The Hyphae of *Uromyces appendiculatus* Within the Leaf Tissue After High Pressure Freezing and Freeze Substitution

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Summary

The fine structure of the intercellular dikaryotic hyphae of the biotrophic fungus *Uromyces appendiculatus* was studied. High pressure freezing and freeze substitution were used to achieve a closer approximation of the native state than with conventional fixation and dehydration techniques. In addition to organelles previously described in rust fungi, heavily decorated multivesicular bodies (star bodies) were found close to the nuclei. Two types of tubular-vesicular complexes were distributed randomly within the cytoplasm of the hyphae. Furthermore, a more or less pronounced brush-like fibrillar layer on the hyphal walls was detected. The possibility that the latter two structures are correlated with the biotrophic phase of this fungus is discussed.

Keywords: *Uromyces appendiculatus*; High pressure freezing; Freeze substitution; Cryoprotection; Fine structure preservation.

Abbreviations: TVC tubular-vesicular complex; MVB multivesicular body; M mitochondrion; N nucleus; NP nuclear pore; S septum; MT microtubule

1. Introduction

After cryo-fixation and freeze substitution a number of new organelles has been detected in fungi (see HOCH 1986, BABA and OSUMI 1987). Observations of freeze substituted germ tubes of *Uromyces phaseoli typica* (HOCH and STAPLES 1983) suggest that, within the leaf parenchyma, the parasitic structures and the host-parasite interfaces of this fungus may also exhibit new details. To verify this, we needed to cryofix the whole parasitic system, i.e., leaf samples of *Phaseolus vulgaris* (approximately 300 µm thick) infected with *Uromyces*

appendiculatus. In previous studies convincing freezing qualities have been obtained only for specimens with a thickness of a few microns, such as single cells (KÄPPELI *et al.* 1984, HIPPE 1985, HIPPE and HERMANN 1986, TOKUNAGA *et al.* 1986, BABA and OSUMI 1987) or hyphae (HEATH *et al.* 1985, HOCH and HOWARD 1980). Therefore, parasitic hyphae and haustoria have been studied only in such cases where they were growing below the cuticle or within the epidermis of the host plant (HIPPE 1985, DAHMEN and HOBOT 1986). High pressure freezing presently is the only practical way to overcome this limitation since samples of up to 600 µm thickness can be cryofixed (RIEHLE and HOECHLI 1973, MÜLLER and MOOR 1984). Therefore we focused on this method to achieve a better preservation of our host-parasite system. Freeze substitution and sectioning were used to study the cytoplasm of the intercellular hyphae and the interface of host and parasite. New details concerning the endomembrane system of the fungus and a brush-like fibrillar layer on the outside of the hyphae were detected. Both structures may be correlated with the establishment of parasitism of this biotrophic fungus.

2. Materials and Methods

Uromyces appendiculatus var. *appendiculatus* on *Phaseolus vulgaris* L. f. sp. Fori was raised in a growth chamber under 10,000 lux (16 h photoperiod), approximately 70% RH and 20°C.

Fourteen day old primary leaves were spray-inoculated with a ure-dospore suspension (ca. 3 mg spores/10 ml distilled water containing 0.05% Tween) and incubated under dim light at 18–20°C and 100% RH for 20–24 h.

Infected areas of the leaves were removed with a 2 mm corkborer 4

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to 6 days after inoculation. In order to remove the air from the intercellular spaces, the samples were infiltrated either with tap water or with distilled water containing 8% (v/v) (2.5 M) methanol at room temperature. When methanol was used, the solution around the samples was renewed after infiltration in order to maintain the correct concentration. Samples routinely remained in 8% methanol for 5–10 min. Then, single samples were placed in an aluminium holder (one half with a hollow of 0.3 mm depth for the sample and the other a flat top) and frozen immediately in the High Pressure Freezer HPM 010 (Balzers Union, Lichtenstein) introduced by MÜLLER and MOOR (1984).

The substitution medium (1.5 ml per specimen) consisted of 2 or 4% osmium tetroxide in acetone which had been dried over calcium chloride. Freeze substitution (MÜLLER *et al.* 1980) was performed at -90°C , -60°C , and -30°C , 8 h for each step, using either a self built device (Dr. M. MÜLLER, ETH Zürich) or the similar Balzers freeze-substitution apparatus FSU 010. Subsequently, the temperature was raised to approximately 0°C within 30 min and the samples were washed in dry acetone for another 30 min.

