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Visualization and characterization of the extracellular matrix of *Bipolaris sorokiniana*

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Extracellular matrix (ECM) surrounding conidia and germlings of *B. sorokiniana* was studied using light microscopy (LM), scanning (SEM) and transmission electron microscopy (TEM). Conidial ECM surrounding dry-inoculated, ungerminated conidia was fluid-like and observed only using a cryo-preparation technique, suggesting that the material was water soluble. ECM enveloping germlings appeared fibrillar in LM, TEM and SEM but amorphous in cryo-SEM, indicating that the structure of the ECM is dependent on the water content of the matrix. Fibrillar ECM formed thread-like structures that extended over long distances on the substrate or towards neighbouring conidia and hyphae. TEM of germlings negatively stained with uranyl acetate revealed the presence of fungal fimbriae. The strong resemblance between the extending organization of fibrillar thread-like ECM structures and fimbriae suggested that fimbriae constitute a basic structural component of the ECM and serve as the aggregation centre for the other ECM components. Histochemical labelling revealed significant differences between ECM surrounding the fungus at different morphological stages. The germ tube ECM was labelled for both proteins and polysaccharides whereas germling ECM consisted of two layers: an inner rich in proteins and an outer composed mainly of polysaccharides. Furthermore, the newly released ECM localized on germ tubes and hyphal tips showed affinity for microspheres carrying any type of surface properties while hyphal ECM had affinity only for negatively charged microspheres. This together suggests that ECM after its release is subjected to structural changes.

INTRODUCTION

Conidia, hyphae, appressoria and haustoria often are surrounded by extracellular matrix (ECM), also referred to as mucilage, extracellular material or sheath. (Jones, 1994; Braun & Howard, 1994a; Nicholson & Kunoh, 1995; Nicholson, 1996). ECM has been found in a wide range of fungi, including aquatic, nematophagous, plant pathogenic and wood-decaying species (Tunlid, Jansson & Nordbring-Hertz, 1992; Nicole *et al.*, 1993; Jones, 1994). Not much is known about the functions or importance of extracellular matrix. It has been suggested that ECM functions as an adhesive, reduces desiccation and provides the fungus with a favourable micro-environment (Moloshok *et al.*, 1993; Jones, 1994; Nicole, Ruel & Ouellette, 1994; Nicholson, 1996). In addition, a number of extracellular lytic enzymes are contained in ECM, including those important for nutrition and pathogenicity (Ruel & Joseleau, 1991; Clement, Butt & Beckett, 1993). The nature and composition of ECM of different fungi may vary substantially (Jones, 1994), so the methods used to observe the ECM of one fungus may not necessarily be applicable to others. ECM is usually transparent; therefore, when using light microscopy (LM), various contrasting agents are utilized.

These include the negative stain Indian ink, various conventional histological stains, colloidal gold and fluorescent probes, like fluorophore-conjugated lectins, antibodies and latex microspheres (Evans, Stempen & Frasca, 1982; Braun & Howard, 1994b; Jones, Bailey & O'Connell, 1995; Kuo & Hoch, 1995; O'Connell *et al.*, 1996). In addition, ECM has been visualized successfully using TEM and SEM techniques (Chaubal, Wilmot & Wynn, 1991; Van Dyke & Mims, 1991; Clement *et al.*, 1993; Braun & Howard, 1994b).

Bipolaris sorokiniana is a plant pathogen that infects economically important crops, such as wheat and barley. Although numerous studies have been conducted on the physiology, pathology and ecology of the fungus, little is known about the properties, composition and role of the ECM of *B. sorokiniana*. There are no reports on mucilage associated with conidia of *Bipolaris* (*Cochliobolus*) species, but a number of microscopical observations have been made on ECM surrounding germlings, hyphae and appressoria (Wheeler & Gantz, 1979; Evans, Stempen & Stewart, 1981; Evans *et al.*, 1982; Carlson *et al.*, 1991b; Braun & Howard, 1994b; Clay, Enkerli & Fuller, 1994; Sugui, Kunoh & Nicholson, 1999).

In our laboratory *B. sorokiniana* has been used to study interactions between a phytotoxin-producing pathogen and its host (Carlson *et al.*, 1991a). The toxin, prehelminthosporol, has been localized to specific organelles inside hyphae

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(Åkesson *et al.*, 1996), as well as to ECM enveloping hyphae. Consequently, we wanted to study this ECM in greater detail. To do so we used various methods to visualize and partly characterize ECM surrounding conidia and germlings of *B. sorokiniana*.

MATERIALS AND METHODS

Organisms

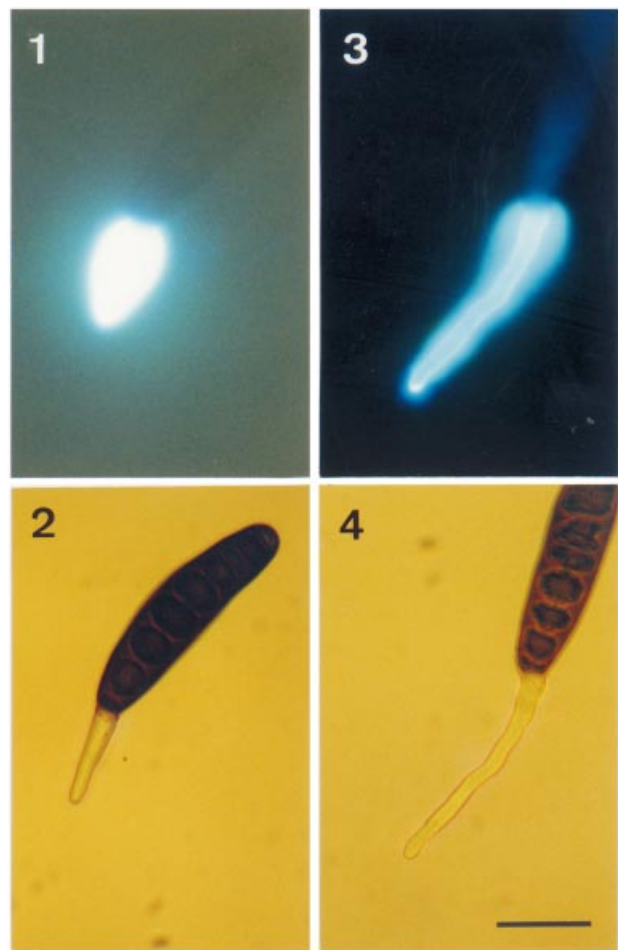
Conidia were collected from 7 to 10 day old colonies of *Bipolaris sorokiniana* [syn. *Helminthosporium sativum*; teleomorph *Cochliobolus sativus*], isolate Tellus, grown on a defined agar medium (Carlson *et al.*, 1991a). The isolate had been maintained at 20 °C on the same substrate and subcultured weekly. A conidial suspension was obtained by flooding the surface of the colony with sterile water and carefully rubbing the surface with a bent glass rod. Hyphal pieces were removed by filtration through a double layer of a nylon net (100 µm mesh size).

