.*, 1991; Welter *et al.*, 1988), of the stem rust *Puccinia graminis* f.sp. *tritici* and leaf rust *Puccinia recondita* on wheat (Hippe-Sanwald, 1993; Hippe-Sanwald *et al.*, 1994; Marticke, 1994) and of the powdery mildew *Erysiphe graminis* f.sp. *hordei* on barley (Hippe, 1985; Hippe-Sanwald *et al.*, 1992; Hippe-Sanwald *et al.*, 1994). The progress obtained by the


9
go
b
er


10
m
MT

application of freeze substitution in preserving the cellular fine structure and the antigenicity of proteins of the host-pathogen interface including the extrahaustorial membrane and the extrahaustorial matrix of biotrophic, foliar pathogens such as powdery mildew and stem rust fungi is discussed in the following paragraph.

In many cases of specimen preparation, the preservation of the plant host tissue appears as a particular challenge. The majority of investigations on the fine structure of large plant tissue samples are mostly done with high pressure frozen material. Even though barley, wheat, *Arabidopsis* and tobacco leaf tissue are difficult to preserve, good results are now available in the literature (Ding *et al.*, 1991; Hippe *et al.*, 1989; Hippe-Sanwald, 1993, 1994; Kiss *et al.*, 1990; Staehelin *et al.*, 1990; Studer *et al.*, 1989). As an example, some aspects of the fine structure of freeze-substituted leaf tissue of *Arabidopsis thaliana* are shown in Figures 5 and 6. The well-preserved mesophyll tissue is characterized by large vacuoles and a high number of chloroplasts embedded in a smoothly contoured cytoplasm (Fig. 5). The stomatal cells of *Arabidopsis* epidermal tissue contain homogenous cytoplasm, small chloroplasts and vacuoles, a nucleus in each cell and mitochondria (Fig. 6).

Fine structure of haustoria of powdery mildew

Powdery mildews are world-wide plant diseases affecting all kinds of plants: cereals, vegetables, grasses, fruit trees and many other plant species. The cytology of the infection stages of the *Erysiphales* in general, and of the host-parasite interaction of *Erysiphe graminis* on cereals in particular, has been investigated extensively (Carver, 1988; Kunoh, 1982). The fungus is characterized by the appearance of patches of a white powder on the plant surface. By cryogenic scanning electron microscopy, the fungus can be excellently identified as a network of hyphae from which conidia are produced in long chains (Fig. 7). Conidia germinate directly on the plant surface, forming short germ tubes and appressoria prior to penetration into the epidermal plant cell. Inside the epidermal cell, the fungus forms haustoria of typical finger-like structure (Fig. 8). As the infection takes place only in the epidermis, superior preservation of the haustorial fine structure following low temperature methods of sample preparation is possible (Figs. 4, 6 and 7) (Hippe, 1985; Dahmen and Hobot, 1986; Hippe-Sanwald *et al.*, 1992).

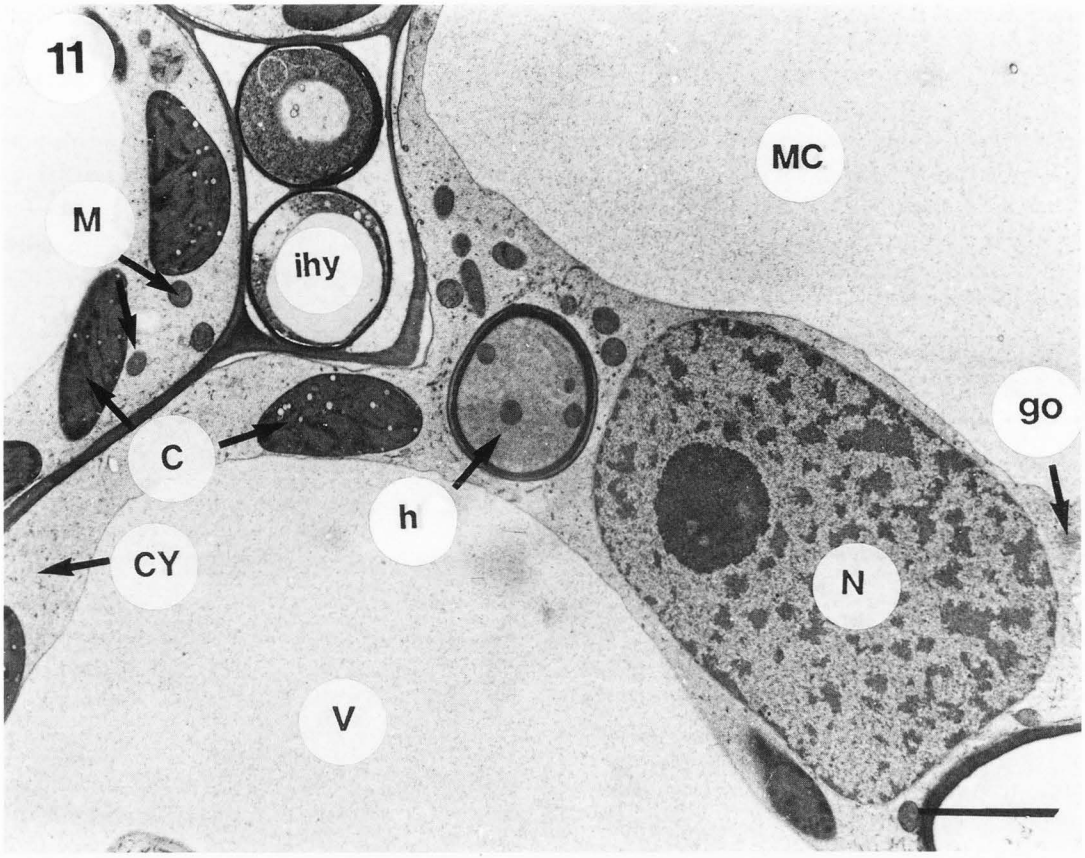
The extrahaustorial matrix contains smooth, fibrillar material accumulated around the haustorium (Fig. 8). It is thought that the extrahaustorial matrix contains nutrients in transit from the host plant to the pathogen (Harder, 1978; Knauf *et al.*, 1989). The identification of the biochemical nature of interfacial components in the extrahaustorial matrix of obligate, biotrophic pathogens by immunocytochemistry is discussed below.