Infiltration with an Epon/Araldite mixture was performed stepwise: 30% resin in acetone at $+4^{\circ}\text{C}$ for up to 7 h, 70% and 100% resin at $+8^{\circ}\text{C}$ for up to 20 h each step and 100% resin at $+18^{\circ}\text{C}$ for approximately 12 h. Samples were then transferred to fresh medium and polymerized at 60°C for 10 h.

Ultrathin sections were cut with glass knives, placed on formvar-coated single hole grids and were post-stained with 3% aqueous uranyl acetate and Reynolds lead citrate for 20 and $2\frac{1}{2}$ min, respectively. Grids were examined with either a Siemens Elmiskop IA or a Zeiss EM 10 C electron microscope, operated at 80 kV.

3. Results

Despite the fact that the commercial high pressure freezer works very reliably with respect to physical performance, the yield in adequately frozen plant samples was approximately 10%, too low to be suitable for the ultrastructural evaluation of a complex biological system such as the interaction of fungus and host. The addition of methanol as a cryoprotectant improved the yield of well frozen specimens considerably, to approximately 80%. 8% methanol was sufficient to suppress detectable effects of ice crystal formation and did not introduce structural alterations as compared to the samples treated with tap water. Micrographs from samples infiltrated with tap water only are included for comparison.

The most obvious improvement over conventional methods of chemical fixation and dehydration was the retention of smooth membranes. The plasma membranes of both plant and fungus followed the contours of the cell walls closely. Similarly, organelles exhibited smooth contours. Patches of membranous structures, subsequently referred to as tubular-vesicular complexes (TVCs), were observed regularly in the fungal cytoplasm. Two types, sometimes found adjacent to each

other, could be distinguished by their structure, the contrast properties of their contents and the association with other organelles.

The more common type (Figs. 1 and 2), referred to as TVC 1, was found in all fungal cell types and consisted of cisternae with an irregular luminal width of 30–70 nm. The contents exhibited intermediate electron opacity. Ribosomes often lined the cisternae but the association may have been coincidental, as the other parts of the cytoplasm were evenly filled with ribosomes. TVC 1 was, as deduced from serial sections, spherical and variable in diameter. Cryoprotection with methanol did not influence its structure (compare with Fig. 3). Frequently, membranous tubes resembling endoplasmic reticulum connected different complexes or radiated from such patches into the surrounding cytoplasm (Fig. 4). In developing spores TVC 1 occupied large areas of the cytoplasm (Fig. 5) and no connections with endoplasmic reticulum were observed. Since the resin regularly separated from the walls of the developing spores, we were not able to follow secretion events as described recently by KNAUF and MENDGEN (1988) after freeze fracturing.

The second type of tubular-vesicular complex, TVC 2, was found in all fungal cell types except in developing spores (Fig. 6). Again, cryoprotection with methanol had no influence on the morphology (compare with Fig. 8). TVC 2 was observed much less frequently than TVC 1 and consisted of small groups of curved or straight tubes (Figs. 6–10), sometimes radiating from a small ring-like tube (Fig. 11). The lumen of the tubes had a regular diameter of approximately 20 nm and stained more intensely than TVC 1. In serial sections, TVC 2 did not extend over more than five sections which indicates that the complex is very small, only up to 450 nm in diameter. Sometimes the TVC, probably TVC 2, displayed special features: in one case it consisted of only one tube (Fig. 12) or, in a developing spore, it resembled a very small dictyosome (Fig. 13).

Single multivesicular bodies (MVBs) were scattered throughout the cytoplasm and occasionally formed loose groups. Frequently the MVBs were found adjacent to the nuclei and/or the plasma membrane (Fig. 14). Straight or curved filaments were attached perpendicularly to some MVBs (Figs. 15 and 16). Heavily decorated MVBs, or “star bodies”, were arranged in clusters. These clusters were in close proximity to tubular structures (Figs. 17 and 18) not seen with conventional methods. Decorated MVBs were always found close to a nucleus (Figs. 15 and 18).