For light microscopy, conidia were suspended in 2.4% (w/v) potato dextrose broth (PDB, Difco) or distilled water and incubated on multiwell glass slides (Kebo, Sweden) for 2 to 24 h at room temperature (20–22°). Growth medium was removed by washing with 3 × 30 ml of tap water.

Seeds of barley (*Hordeum vulgare* cv. Harry) were surface sterilized in 4.4% NaClO (pH 12.8) and incubated for 2 d on moist filter paper. Resulting seedlings were placed in glass beakers on top of moistened, sterile sand. Plants were grown aseptically for 14 d in a growth chamber using a 16 h light (18°)/8 h dark (16°) photoperiod/temperature regime. The abaxial surface of the second leaf of barley was inoculated with dry conidia using a soft sterilized paint brush, or a piece of the leaf was cut, placed on the surface of water agar and inoculated with 10 µl droplets of the conidial suspension. After incubation for 14 h the material was used for cryo-SEM studies.

Histochemical labelling

Germlings attached to the glass slides were stained with one of the following: 1% (w/v) acid fuchsin in distilled water, 1% (w/v) alcian blue in 3% acetic acid, 0.01% (w/v) aniline blue in 0.15 M K₂HPO₄, 0.1% calcofluor white (CFW) in distilled water, 0.2% (w/v) Coomassie Brilliant Blue G-250 (CBB) in 40% methanol and 10% acetic acid, 0.3% (w/v) fluorescein isothiocyanate (FITC) in 10 mM Tris buffer (pH 9), 2% (w/v) nigrosin in distilled water, 0.5% (w/v) phloxine in distilled water, 0.1% (w/v) trypan blue in 10% acetic acid, 25% (v/v) black Indian ink (Winsor & Newton, England) in distilled water, 1% (w/v) amido black in 7% acetic acid, 0.1% (w/v) methylene blue in distilled water of 0.1% (w/v) Nile red in 0.1% (w/v) dimethylsulphoxide. Acridine orange and fluorescent periodic acid-Schiff (PAS) reagent were prepared according to Clark (1981). After staining, a cover slip was placed on top of the sample and sealed with paraffin, whereupon specimens were observed under light or fluorescence microscopes.



Figs 1–4. Fluorescence of ECM labelled with CFW (**Figs 1, 3**) and corresponding light micrographs (**Figs 2, 4**) of *Bipolaris sorokiniana* incubated in 2.4% PDB for 3 h on a glass surface. Germ tube (**Figs 1–2**). Germling (**Figs 3–4**). Bar = 25 µm.