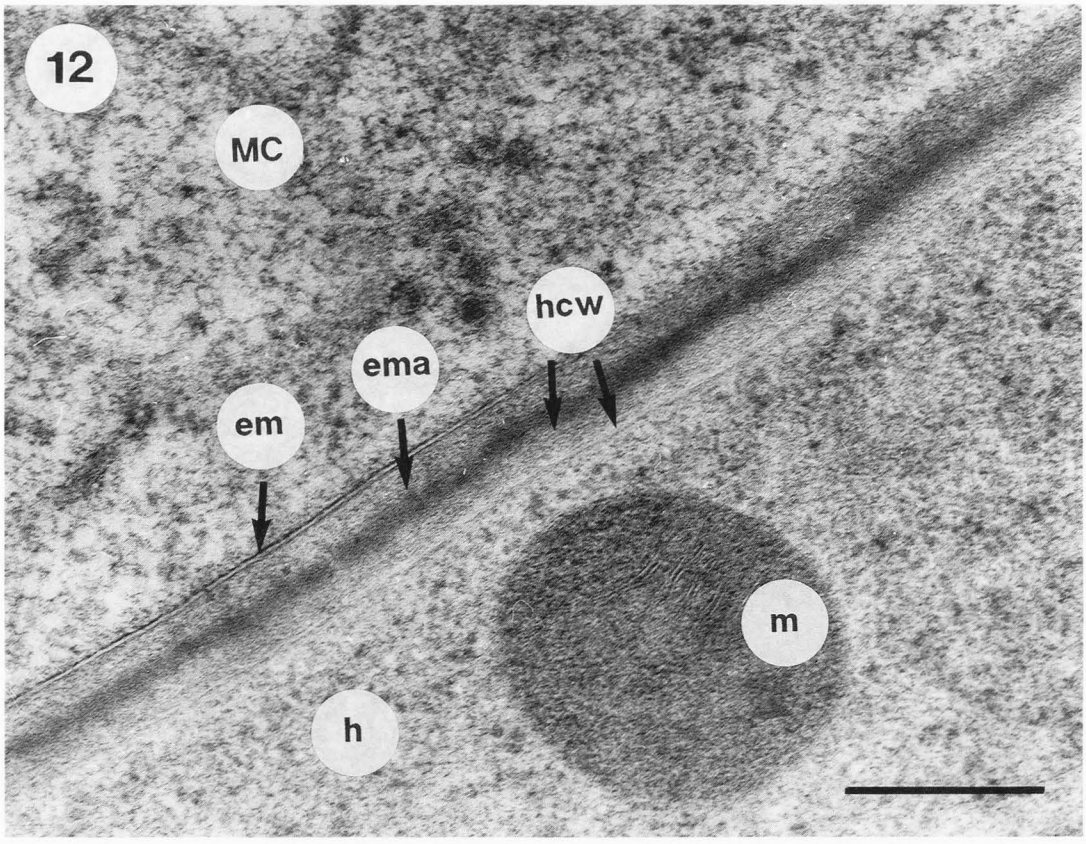
Figure 11. Infection site containing a haustorium (h) of stem rust (*Puccinia graminis* f.sp. *tritici*) and cross-sectioned intercellular hyphae (ihy) located outside of the mesophyll cell (MC) of the incompatible wheat line Prelude-Sr5. No hypersensitive host cell response is apparent in this case. The fine structural preservation is according to Hippe-Sanwald *et al.* (1994). The haustorium is enclosed by smooth host cytoplasm (CY). A round-shaped haustorium (h) occurs inside the mesophyll cell. The haustorial cell wall and the extrahaustorial matrix are darkly stained. In the vicinity of the fungal haustorium, a large host nucleus (N), vacuoles (V), chloroplasts (C), small mitochondria (M) and a Golgi-body (go) are seen. Bar = 3 μm.

Figure 12. Plant-pathogen interface of *Puccinia graminis* f.sp. *tritici* haustoria (h) on compatible wheat cultivar, Little Club, processed by high pressure freezing, freeze substitution and subsequent Lowicryl HM20 embedding according to Hippe-Sanwald *et al.* (1994). The host cytoplasm of the mesophyll cell (MC) is separated from the biotrophic pathogen by the extrahaustorial matrix (ema) and the distinctly resolved extrahaustorial membrane (em). The extrahaustorial matrix (ema) is closely attached to the haustorial cell wall (hcw). The haustorial cell wall is composed of two layers, an inner electron translucent layer and an outer electron dense layer. The fungal plasma membrane as well as a mitochondrion (m) are distinctly resolved. Bar = 0.25 μm.