Figs. 1-29. Sections of *U. appendiculatus* infected bean leaves, prepared by high pressure freezing and freeze substitution. Except where otherwise stated, the samples were cryoprotected with 8% methanol and the micrographs represent intercellular hyphae. *TVC* Tubular-vesicular complex; *MVB* multivesicular body; *M* mitochondrion; *N* nucleus; *NP* nuclear pore; *S* septum; *MT* microtubule

Figs. 1-5. Tubular-vesicular complexes, type 1 (TVC 1)

Fig. 1. Several clusters of TVC 1 in a longitudinal section through a hypha ($\times 20,000$)

Fig. 2. The TVC is clearly differentiated from the surrounding cytoplasm ($\times 27,200$)

Fig. 3. Small TVC 1 from a sample, not cryoprotected with methanol ($\times 30,000$)

Fig. 4. Tubes, perhaps endoplasmic reticulum cisternae, may connect the TVCs or radiate from the TVC into the cytoplasm ($\times 36,000$)

Fig. 5. Large TVC 1 is a developing spore ($\times 17,600$)

Figs. 6–13. Tubular-vesicular complexes, type 2 (TVC2)

Figs. 6 and 7. Groups of TVC 1 and TVC 2 adjacent to each other, illustrating their different structure and contrast properties (Fig. 6 $\times 43,000$; Fig. 7 $\times 60,000$)

Fig. 8. TVC2 from a sample not cryoprotected with methanol ($\times 60,000$)

Figs. 9–13. The different shapes of TVC 2, depending on the plane of section: irregular (Fig. 9 $\times 60,000$), ringed (Fig. 10 $\times 60,000$), ringed with extensions (Fig. 11 $\times 60,000$), only one cisterna (Fig. 12 $\times 64,000$) or even resembling a small dictyosome (Fig. 13 $\times 61,000$)

Bundles of microfilaments (Fig. 19) were found only in senescent regions of sporogeneous hyphae with extensive vacuolization.

Microtubules were usually oriented parallel to the long axis of the cell. They were straight or gently curved (Figs. 19 and 20) and, in cross sections, exhibited the typical rounded shape (Fig. 28). An increased number of microtubules was observed in areas of contact with other cells (not shown).

Filasomes, which consist of a central vesicle surrounded by a filamentous coat, as described by HOCH and HOW-

ARD (1980), were generally found close to the plasma membrane (Fig. 20).

Microvesicles, 60–90 nm in diameter, were randomly distributed throughout the cytoplasm (Figs. 21 and 22). They had a clearly visible membrane and the contents were of low electron opacity.

Microbodies were distributed randomly in the cytoplasm except near the septum, where they were arranged in a semicircle close to the septal pore (Fig. 23). The hyphal walls varied in thickness (Figs. 24–27), but only one layer was distinguishable. The walls were cov-

Fig. 14. Group of MVBs close to the nucleus ($\times 60,000$)

Figs. 15 and 16. Straight or curved filamentous structures, attached perpendicular to the MVBs membrane. These MVBs are also close to the nucleus (Fig. 15 $\times 32,000$, Fig. 16 $\times 40,000$)

Figs. 17 and 18. Clusters of heavily decorated MVBs ("star bodies") in close neighbourhood to tubular structures (arrows) in the neighbourhood of the nucleus (Fig. 17 $\times 60,000$, Fig. 18 $\times 40,000$)

ered by a layer of long fibrils (Figs. 24–27). This brush-like fibrillar material was occasionally seen on young hyphae growing at the edge of the rust pustule and was of modest length (Fig. 24). Close to the pustule, where hyphae began to aggregate under the epidermis, the brush-like fibrils were more frequently seen and were longer, approximately $300\ \mu\text{m}$ (Figs. 25–27). Figure 25 shows a hypha from a sample not cryoprotected with

methanol. Sometimes the fibrils seemed to be intertwined (Fig. 26). In regions of contact between host and parasite (Figs. 28 and 29), the fibrillar material formed a dense net which seemed to be interwoven with the host cell wall material (Fig. 28). Usually, the fungal and host walls could be distinguished by their staining properties (Fig. 28), although not always (Fig. 29).

4. Discussion

High pressure freezing has been used successfully with mammalian tissues (MOOR *et al.* 1980, HUNZIKER *et al.* 1984, MÜLLER and MOOR 1984, MOOR 1987). Leaf tissue, however, is difficult to handle since the intercellular space is filled with air which is compressible. This air must be removed by infiltration before the application of high pressure freezing. With the use of tap water ice crystals developed upon freezing and caused damage to the cytoplasm and cracked the cell walls. During subsequent freeze substitution, when the ice was removed from the tissue, many specimens disintegrated. Therefore a cryoprotectant was needed to adjust the freezing properties within the tissue. Methanol (8%) was selected as cryoprotectant since it penetrates cells rapidly (MORRIS 1980). Good results have been obtained with mammalian cells (HEUSER and KIRSCHNER 1980, WILLIAMS *et al.* 1984), some parasites (JAMES 1980) and some algae (MORRIS *et al.* 1980). The toxicity of methanol is time, concentration and temperature dependent (WILLIAMS *et al.* 1984). *Euglena gracilis* may recover after a treatment with 10% methanol for 15 min (MORRIS and CANNING 1978) although physiological alterations may result from this treatment (MORRIS *et al.* 1980). As such changes may be visible, we compared our methanol treated samples with the samples adequately frozen after infiltration with tap water only. In addition, we consulted previous cytological investigations after cryo-fixation without the use of cryoprotectants. Morphological damage due to the methanol treatment was not observed in our samples. However, physiological alterations can not be excluded.