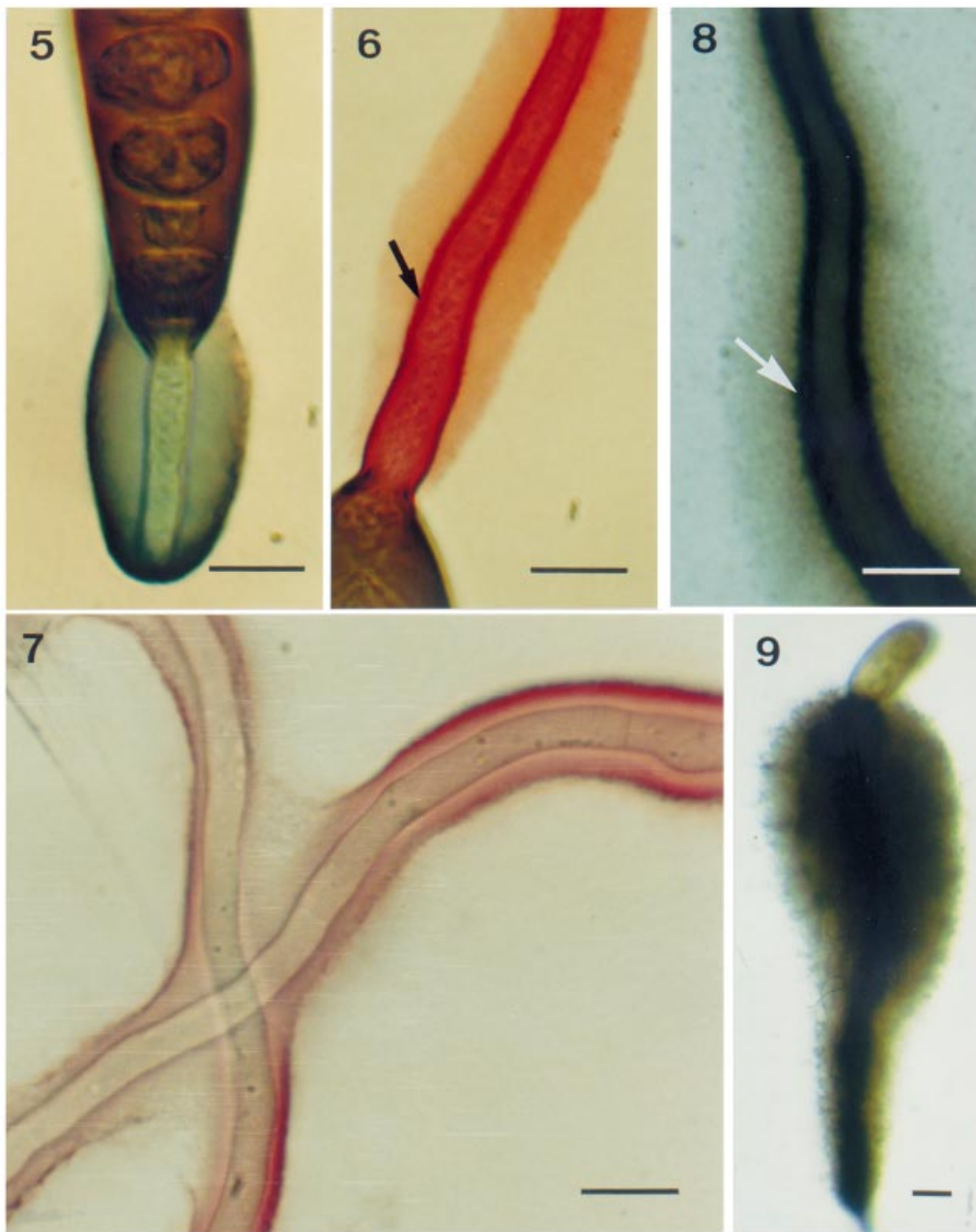
Colloidal gold labelling

Germlings of *B. sorokiniana* attached to glass slides were fixed in glutaraldehyde (GA) and thereafter incubated with 30 µl of colloidal gold (20 nm diam., Sigma) solution for 1 h on a rotary shaker (100 rpm) (Jones *et al.*, 1995). For silver enhancement of gold labelling we followed the protocol of Jones *et al.* (1995), apart from the termination of silver enhancement with sodium thiosulphate. Excesses of reagents were removed with distilled water.

To determine whether hydrophobic or ionic forces were responsible for the affinity of colloidal gold to the ECM, the non-ionic detergent Tween 20, was added to a final concentration of 2% (v/v) or the pH of the gold solution was adjusted to 2 or 10 using 0.2 M HCl or KOH, respectively.

Polystyrene microspheres

Unmodified polystyrene microspheres (0.8 µm diam.), carboxylate microspheres (0.8 µm diam.), bearing negative surface charges, and amino microspheres (0.5 µm diam.) bearing positive surface charges, (Serva, and Polysciences Europe, Germany), were used with the aim of determining



Figs 5–9. Colloidal gold labelling of ECM of *Bipolaris sorokiniana* incubated on a glass slide (**Figs 5–7**). **Fig. 5.** ECM of a germ tube. **Fig. 6.** Two-layered ECM of a germling. Inner layer (arrow). **Fig. 7.** ECM surrounding 1-day-old hyphae. Labelling of ECM of *B. sorokiniana* using colloidal gold followed by silver enhancement (**Figs 8–9**). **Fig. 8.** Two-layered germling ECM. Note, fibrillar structure of the outer layer. Inner layer (arrow). **Fig. 9.** Copious appearance of ECM. Bars = 10 μm .

their capacity to bind to ECM of different structures of *B. sorokiniana*. Stock solutions (5 μl) of microspheres were diluted with 1 ml of 10 mM 3-(*N*-morpholino) propanesulphonic acid (MOPS) buffer (pH 7.2) or distilled water. Thirty microlitres of the microsphere suspension were added to pre-washed germlings, which had adhered to the glass slides. The slides were then agitated (100 rpm) for 1 h at room temperature. After unattached microspheres had been removed with tap water, sites to which microspheres had attached (conidia, ECM of germ-tubes, hyphal tips, hyphae) were recorded using LM.

Lectins

FITC-labelled lectins used to detect the presence of carbohydrates in the cell wall and germling ECM included *Bandeiraea simplicifolia* agglutinin (BS-1), concanavalin A (Con A), *Helix pomatia* agglutinin (HPA), *Lens culinaris* agglutinin (LCA), *Ricinus communis* agglutinin (RCA₁₂₀), *Tetragonolobus purpurea* agglutinin (TPA), wheat germ agglutinin (WGA). All were purchased from Sigma. Binding specificities of lectins are reported in Table 3. Germlings attached to glass slides were exposed to lectin solutions (100 $\mu\text{g ml}^{-1}$) in phosphate-

Table 1. Histochemical labelling of the ECM of *B. sorokiniana* germlings

	Inner ECM	Outer ECM
LM		
<i>Protein</i>		
Acid fuchsin	+	–
Amido black	–	–
CBB	+	–
FITC	+	–
Phloxine	+	–
Trypan blue	+	–
Nigrosin	+	–
<i>Polysaccharide</i>		
Acridine orange	–	–
Alcian blue	–	–
Aniline blue	–	–
CFW	±	+
PAS	–	–
Methylene blue	–	–
<i>Others</i>		
Colloidal gold	+	+
Indian ink ¹	–	+
Nile red	–	–
TEM		
RR	–	+

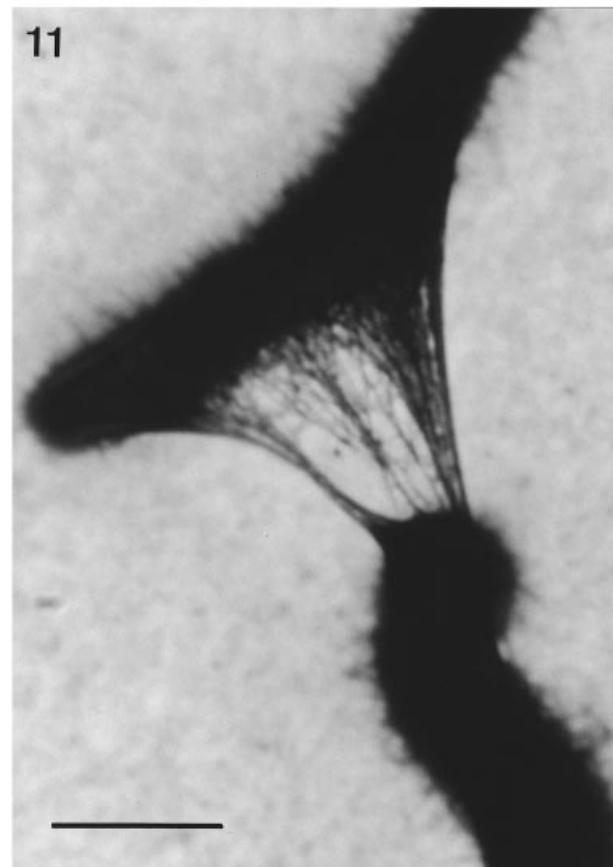
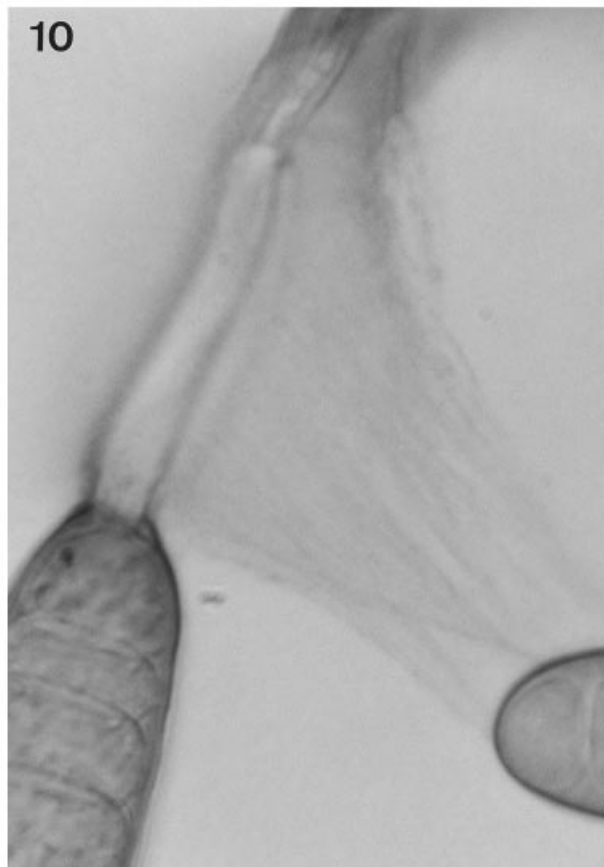
+ = Labelling, – = no labelling, ± = occasional labelling.