The haustorial cytoplasm appears well-defined and smoothly contoured. Mitochondria appear sharply resolved and small ring-like or rod-like Golgi-equivalents are visible (Figs. 9 and 10). One striking feature of young haustoria is the proliferation of vesicular bodies primarily in the periphery of the haustorial central body and in the tips of the haustorial fingers (Figs. 9 and 10). A direct relationship between the vesicles and the endoplasmic reticulum can be inferred from the close association of these two membrane systems (Fig. 9) (Hippe-Sanwald *et al.*, 1992). These vesicular aggregates are reminiscent of the tubular-vesicular complexes observed in haustoria of the bean rust pathogen following high-pressure freezing and freeze-substitution (Knauf *et al.*, 1989).

With the advent of improved, low temperature methods of sample preparation, the incompatible interaction between barley carrying the *Ml-a 1* resistance gene and *Erysiphe graminis hordei* was re-evaluated (Hippe-Sanwald *et al.*, 1992). A striking degradation of haustoria was found in the resistant barley, comprising clearing of the matrix and loss of cristae in the


11
MC
M
ihy
go
C
h
CY
N
V


12
MC
hcw
ema
em
m
h

haustorial mitochondria, absence of distinct endoplasmic reticulum and Golgi-like cisternae, the formation of vacuoles, and the occurrence of a distended sheath. As a clear result, alterations in the mitochondrial ultrastructure preceded all other signs of degradation in haustoria found in the resistant barley line. Possibly, an early event in the expression of *Ml-a 1* resistance in barley may be a disruption of mitochondrial function leading to other degradative, fine structural changes outlined above (Hippe-Sanwald *et al.*, 1992).

In comparison, the haustoria that develop in susceptible barley lines appear highly metabolically active. A striking electron dense matrix surrounding the cristae membranes is a typical feature found in the haustorial mitochondria of powdery mildew in the compatible interaction (Fig. 10) (Hippe-Sanwald *et al.*, 1992).

Fine structure of haustoria of rust fungi

The stem rust fungus *Puccinia graminis* f.sp. *tritici* (Pgt) attacks all above ground parts of the wheat plant. Both incompatible and compatible interactions between the stem rust fungus *Puccinia graminis* f.sp. *tritici* and wheat, as well as compatible interactions between leaf rust *Puccinia recondita* and wheat, have been investigated at the electron microscopic level using low temperature preparation techniques (Hippe-Sanwald *et al.*, 1994; Hippe-Sanwald, 1993; Particke *et al.*, in preparation). It must be emphasized that for satisfactory preservation of fungi such as rust growing deeper in the parenchymal plant tissue, freeze substitution is only worthwhile using high pressure frozen material (Hippe-Sanwald, 1993; Mendgen *et al.*, 1991).

Results on small areas of adequately preserved fungal infection structures of *Pgt* in parenchymal leaf tissue of the resistant Prelude-Sr 5 wheat plant are presented (Fig. 11). Occasionally, spaces resulting from a slight shrinkage of the plant tissue are observed between the plant cell wall and the plasma membrane and should be considered as artifactual. The fungus is growing in the intercellular space. Cross-sectioned hyphae are visualized in the micrograph. The pathogen also penetrates into the mesophyll cells and forms haustoria. Haustoria found in the incompatible interaction are generally smaller in size and fewer in number than the haustoria that develop in the near-isogenic, susceptible wheat plant (Hippe-Sanwald *et al.*, 1994). Comprehensive descriptions of rust haustoria by Harder and Chong (1991), Mendgen *et al.* (1991) and Particke (1994) are based on LT-preparation.

In the compatible and incompatible interaction, the rust haustoria are surrounded by smooth host cytoplasm containing chloroplasts, mitochondria, Golgi-bodies, endoplasmic reticulum and a large nucleus (Fig. 11). Strikingly, the fungal cytoplasm is darker stained than the plant cytoplasm. The host cytoplasm and the rust haustorium are separated by the extrahaustorial membrane and the extrahaustorial matrix (Fig. 12). Low temperature preparation techniques, including HP-freezing, freeze-substitution, and LT-embedding in Lowicryl HM20, provide excellent visualization of the host-parasite interface (Fig. 12). The extrahaustorial membrane of stem rust is not undulated. The fibrillar extrahaustorial matrix, which is closely attached to the extrahaustorial membrane and the haustorial cell wall is composed of fibrillar material and is also clearly preserved. Similar to the stem rust and leaf rust system, the extrahaustorial matrix of bean rust, *Uromyces appendiculatus*, is also characterized by a fibrillous, extrahaustorial matrix, and the extrahaustorial membrane is clearly defined after freeze-substitution (Hippe-Sanwald *et al.*, 1994; Mendgen *et al.*, 1991).