Freeze substitution results in superior structural preservation as compared to conventional preparative procedures. The membranes are smooth and the plasma membrane follows the cell wall closely. This has been repeatedly shown after cryo-fixation of fungi (HOWARD and AIST 1979, HOCH and HOWARD 1980, HOWARD 1981, HOCH and STAPLES 1983, NEWHOUSE *et al.* 1983, HIPPE 1985, DAHMEN and HOBOT 1986, TOKUNAGA *et al.* 1986).

Fig. 19. Microfilament bundle and microtubules (arrows) ($\times 50,000$)

Fig. 20. Filasomes (large arrows) and a microtubule (small arrow) near the plasmamembrane ($\times 70,000$)

Figs. 21 and 22. Microvesicles (arrows) ($\times 64,000$)

Fig. 23. Microbodies arranged in a semicircle close to the septal pore ($\times 37,000$)

Figs. 24–27. Brush-like fibrillar material on the hyphae of *Uromyces appendiculatus*

Fig. 24. Young hypha at the edge of the rust pustule with a quite modest layer of extracellular material ($\times 39,000$)

Figs. 25–27. Aggregated hyphae under the epidermis with high amounts of extracellular brush-like fibrils

Fig. 25. Brush-like material in a sample not cryoprotected with methanol ($\times 40,000$)

Fig. 26. The fibrils are sometimes intertwined at their ends ($\times 40,000$)

Fig. 27. In a few cases the fibrils appear completely interwoven ($\times 40,000$)

Fig. 28. Dense net of fibrils, interwoven with the host cell wall material (arrows). Both walls are discernible by their staining properties ($\times 32,000$)

Fig. 29. Sometimes the walls of host and parasite have quite similar staining properties with the techniques used ($\times 40,000$)

We found tubular-vesicular complexes which were not detectable in *U. appendiculatus* after conventional preparative techniques. TVC 1 corresponds to the TVC described in haustoria (KNAUF *et al.* 1989). It is especially prominent in developing spores (KNAUF and MENDGEN 1988). It is not clear why this complex has not been detected in most other fungi after cryo-fixation. TVC 1 may be differentiated only during the growth of this biotrophic fungus within the leaf parenchyma. Only two other biotrophic fungi have been studied using cryo-fixation. In the haustoria of *Erysiphe graminis*, DAHMEN and HOBOT (1986) describe "grey staining areas" which had a tubular structure closely resembling the TVC 1 in the present study. Similar structures have not been found in the germ tubes of *U. phaseoli* var. *typica* (HOCH and STAPLES 1983). The function of the TVC 1 is not known, but it is likely an aggregation of rough endoplasmic reticulum with special tasks.

TVC 2 is similar to the presumed Golgi bodies described after cryofixation in germ tubes of the bean rust fungus (HOCH and STAPLES 1983) and other fungi (DAHMEN and HOBOT 1986, HOCH and HOWARD 1980, HOWARD 1981, HOWARD and AIST 1979, NEWHOUSE *et al.* 1983) and after conventional preparation (GROVE and BRACKER 1970). In some instances the TVC 2 (Figs. 12 and 13) were similar to the dictyosome-like organelles found in oomycetous fungi (HEMMES and RIBEIRO 1977, HEATH *et al.* 1985). However, secretory functions have not been proven for all the presumed Golgi bodies or dictyosomes.

TVC 1 and 2 were often found adjacent to each other but were never in direct contact, similar to the results for *E. graminis* (DAHMEN and HOBOT 1986). Therefore, these two structures may have specific and different tasks. Cytochemical studies are underway to clarify the content of the tubules and vesicles.

Filamentous structures on MVBs were previously found in *U. appendiculatus* and other rust fungi and have been interpreted either to be rod-shaped virus particles (LITTLEFIELD and HEATH 1979) or microfilaments (HOCH and STAPLES 1983). Our results do not clarify which interpretation is correct.