¹ Negative stain.

buffered saline (PBS, 0.01 M sodium phosphate buffer, pH 6.8, 0.15 M NaCl, containing 0.1 mM Ca²⁺ for BS-1 and 0.1 mM Ca²⁺, Mn²⁺ for Con A, LCA solutions) for 30 min at room temperature in darkness, washed in buffer, covered with a coverslip, and immediately examined under a fluorescence microscope equipped with FITC filters. To create a negative control, lectins labelling the cell wall were exposed for 1 h to the sugar haptens before labelling. Thus, WGA was exposed to 1 mg ml⁻¹ of *N,N*-diacetylchitobiose, RCA₁₂₀ to 100 mg ml⁻¹ D(+)-galactose, and Con A and LCA to 2 mg ml⁻¹ and 4 mg ml⁻¹ of D(+)-mannose, respectively. Hapten sugars were used in amounts recommended by the manufacturer.

TEM of the ECM

Surfaces of polycarbonate membranes of 12-mm Transwell inserts (Costar, Cambridge) were covered with a thick layer of conidia suspended in 2.4% (w/v) PDB and incubated for 3.5 h at room temperature. Adhered germlings were washed with 0.2 M sodium cacodylate (SCC) buffer (pH 7.2), GA fixed and stained with 3.3% (w/v) Ruthenium red (RR) or 4% (w/v) tannic acid (TA) according to Handley, Hargreaves & Harty (1988) and Hayat (1981), respectively. Controls were prepared



Figs 10–11. Extensions of germling ECM of *Bipolaris sorokiniana* incubated on a glass slide for 3 h. **Fig. 10.** ECM extending to neighbouring conidium. Colloidal gold labelling. **Fig. 11.** ECM extending to neighbouring hyphal tip. Colloidal gold-silver labelling. Bar = 15 µm.

by excluding the RR or TA from the treatments. Preparations were post-fixed with OsO₄ and RR, or OsO₄ alone in SCC buffer (Hayat, 1981; Handley *et al.*, 1988). Samples then were washed with 0.15 M SCC buffer and dehydrated in a graded ethanol series, embedded in Epon resin and polymerized for 24 h at 60°. Ultrathin sections were cut with a diamond knife and mounted on Formvar-coated copper grids, whereupon they were post-stained with uranyl acetate and lead citrate. Sections were examined in a Jeol 1200 EX electron microscope operating at 60 kV.

SEM

Newly collected and washed conidia were resuspended in 2.4% (w/v) PDB or sterile distilled water, applied to polycarbonate membranes and incubated for 3 and 6 h, respectively. Samples were washed with sterile distilled water, labelled with colloidal gold and fixed in 2.5% (v/v) GA in water for 2 h. Gold-labelling was excluded from controls. Thereafter, samples were washed with water, dehydrated in a graded ethanol series and critical-point dried. Specimens were mounted on aluminium stubs, sputter-coated with gold, and examined in a Philips 515 scanning electron microscope.

Cryo-SEM

Pieces of infected leaves or polycarbonate membranes to which conidia and germlings had attached were applied to a specimen holder and frozen by plunging them into a liquid nitrogen slush. Frozen specimens were transported under vacuum in a transfer chamber from the slush freezer to the cryo stage (Oxford CT 1500) mounted on a Philips 515 scanning electron microscope. Contaminating ice was removed by sublimation at -85° for 5 min. Specimens were transferred to the preparation chamber and sputter-coated with gold before being returned to the SEM stage and examined at 20 kV at -150°.

TEM of fimbriae

A 4- μ l droplet of a dense conidial suspension in 2.4% (w/v) PDB was placed on top of Formvar- or carbon-coated copper grids and incubated until the conidia started to germinate. Once germ tubes had reached one to three conidial lengths, grids were washed three times with a drop of water and stained in 2% (w/v) sodium phosphotungstate or 2% (w/v) uranyl acetate water solutions for 30 s. After blotting away surplus stain, grids were air-dried and examined in a Philips CM-10 transmission electron microscope operating at 60 kV.

RESULTS

Histochemical and colloidal gold labelling – LM

Both germ tubes and germlings were covered with ECM that could be visualized with various stains using LM. Most of the samples were visualized after 3 h, occasionally samples were visualized over a longer time period (2–24 h). At the sampling time 3 h about 90% of conidia had germinated, and the

Table 2. Attachment of colloidal gold and polystyrene microspheres to ECM of different structures of *B. sorokiniana*

Surface characteristics	Germ tubes	Germlings, hyphae	Hyphal tips
<i>Colloidal gold</i>			
Hydrophobic, negative	++	++	++
<i>Microspheres</i>			
Negative	++	++	++
Positive	+	–	+
Unmodified	+	–	+

+ = Attachment of colloidal gold or microspheres to ECM.
++ = Strong attachment, – = no attachment.