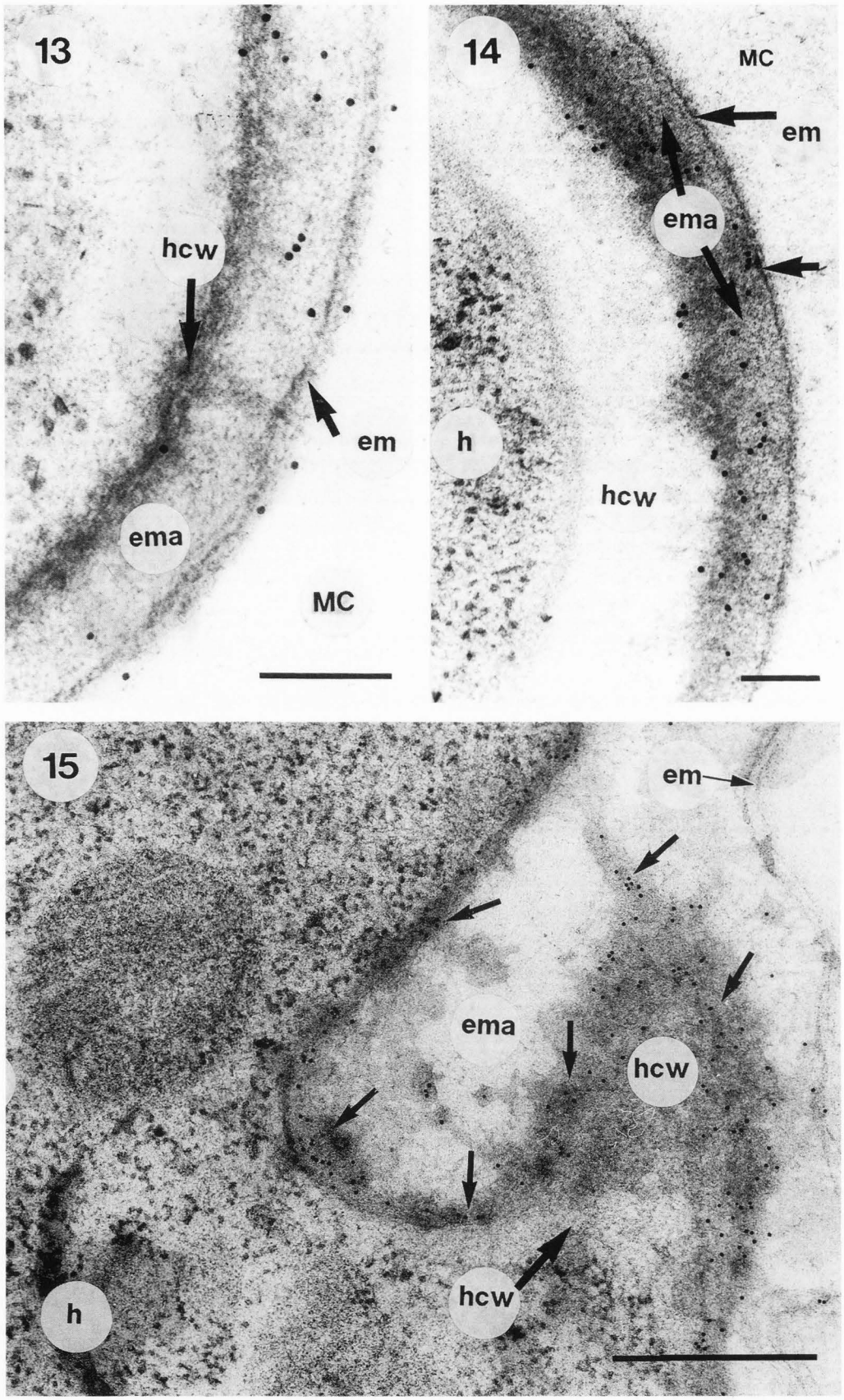
Figures 13 and 14. Evidence for the localization of elicitor-active glycoproteins in the outer haustorial cell wall layer (hcw) of haustoria (h) of *Puccinia graminis* f.sp. *tritici* in a compatible (Fig. 12) and incompatible (Fig. 13) wheat stem rust interaction. In both systems, there is also evidence for the release of the elicitor glycoprotein into the extrahaustorial matrix (ema) never passing the extrahaustorial membrane (em) into the mesophyll cell (MC). Bar = 0.25 μm.

Figure 15. Immunocytochemical localization of stem rust elicitor-like epitopes in the outer haustorial cell wall layer (hcw) of haustoria (h) of *Erysiphe graminis* f.sp. *hordei*, in a barley epidermal cell. The extension of this layer into the extrahaustorial matrix (ema) is demonstrated. No cross-reaction is found to the haustorial cytoplasm. The extrahaustorial membrane (em; arrow) separates the pathogen and the host. Bar = 0.25 μm.

Immunocytochemistry

The extrahaustorial matrix is a highly specialized interface, presumably of plant origin, that separates the host and the biotrophic fungus. Knowledge of the composition of the extrahaustorial matrix and of any changes during the development of the haustoria is fragmentary. The detection of different polysaccharides or glycoproteins within the matrix of various pathogens visualized by cytochemistry or immunogold labelling can aid understanding of the structural and physiological meanings of interfacial material at the host-parasite interface. (Chong *et al.*, 1986; Harder and Chong, 1991; Hippe-Sanwald *et al.*, 1994; Particke *et al.*, in preparation).