Microfilaments, microtubules, filosomes, microvesicles and microbodies have already been described for several fungi after cryo-fixation, and their physiological functions have been discussed (HOCH and HOWARD 1980, HOCH and STAPLES 1983, HOWARD 1981).

The extracellular brush-like fibrils of *U. appendiculatus* are visible to the extent seen in this study only after cryo-fixation (SCHNEIDER 1986). With conventional techniques, remnants of such material are only visible

at contact sites with the host wall (see LITTLEFIELD and HEATH 1979). Similar fibrillar material has been documented after cryo-fixation of various yeasts (WALTHER *et al.* 1984, KÄPPELI *et al.* 1984, TOKUNAGA *et al.* 1986, BABA and OSUMI 1987). This extracellular material varied in thickness, structure and properties under different growth conditions, *e.g.* sugars supplied to the nutrient media (KÄPPELI *et al.* 1984, MCCOURTIE and DOUGLAS 1981). Therefore, the discontinuity in the appearance of the fibrils on the bean rust hyphae and the variation in their thickness may be due to the nutritional status of the hyphae in the leaf tissue. On the other hand hyphal differentiation during development of the pustule may influence the structure of the extracellular fibrils. Germ tubes of *U. phaseoli* var. *typica* also exhibit extracellular material after cryo-fixation (HOCH and STAPLES 1983) but it resembles slime and is not in the brush-like order which occurs on the hyphae of this fungus when it grows within the leaf. Cytochemical studies also suggest a difference between surface molecules of various fungal structures. Germ tubes have a high affinity to wheat germ agglutinin suggesting the presence of chitin and terminal N-acetylglucosamine whereas the infection hypha, an intercellular hypha, has a high affinity for different glucanases suggesting the presence of β -1-3 and β -1-6 linked glucans (MENDGEN *et al.* 1985). We suggest that this material has some tasks in the regulation of the host parasite interaction (MENDGEN *et al.* 1988).

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References

- BABA M, OSUMI M (1987) Transmission and scanning electron microscopic examination of intracellular organelles in freeze-substituted *Kloeckera* and *Saccharomyces cerevisiae* yeast cells. *J Electr Microsc Tech* 5: 249–261
- DAHMEN H, HOBOT JA (1986) Ultrastructural analysis of *Erysiphe graminis* haustoria and subcuticular stroma of *Venturia inaequalis* using cryosubstitution. *Protoplasma* 131: 92–102
- GROVE SN, BRACKER CE (1970) Protoplasmic organization of hyphal tips among fungi: vesicles and Spitzenkörper. *J Bacteriol* 104: 989–1009
- 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
- HEMMES DE, RIBEIRO OK (1977) Electron microscopy of early gametangial interaction in *Phytophthora megasperma* var. *sojae*. *Can J Bot* 55: 436–447
- HEUSER JE, KIRSCHNER MW (1980) Filament organization in cytoskeletons. *J Cell Biol* 86: 212–234
- HIPPE S (1985) Ultrastructure of haustoria of *Erysiphe graminis* f.