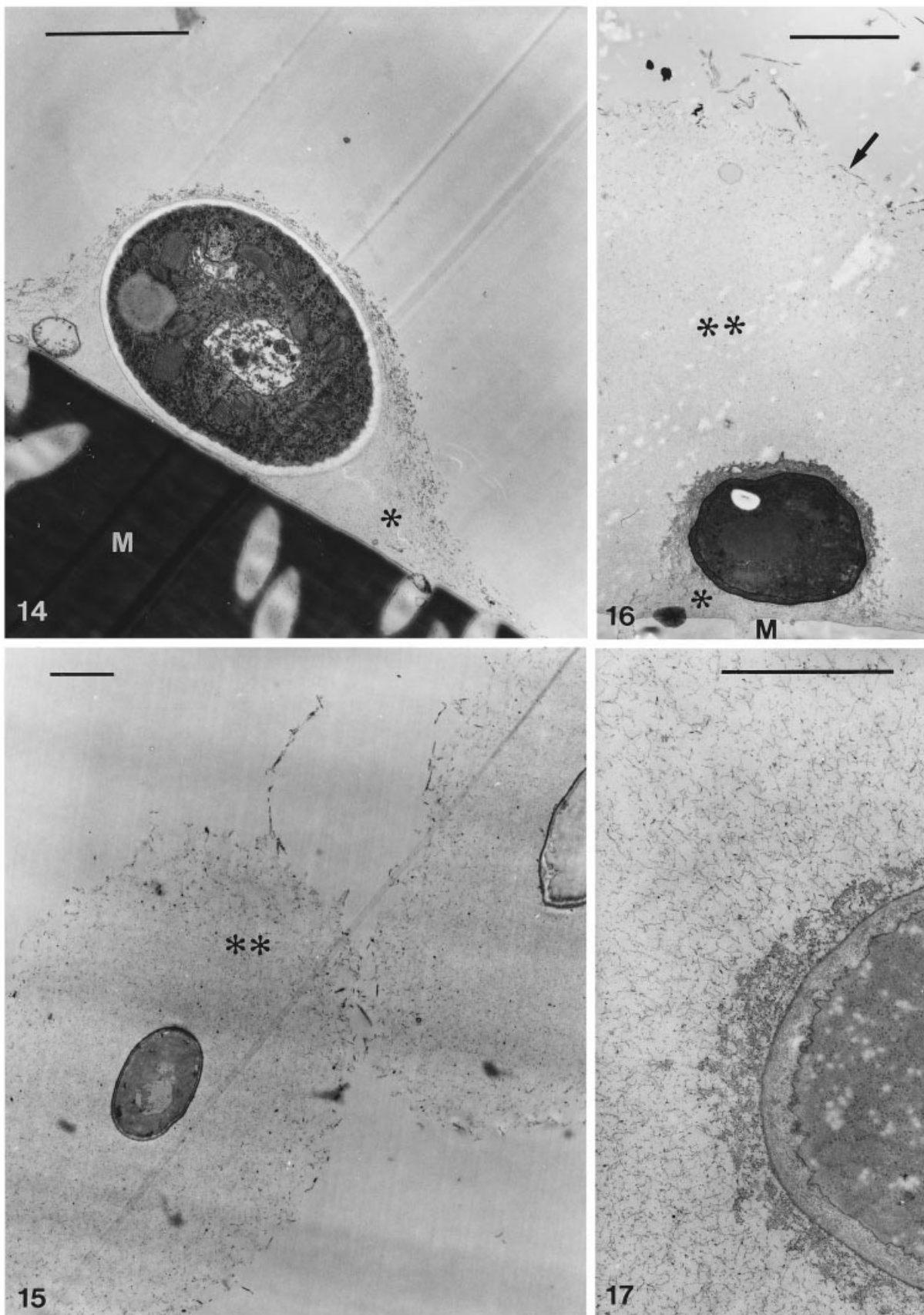


Figs 12–13. Attachment of polystyrene microspheres to ECM of *Bipolaris sorokiniana*. The fungus was incubated on a glass slide for 3 h. **Fig. 12.** Negatively charged microspheres attached to the surface of ECM, revealing its borders. **Fig. 13.** Positively charged microspheres attached to the ECM of hyphal tips. Bar = 25 μ m.