Immunogold electron microscopy has recently been used to determine the subcellular distribution of various


13
hcw
em
ema
MC
14
MC
em
ema
h
hcw
15
em
ema
hcw
h
hcw

glycoproteins in the extrahaustorial matrix of rust fungi and powdery mildew (Hippe-Sanwald *et al.*, 1994). In this context, investigations on the localization of an elicitor-active glycoprotein have been performed in the compatible and incompatible interaction. Infection sites of stem rust and leaf rust on primary leaves of wheat as well as of powdery mildew on barley were probed with a monoclonal antibody to the elicitor glycoprotein (Marticke *et al.*, in preparation).

Investigations were performed on various infection structures such as the germ tubes, the intercellular mycelium, the haustorial mother cells and the haustoria to elucidate the subcellular localization of elicitor proteins. According to immunogold labeling of the haustorial cell wall and the extrahaustorial matrix, elicitor glycoproteins have been clearly identified as interface components in the extrahaustorial matrix of rust fungi on wheat in the compatible and incompatible interaction (Hippe-Sanwald, 1993; Marticke, 1994).

Details of the haustorial cell wall and the extrahaustorial matrix of stem rust are shown in Figures 13 and 14. The haustorial cell wall of stem rust in the compatible interaction is primarily electron dense (Fig. 13). In a few cases the double-layered structure of the haustorial cell wall is observed. The outer layer, which is associated with the extrahaustorial matrix, is darkly stained, whereas the inner layer generally failed to stain and appears translucent. In the incompatible interaction, both cell wall layers are strikingly thicker but only the outer, dark cell wall layer appears immunogold stained (compare Figs. 13 and 14). There is also evidence for the release of the elicitor glycoprotein into the extrahaustorial matrix since distinct labeling of the extrahaustorial matrix is visible (Figs. 13 and 14).

A considerable fixation sensitivity has been found in studies on the localization of an elicitor-active glycoprotein in the haustorial cell wall and the extrahaustorial matrix of the stem rust *Puccinia graminis* f.sp. *tritici* infecting wheat leaves. By using the progressive lowering temperature (PLT)-technique instead of freeze substitution fewer antigenic sites have been detected. PLT-technique is based on chemical fixation at room temperature prior to progressive lowering of the temperature for Lowicryl embedding at low temperature conditions. In all samples, the density of detectable antigenic sites is considerably increased using freeze substitution (Hippe-Sanwald, 1993).

The results show that molecular components of the extrahaustorial matrix originate from the haustorial cell walls of the rust fungus (Marticke, 1994). However, evidence exists that similar glycoproteins, like the stem rust elicitor molecules, may also occur in the cell walls of other biotrophic fungal pathogens such as powdery mildew on barley. As shown in Figure 15, monoclonal anti-stem rust elicitor antibodies cross-reacted with similar epitopes occurring in the extrahaustorial matrix and outer haustorial cell wall layer of powdery mildew on barley. Presumably, various biotrophic pathogens share common elicitor epitopes which may play an important role in the plant pathogen interaction by triggering the plant resistance response in a still unknown way.

Conclusion and Perspectives

Low temperature preparation techniques can be regarded as important tools in biological electron microscopy. In plant pathology, these combined methods of ultrarapid freezing, freeze substitution and low temperature embedding offer a deeper insight into the architecture and the molecular substructure of the host parasite interface. On the basis of these methods, new perspectives are offered for a combined cell biological and molecular analysis. The fate of a protein in the cell as well as the spatial identification of compounds being possibly involved in the cellular signal transduction during a host-parasite interaction, can be analyzed on the subcellular level. A deeper understanding of the structure-function relationship in biology, and in particular, in plant biology with regard to the plant parasite interaction, can be achieved.

Acknowledgements

Financial support of the Dr. Christian Dräger Stiftung (Lübeck; Germany) and the Korea University, Seoul, Republic of Korea is gratefully acknowledged. Thanks are due to Dr. S. Somerville (Carnegie Institution of Washington, Stanford, CA, USA) for the good cooperation during the preparation of powdery mildew on barley for low temperature scanning electron microscopy.

References

Aist JR, Bayles CJ (1991a) Ultrastructural basis of mitosis in the fungus *Nectria haematococca* (sexual stage of *Fusarium solani*). I. Asters. Protoplasma **161**: 111-122.

Aist JR, Bayles CJ (1991b) Ultrastructural basis of mitosis in the fungus *Nectria haematococca* (sexual stage of *Fusarium solani*). II. Spindles. Protoplasma **161**: 123-136.

Baba M, Osumi M (1987) Transmission and scanning electron microscopic examination of intracellular organelles in freeze-substituted *Kloeckera* and *Saccharomyces cerevisiae* yeast cells. J Electron Microsc Tech **5**: 249-261.

Bendayan M (1984) Protein A-gold electron micro-

scopic immunocytochemistry: Methods, applications, and limitations. J Electron Microsc Tech **1**: 243-270.

Bergstrom GC, Nicholson RL (1981) Invertase in the spore matrix of *Colletotrichum*. Physiol Molec Plant Path **36**: 397-407.

Bourett TM, Hoch HC, Staples RC (1987) Association of the microtubule cytoskeleton and the thigmotropic signal for appressorium formation in *Uromyces*. Mycologia **79**: 540-545.

Bourett TM, Howard RJ (1990) *In vitro* development of penetration structures in the rice blast fungus, *Magnaporthe grisea*. Can J Bot **68**: 329-342.

Bourett TM, Howard RJ (1992) Actin in penetration pegs of the fungal rice blast pathogen, *Magnaporthe grisea*. Protoplasma **168**: 20-26.