- sp. *hordei* preserved by freeze-substitution. *Protoplasma* 129: 52–61
- HERMANN M (1986) Improved structural preservation in freeze-substituted sporidia of *Ustilago avenae* – a comparison with low-temperature embedding. *Protoplasma* 135: 19–30
- HOCH HC (1986) Freeze-substitution of fungi. In: ALDRICH HC, TODD WJ (eds) *Ultrastructure techniques for microorganisms*. Plenum, New York, pp 183–211
- HOWARD RJ (1980) Ultrastructure of freeze-substituted hyphae of the basidiomycete *Laetisaria arvalis*. *Protoplasma* 103: 281–297
 - STAPLES RC (1983) Ultrastructural organization of the non-differentiated uredospore germling of *Uromyces phaseoli typica*. *Mycologia* 75: 795–824
- 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
- AIST JR (1979) Hyphal tip cell ultrastructure of the fungus *Fusarium*: improved preservation by freeze-substitution. *J Ultrastruct Res* 66: 224–234
- HUNZIKER EB, HERRMANN W, SCHENK RK, MÜLLER M, MOOR H (1984) Cartilage ultrastructure after high pressure freezing, freeze substitution, and low temperature embedding. I. Chondrocyte ultrastructure – implications for the theories of mineralization and vascular invasion. *J Cell Biol* 98: 267–276
- JAMES ER (1980) Cryopreservation of *Schistosoma mansoni* schistosomula using 40% v/v (10%) methanol and rapid cooling. *Cryo Lett* 1: 535–544
- KÄPPELI O, WALTHER P, MÜLLER M, FIECHTER A (1984) Structure of the cell surface of the yeast *Candida tropicalis* and its relation to hydrocarbon transport. *Arch Microbiol* 138: 279–282
- KNAUF G, MENDGEN K (1988) Secretion-systems and membrane-associated structures in rust fungi after high pressure freezing and freeze-fracturing. *Biol Cell*, in press
- KNAUF GM, WELTER K, MÜLLER M, MENDGEN K (1989) The haustorial host-parasite interface in rust-infected bean leaves after high pressure freezing. *Physiol Mol Plant Pathol*, in press
- LITTELFIELD LJ, HEATH MC (1979) *Ultrastructure of rust fungi*. Academic Press, New York, pp 244–248
- MCCOURTIE J, DOUGLAS LJ (1981) Relationship between cell surface composition of *Candida albicans* and adherence to acrylic after growth on different carbon sources. *Infect Immun* 32: 1234–1241
- MENDGEN K, LANGE M, BRETSCHNEIDER K (1985) Quantitative estimation of the surface carbohydrates on the infection structures of rust fungi with enzymes and lectins. *Arch Microbiol* 140: 307–311
- SCHNEIDER A, STERK M, FINK W (1988) The differentiation of infection structures as a result of recognition events between some biotrophic parasites and their hosts. *J Phytopathol* 123: 259–272
- MOOR H (1987) Theory and practice of high pressure freezing. In: STEINBRECHT RA, ZIEROLD K (eds) *Cryotechniques in biological electron microscopy*. Springer, Berlin Heidelberg New York Tokyo, pp 175–191
- BELLIN G, SANDRI C, AKERT K (1980) The influence of high pressure freezing on mammalian nerve tissue. *Cell Tissue Res* 209: 201–216
- MORRIS GJ (1980) Plant cells. In: ASHWOOD-SMITH MJ, FARRANT J (eds) *Low temperature preservation in medicine and biology*. Pitman Medical, Tunbridge Wells, pp 253–283
- CANNING CE (1978) The cryopreservation of *Euglena gracilis*. *J Gen Microbiol* 108: 27–31
 - CLARKE A, FULLER BJ (1980) Methanol as a cryoprotective additive for *Chlorella*. *Cryo Lett* 1: 121–128
- MÜLLER M, MOOR H (1984) Cryofixation of thick specimens by high pressure freezing. In: REVEL J-P, BARNARD T, HAGGIS GH (eds) *The science of biological specimen preparation for microscopy and microanalysis*. Proceedings 2nd Pfefferkorn Conference. AMF O'Hare, Chicago, Illinois, pp 131–138
- MARTI T, KRIZ S (1980) Improved structural preservation by freeze substitution. In: BREDEROO P, DE PRIESTER W (eds) *Proceedings 7th European Congress Electron Microscopy, The Hague, vol 2*. European Congress on Electron Microscopy Foundation, Leiden, The Netherlands, pp 720–721
- 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
- RIEHLE U, HOECHLI M (1973) The theory and technique of high pressure freezing. In: BENEDETTI EL, FAVARD P (eds) *Freeze-etching techniques and applications*. Société Française de Microscopie Électronique, Paris, pp 31–61
- SCHNEIDER A (1986) *Feinstruktur des Wirt-Parasit-Systems Puccinia coronata f. sp. avenae/Avena sativa nach Anwendung physikalischer und chemischer Fixierungsverfahren*. Diss, Universität Konstanz, Federal Republic of Germany
- TOKUNAGA M, KUSAMICHI M, KOIKE H (1986) Ultrastructure of outermost layer of cell wall in *Candida albicans* observed by rapid-freezing technique. *J Electron Microsc* 35: 237–246
- WALTHER P, MÜLLER M, SCHWEINGRUBER ME (1984) The ultrastructure of the cell surface and plasma membrane of exponential and stationary phase cells of *Schizosaccharomyces pombe*, grown in different media. *Arch Microbiol* 137: 128–134
- WILLIAMS RJ, BROSS J, TAKAHASHI T, MERYMAN HT (1984) Erythrocyte metastability: the “toxicity” of alcohols and their efficiency as surfactants. *Cryo Lett* 5: 269–276