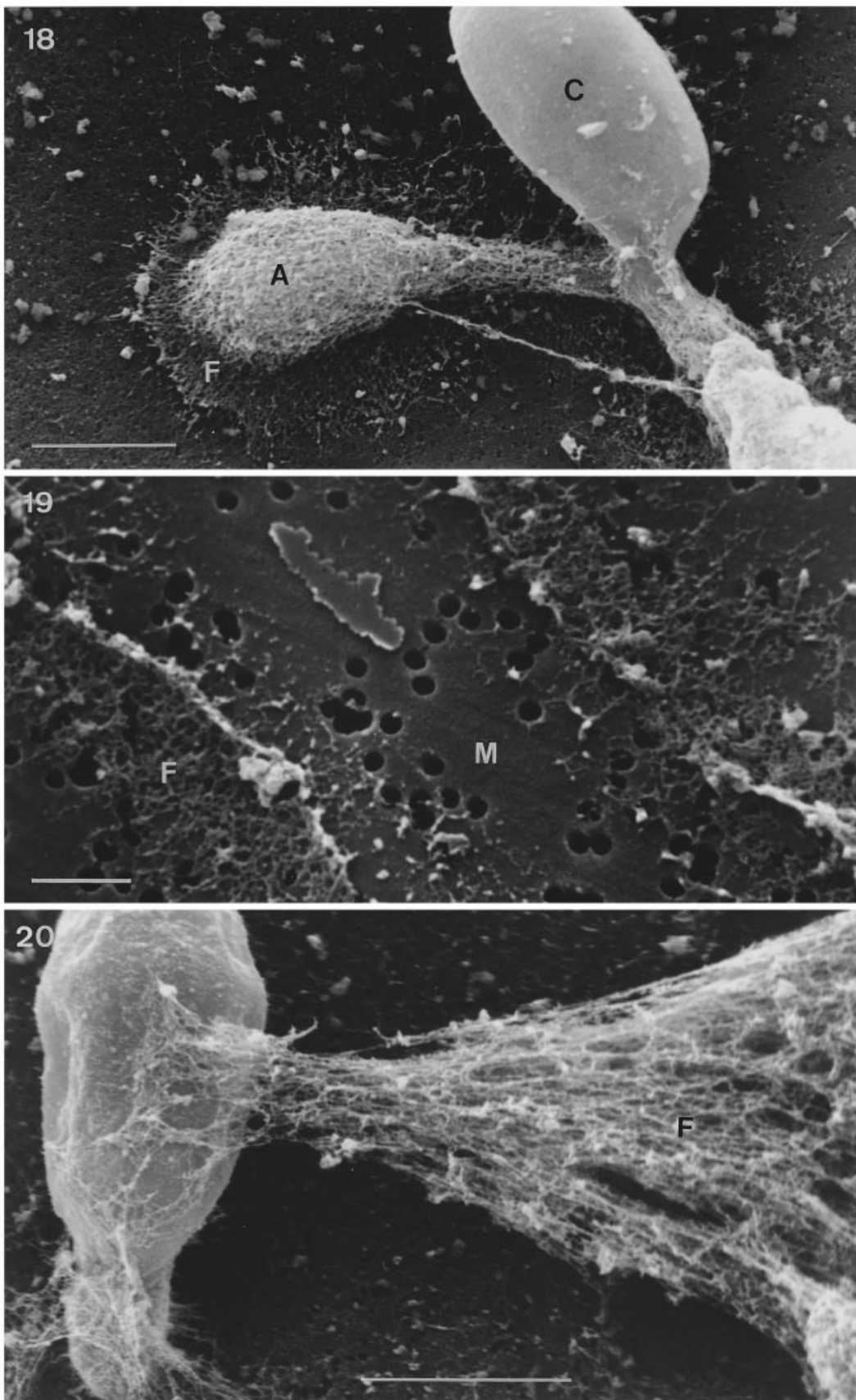
Table 3. Labelling of *B. sorokiniana* using FITC-conjugated lectins

	Specificity	Germling cell wall	ECM
WGA	β -(1–4) (glcNAc) ₂	+	–
Con A	α -man, α -glc	+	–
LCA	α -man, α -glc	+	–
RCA ₁₂₀	β -gal (terminal)	–	–
HPA	α -galNAc	–	–
BS-I	α -gal, α -galNAc	–	–
TPA	α -l-fuc	–	–

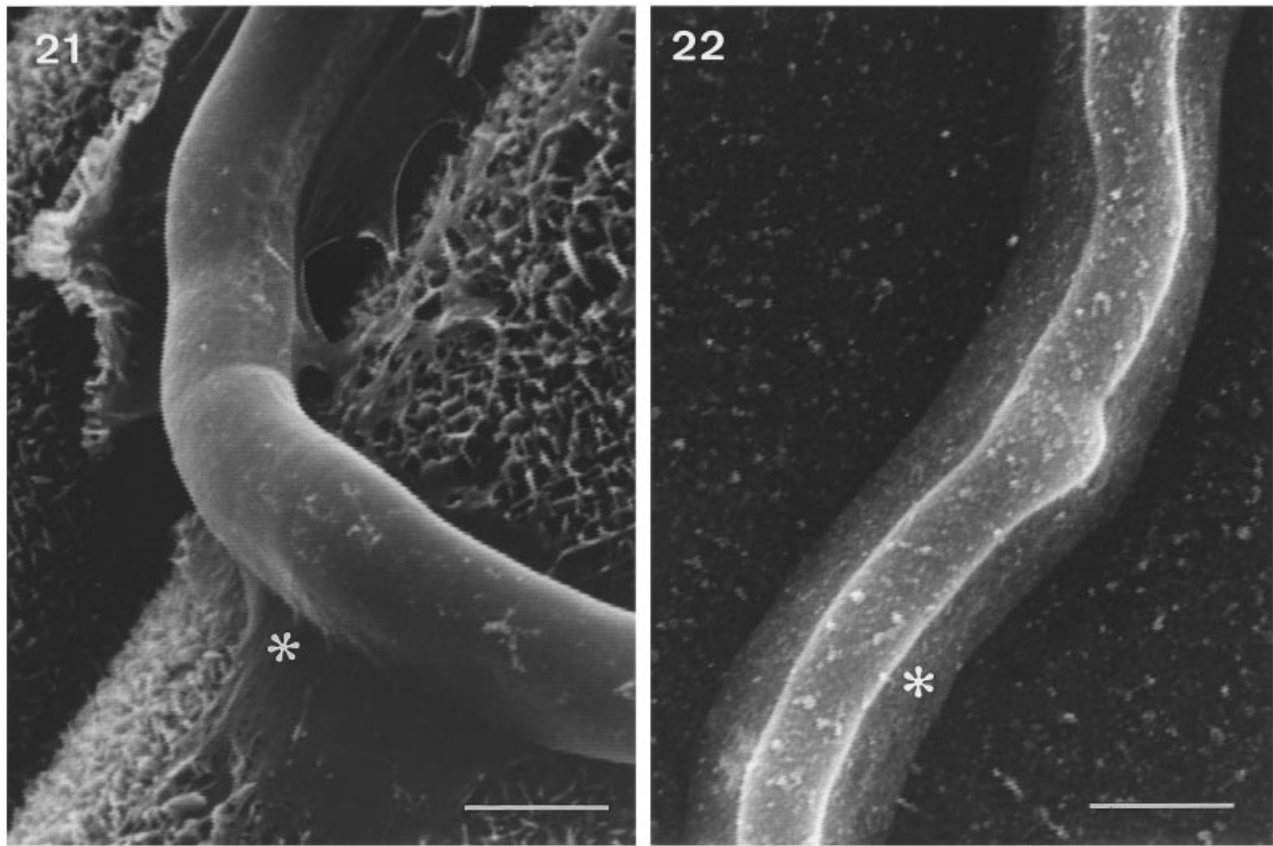
+ = Labelling, – = no labelling.
Lectin concentration was 100 μ g ml⁻¹ in 10 mM PBS buffer (pH 6.8).