Carver TLW (1988) Pathogenesis and host-parasite interaction in cereal powdery mildew. In: Experimental and Conceptual Plant Pathology. Vol. 2. Hess W, Singh R, Singh U, Weber D (eds.). Gordon and Beach, New York. pp. 351-381.

Carlemalm E, Villiger W, Acetarin JD, Kellenberger E (1986) Low temperature embedding. In: The Science of Biological Specimen Preparation, 1985. Müller M, Becker RP, Boyde A, Wolosewick JJ (eds.). Scanning Electron Microscopy, Inc., AMF O'Hare, IL 60666. pp. 147-154.

Chong J, Harder DE, Rohringer R (1986) Cytochemical studies on *Puccinia graminis* f. sp. *tritici* in a compatible wheat host. II. Haustorium mother cell walls at the host cell penetration site, haustorial walls and the extrahaustorial matrix. Can J Bot **64**: 2561-2575.

Clay RP, Enkerli J, Fuller MS (1994) Induction and formation of *Cochliobolus sativus* appressoria. Protoplasma **178**: 34-47.

Clement JA, Martin SG, Porter R, Butt TM, Beckett A (1993a) Germination and the role of extracellular matrix in adhesion of urediniospores of *Uromyces viciae-fabae* to synthetic surfaces. Mycol Res **97**: 585-593.

Clement JA, Butt TM, Beckett A (1993b) Characterization of the extracellular matrix produced *in vitro* by urediniospores and sporelings of *Uromyces viciae-fabae*. Mycol Res **97**: 594-602.

Dahmen H, Hobot JA (1986) Ultrastructural analysis of *Erysiphe graminis* haustoria and subcuticular stroma of *Venturia inaequalis* using cryosubstitution. Protoplasma **131**: 92-102.

Ding B, Turgeon R, Parthasarathy MV (1991) Microfilament organization and distribution in freeze substituted tobacco plant tissues. Protoplasma **165**: 96-105.

Epstein L, Laccetti L, Staples RC, Hoch HC, Hoose WA (1985) Extracellular proteins associated with induction of differentiation in bean rust uredospore germlings. Phytopathology **75**: 1073-1076

Epstein L, Laccetti L, Staples RC, Hoch HC, Hoose WA (1987) Cell-substratum adhesive protein involved in surface contact responses of the bean rust fungus. Physiol Mol Pant Pathol **30**: 373-388.

Gilkey JC, Staehelin LA (1986) Advances in ultrarapid freezing for the preservation of cellular ultrastructure. J Electron Microsc Tech **3**: 177-210.

Grove SN, Bracker CE (1970) Protoplasmic organization of hyphal tips among fungi: Vesicles and Spitzenkörper. J Bacteriol **104**: 989-1009.

Hamer JE, Howard RJ, Chumley FG, Valent B (1988) A mechanism for surface attachment in spores of a plant pathogenic fungus. Science **239**: 288-290.

Harder DE (1978) Comparative ultrastructure of the haustoria in uredial and pycnial infections of *Puccinia coronata avenae*. Can J Bot **67**: 2043-2051.

Harder DE, Chong J (1991) Rust haustoria. In: Electron Microscopy of Plant Pathogens. Mendgen K, Lesemann DE (eds.). Springer, Berlin. 235-250.

Heath IB, Rethoret K (1982) Mitosis in the fungus *Zygorhynchus moelleri*: Evidence for stage specific enhancement of microtubules by freeze substitution. Eur J Cell Biol **28**: 180-189.

Heath IB, Rethoret K, Moens PB (1984) The ultra structure of mitotic spindles from conventionally fixed and freeze-substituted nuclei of the fungus *Saprolegnia*. Eur J Cell Biol **35**: 284-295.

Heath IB, Rethoret K, Arsenault AL, Ottensmeyer FP (1985) Improved preservation of the form and contents of wall vesicles and the Golgi apparatus in freeze substituted hyphae of Saprolegnia. Protoplasma **128**: 81-93.

Hippe S (1984) Electron microscopic investigations on the cytology of *Ustilago avenae* as influenced by the systemic fungicides triadimefon, nuarimol, and imazalil-nitrate. A thin-section and freeze-fracture study. Pest Biochem Physiol **21**: 170-183.

Hippe S (1985) Ultrastructure of haustoria of *Erysiphe graminis* f.sp. *hordei* preserved by freeze-substitution. Protoplasma **129**: 52-61.

Hippe S, Hermanns M (1986) Improved structural preservation in freeze-substituted sporidia of *Ustilago avenae* - A comparison with low temperature embedding. Protoplasma **135**: 19-30.

Hippe S, Düring K, Kreuzaler F (1989) *In situ* localization of a foreign protein in transgenic plants by immunoelectron microscopy following high pressure freezing, freeze substitution and low temperature embedding. Eur J Cell Biol **50**: 230-234.

Hippe-Sanwald S. (1993) The impact of freeze-substitution on biological electron microscopy. Microsc Res Tech **24**: 400-422.

Hippe-Sanwald S, Hermanns M, Somerville SC (1992) Ultrastructural comparison of incompatible and

compatible interactions in the barley powdery mildew disease. Protoplasma **168**: 27-40.