Figs 14–17. TEM of *Bipolaris sorokiniana* incubated in 2.4% PDB for 3.5 h on polycarbonat membrane (M). Cross-sections of hyphae. **Fig. 14.** Appearance of the inner-ECM (asterisk) after conventional sample preparation (control). Bar = 2 μ m. **Fig. 15.** Outer-ECM (two asterisks) made visible with Ruthenium red labelling. Bar = 2 μ m. **Fig. 16.** Two-layered ECM. Arrow shows the border of the outer layer. Bar = 2 μ m. **Fig. 17.** Two-layered ECM at higher magnification. Bar = 1 μ m.



Figs 18–20. SEM of colloidal gold-silver stained fibrillar ECM of *Bipolaris sorokiniana* incubated in water on polycarbonate membrane. GA fixation. F = Fibrillar ECM. **Fig. 18.** A = Appressorium. C = Conidium. Bar = 10 μm . **Fig. 19.** ECM fibrils remained on the polycarbonate membrane (M) after removal of the hyphae. Bar = 2 μm . **Fig. 20.** Fibrillar ECM extending to conidium. Bar = 10 μm .



Figs 21–22. Cryo-SEM of hyphae of *Bipolaris sorokiniana* incubated in water for 12 h on barley leaf and polycarbonate membrane. Note amorphous ECM (asterisk) spreading on interface between the hyphae and surface. **Fig. 21.** *B. sorokiniana* water drop inoculated for 12 h on barley leaf surface. **Fig. 22.** Detail of ECM enveloping hyphae on polycarbonate membrane. Bars = 5 µm.

preparation contained all from just appearing germ tubes to germlings of 150 µm length. A hypha just emerged from a conidium (about a half of conidial length) was considered as a germ tube, while those hyphae reaching up to 2–3 conidial length were called germlings. Fungi grown longer than that usually were considered as hyphae.

The ECM surrounding germ tubes labelled intensively for β-glucans with CFW (Fig. 1) and for proteins with colloidal gold (Fig. 5). The staining intensity of the ECM covering germlings was considerably weaker (Figs 3 and 6). The ECM was not visible in unstained preparations (Figs 2 and 4). Furthermore, gold-labelling revealed the presence of two layers in the ECM of germlings, a narrow layer adjacent to the cell wall and an outer less-intensively labelled layer (Fig. 6). The narrow ECM layer was stained with a number of other protein-specific stains (Table 1). In contrast, the inner layer was only occasionally resolved by CFW. Thus, the germling ECM that stained modestly with CFW was probably the outer layer. In addition, the outer layer was visualized by the negative stain Indian ink (Table 1).

When the outer layer was barely detectable silver particles were precipitated onto the gold to enhance labelling and improve visualization of ECM (Figs 8 and 9). After silver enhancement the outer ECM appeared fibrillar (Figs 8 and 9). Colloidal gold also labelled an ECM layer surrounding older hyphae (Fig. 7). The appearance of the hyphal ECM varied and

the two-layered structure which was typical for the germling ECM was not visualized.

Frequently gold-labelling, especially after silver enhancement, revealed thread-like ECM extensions from hypha to conidium and from hypha to hypha (Figs 10–11). The frequency and degree of extension of the thread-like structures were greater for germ tubes that had grown in distilled water than for those grown in a nutrient medium. The ECM threads established contact with material of fungal origin as well as non-living materials, e.g. ion exchange chromatography beads (Bio-Rad DEAE) (not shown).

Labelling of ECM with colloidal gold was inhibited by a low (2) or high (10) gold-solution pH or by adding the non-ionic detergent Tween 20. These results suggested that ionic and hydrophobic interactions were involved in the binding of anionic colloidal gold to the ECM.

Polystyrene microspheres

Negatively charged microspheres attached to the surface of ECM enveloping all germ tubes, hyphal tips, germlings and hyphae (Table 2; Fig. 12). Unmodified and positively charged microspheres attached only to ECM of germ tubes and hyphal tips (Table 2, Fig. 13). This result indicated that negatively charged microspheres had affinity for both newly released and

older ECM, while the positive and unmodified microspheres adhered only to newly released material.

Lectins

None of the lectins studied bound to the ECM. WGA, Con A and LCA bound only to the cell walls of germlings and hyphae of *B. sorokiniana* (Table 3). In the control treatments the sugar haptens blocked the lectin labelling of the cell wall.

TEM of the ECM

ECM observed after conventional chemical fixation with GA and OsO₄ (Fig. 14). Ruthenium red addition during GA and OsO₄ fixation revealed the outer layer (Table 1, Figs 15–17). All hyphal cross-sections of RR-treated material examined were surrounded by an outer ECM 4–8 μm wide. Approximately 50% of the cross-sectioned hyphae possessed the inner layer (Figs 16, 17). This was true for OsO₄, TA and RR preparations. A reason for the frequent absence of inner layer might be either that it was not well preserved during conventional sample preparation or OsO₄ was poorly contrasting the ECM proteinaceous components. Labelling of specimens with TA did not improve visualization of the ECM.

SEM and cryo-SEM

SEM of germlings of *B. sorokiniana* grown on polycarbonate membranes, with or without colloidal gold-labelling, showed that the sheath had a fibrillar structure. The use of gold-labelling improved the degree of visualization of the fibrils. Thick nets of fibrils surrounded the surface of germlings and appressoria (Fig. 18). The sheath extensively expanded at sites where hyphae were in direct contact with the surface. The net of fibrils surrounding germlings was sometimes up to 10 μm wide. After hyphae had been removed from the surface, some fibrillar material remained attached to the polycarbonate membrane, indicating its involvement in hyphal adhesion to the surface (Fig. 19). Similarly, as observed using the light microscope and gold-silver labelling (Figs 10–11), SEM showed that ECM frequently radiated out from the hyphal periphery to neighbouring structures (Fig. 20). Cryo-SEM of *B. sorokiniana* attached to barley leaves or polycarbonate membranes revealed amorphous ECM surrounding the hyphae (Figs 21–22).