Hippe-Sanwald S, Marticke KH, Kieliszewski MJ, Somerville SC (1994) Immunogold localization of THRGP-like epitopes in the haustorial interface of biotrophgic fungi on monocots. Protoplasma **178**: 138-155.

Hoch HC (1986) Freeze-substitution of fungi. In: Ultrastructure Techniques for Microorganisms. Aldrich HC, Todd WJ (eds.). Plenum, New York. pp. 183-212.

Hoch HC, Howard RJ (1980) Ultrastructure of freeze-substituted hyphae of the basidiomycete *Laetisaria arvalis*. Protoplasma **103**: 281-297.

Hoch HC, Staples RC (1983) Ultrastructural organization of the non-differentiated uredospore germling of *Uromyces phaseoli* variety *typica*. Mycologia **75**: 795-824.

Hoch HC, Staples RC (1991) Signaling for infection structure formation in fungi. In: The Fungal Spore and Disease Initiation in Plants and Animals. Cole GT, Hoch HC (eds.). Plenum, New York. pp. 25-42.

Horowitz RA, Giannasca PJ, Woodcock CL (1990) Ultrastructural preservation of nuclei and chromatin: Improvement with low-temperature methods. J Microsc **157**: 205-224.

Howard RJ (1981) Ultrastructural analysis of hyphal tip cell growth in fungi: Spitzenkörper, cytoskeleton and endomembranes after freeze-substitution. J Cell Sci **48**: 89-103.

Howard RJ, Aist JR (1979) Hyphal tip cell ultrastructure of the fungus *Fusarium*: Improved preservation by freeze-substitution. J Ultrastruct Res **66**: 224-234.

Humbel BM, Müller M. (1986) Freeze substitution and low temperature embedding. In: The Science of Biological Specimen Preparation, 1985. Müller M, Becker RP, Boyde A, Wolosewick JJ (eds.). *op. cit.* pp. 175-183.

Hyde GJ, Gubler F, Hardham AR (1991a) Ultrastructure of zoosporogenesis in *Phytophthora cinnamomi*. Mycol Res **95**: 577-591.

Hyde GJ, Lancelle SA, Hepler PK, Hardham AR (1991b) Freeze substitution reveals a new model for sporangial cleavage in *Phytophthora*, a result with implications for cytokinesis in other eukaryotes. J Cell Sci **100**: 735-746.

Hyde GJ, Lancelle SA, Hepler PK, Hardham AR (1991c) Sporangial structure in *Phytophthora* is disrupted after high pressure freezing. Protoplasma **165**: 203-208.

Hyde GJ, Hepler PK, Gubler F, Lancelle SA, Hardham AR (1993) Electron microscopic approaches to the study of zoospore formation in *Phytophthora*. J Computer-Assisted Microsc **5**: 37-41.

Jensen CG, Aist JR, Bayles CJ, Bollard SM, Jensen LCW (1991) Ultrastructural basis of mitosis in the fungus *Nectria haematococca* (sexual stage of *Fusarium solani*). III. Intermicrotubule bridges. Protoplasma **161**: 137-149.

Kellenberger E, Dürrenberger M, Villiger W, Carlemalm E, Wurtz M (1987) The efficiency of immunolabel on Lowicryl sections compared to theoretical predictions. J Histochem Cytochem **35**: 959-969.

Kiss JZ, Giddings Jr TH, Staehelin LA, Sack FD (1990) Comparison of the ultrastructure of conventionally fixed and high pressure frozen/freeze substituted root tips of *Nicotiana* and *Arabidopsis*. Protoplasma **157**: 64-74.

Klecan AL, Hippe S, Somerville SC (1990) Reduced growth of *Erysiphe graminis* f.sp. *hordei* induced by *Tilletiopsis pallescens*. Phytopathology **80**: 325-331.

Knauf GM, Welter K, Müller M, Mendgen K (1989) The haustorial host-parasite interface in rust-infected bean leaves after high-pressure freezing. Physiol Molec Plant Pathol **34**: 519-530.

Kunoh H (1982) Primary germ tubes of *Erysiphe graminis* conidia. In: Plant infection: The Physiological and Biochemical Basis. Asada Y, Bushnell WR, Ouchi S, Vance CP (eds.). Japan Scientific Societies Press, Tokyo. pp. 45-59.

Kunoh H, Nicholson RL, Yosioka H, Yamaoka N, Kobayashi I (1990) Preparation of the infection court by *Erysiphe graminis:* Degradation of the host cuticle. Physiol Molec Plant Path **36**: 397-407.

Kwon YH, Hoch HC, Aist JR (1991) Initiation of appressorium formation in *Uromyces appendiculatus*: organization of the apex, and the responses involving microtubules and apical vesicles. Can J Bot **69**: 2560-2573.

Lancelle SA, Callham DA, Hepler PK (1986) A method for rapid freeze fixation of plant cells. Protoplasma **131**: 153-165.

Marticke KH (1994) Immunocytochemical investigations on the importance of a glycoprotein elicitor of *Puccinia graminis* f.sp. *tritici* and extensin-like molecules for the interaction of rust with wheat. Doctoral Dissertation, RWTH Aachen, Germany.

McKerracher LJ, Heath IB (1985) Microtubules around migrating nuclei in conventionally-fixed and freeze-substituted cells. Protoplasma **125**: 162-172.

Mendgen K, Welter K, Scheffold F, Knauf-Beiter G (1991) High pressure freezing of rust infected plant leaves. In: Electron Microscopy of Plant Pathogens. Mendgen K, Lesemann DE (eds.). Springer, Berlin. pp. 31-42.

Mims CW. (1991) Using electron microscopy to study plant pathogenic fungi. Mycologia **83**: 1-19.

Mims CW, Richardson EA (1989) Ultrastructure of appressorium development by basidiospore germlings of the rust fungus *Gymnosporangium juniperi-virginianae*. Protoplasma **148**: 111-119.

Mims CW, Richardson EA. (1990) Ultrastructure of

secondary spore formation in the rust *Gymnosporangium juniperi-virginianae*. Mycologia **82**: 236-242.

Mims CW, Richardson EA, Timberlake WE (1988) Ultrastructural analysis of conidiophore development in the fungus *Aspergillus nidulans* using freeze-substitution. Protoplasma **144**: 132-141.

Mims CW, Richardson EA, and Kimbrough JW (1990) Ultrastructure of ascospore delimitation in freeze substituted samples of *Ascodesmis nigricans* (Pezizales). Protoplasma **156**: 94-102.

Monaghan P, Robertson D (1990) Freeze-substitution without aldehyde or osmium fixatives: Ultrastructure and implications for immunocytochemistry. J Microsc **158**: 355-363.

Newhouse JR, Hoch HC, MacDonald WL (1983) The ultrastructure of *Endothia parasitica*. Comparison of a virulent with a hypovirulent isolate. Can J Bot **61**: 389-399.

Newhouse JK, MacDonald WL, Hoch HC (1990) Virus-like particles in hyphae and conidia of European hypovirulent (dsRNA-containing) strains of *Cryphonectria parasitica*. Can J Bot **68**: 90-101.

O'Donnell K (1992) Ultrastructure of meiosis and the spindle pole body cycle in freeze-substituted basidia of the smut fungi *Ustilago maydis* and *Ustilago avenae*. Can J Bot **70**: 629-638.

Pringle RB (1981) Nonspecific adhesion of *Bipolaris sorokiniana* sporelings. Can J Plant Path **3**: 9-11.

Read ND (1991) Low-temperature scanning electron microscopy of fungi and fungus-plant interactions. In: Electron Microscopy of Plant Pathogens. Mendgen K, Lesemann DE (eds.). Springer, Berlin. pp. 17-29.

Robards AW, Sleytr UB (1985) Low temperature methods in biological electron microscopy. In: Practical Methods in Electron Microscopy, Vol. 10. Glauert AM (ed.). Elsevier, Amsterdam. pp. 461-493.

Roberson RW (1992) The actin cytoskeleton in hyphal cells of *Sclerotium rolfsii*. Mycologia **84**: 41-51.

Roberson RW, Fuller MS (1988) Ultrastructural aspects of the hyphal tip of *Sclerotium rolfsii* preserved by freeze substitution. Protoplasma **146**: 143-149.

Snetselaar KM, Mims CW (1994) Light and electron microscopy of *Ustilago maydis* hyphae in maize. Mycol Res **98**: 347-355.

Staehelin LA, Giddings Jr TH, Kiss JZ, Sack FD. (1990) Macromolecular differentiation of Golgi stacks in root tips of *Arabidopsis* and *Nicotiana* seedlings as visualized in high pressure frozen and freeze-substituted samples. Protoplasma **157**: 75-91.

Studer D, Michel M, Müller M. (1988) Cryofixation of plant tissue by high pressure freezing. Inst Phys Conf Ser **3**: 19-20.

Studer D, Michel M, Müller M. (1989) High pressure freezing comes of age. Scanning Microsc Suppl **3**: 253-269.

Swann EC, Mims CW. (1991) Ultrastructure of freeze-substituted appressoria produced by aeciospore germlings of the rust fungus *Arthuriomyces peckianus*. Can J Bot **69**: 1655-1665.

Tiburzy R, Rogner UC, Fischer R, Beißmann B, Kreuzaler FM, Reisener HJ (1991) Detection of an elicitor of infection structures of *Puccinia graminis* using monoclonal antibodies. Eur J Cell Biol **55**: 174-178.

Tunlid A, Johansson T, Nordbing-Hertz B (1991) Surface polymers of the nematode-trapping fungus *Arthrobotrys oligospora*. J Gen Microbiol **137**: 1231-1240.

Van Dyke CG, Mims CW (1991) Ultrastructure of conidia, conidium germination, and appressorium development in the plant pathogenic fungus *Colletotrichum truncatum*. Can J Bot **69**: 2455-2467.

Vargas MM, Aronson JM, Roberson RW (1993) The cytoplasmic organization of hyphal tip cells in the fungus *Allomyces macrogynus*. Protoplasma **176**: 43-52.

Welter K, Müller M, Mendgen K. (1988) The hyphae of *Uromyces appendiculatus* within the leaf tissue after high-pressure freezing and freeze substitution. Protoplasma **147**: 91-99.

Wessels JGH (1990) Role of cell wall architecture in fungal tip growth generation. In: Tip Growth in Plant and Fungal Cells. Heath IB (ed.). Academic Press, New York. pp. 1-29.

Wessels JGH (1993) Wall growth, protein excretion and morphogenesis in fungi. New Phytol **123**: 397-413.

Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no **Discussion with Reviewers.**