Conidial ECM

A fluid-like mucilage surrounding conidia placed on barley leaves was visible using cryo-SEM. The material was observed best at the interface between the conidium and the leaf surface (Fig. 23) or between conidia. Since the material was fluid, detection was possible only at the interface between the conidium and the substratum. Therefore, it was not possible to determine whether the material surrounded the entire conidium or only the part in contact with the surface. Mucilage was observed on dry-inoculated conidia, but not on those inoculated in a drop of water. After germination, conidia appeared to have lost the mucilage. None of the other methods used in this study visualized conidial ECM.

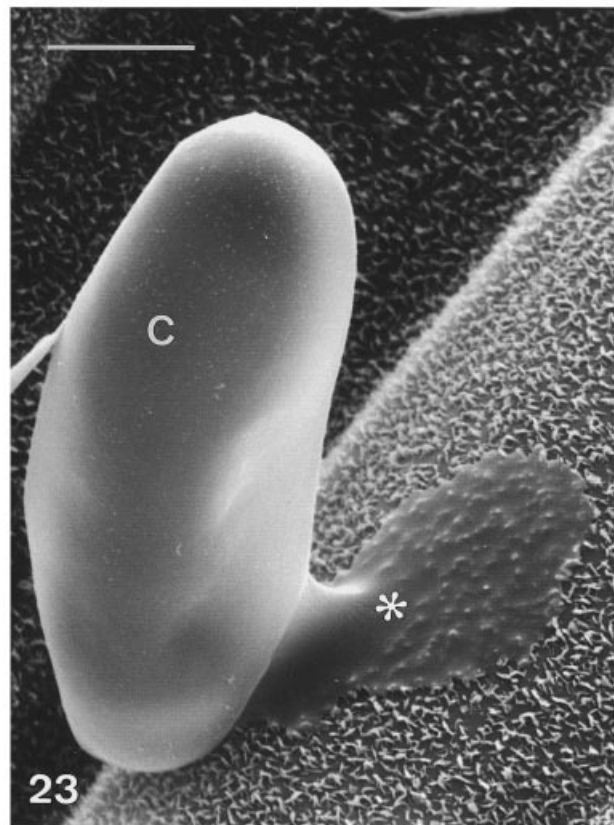


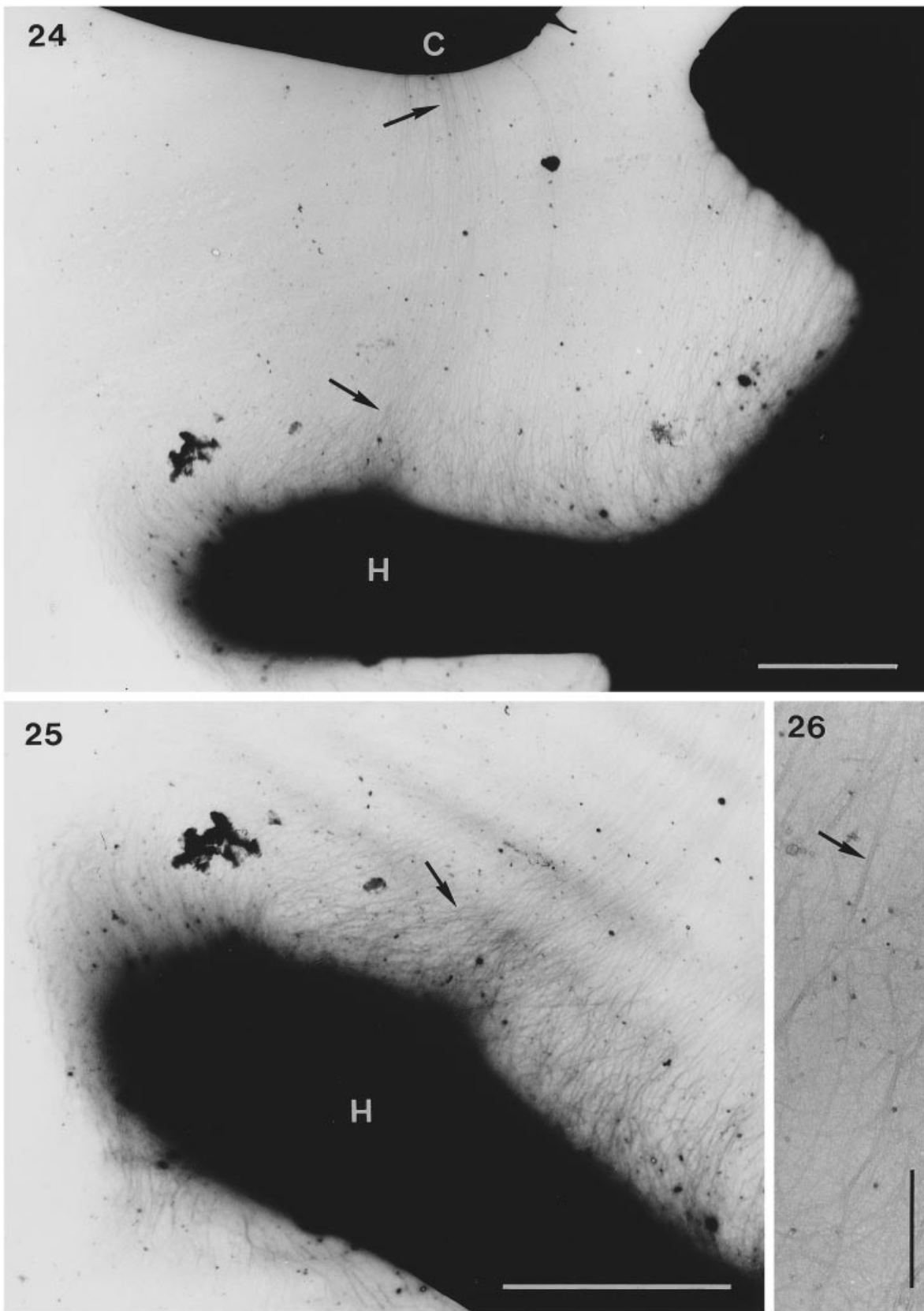
Fig. 23. Cryo-SEM of conidial mucilage (asterisk). The barley leaf was dry inoculated with conidia of *Bipolaris sorokiniana* and incubated for 14 h. C = Conidium. Bar = 10 μm.

TEM of fimbriae

Negative staining of germlings attached to Formvar- or carbon-coated TEM grids using sodium phosphotungstate or uranyl acetate revealed filaments covering germlings and hyphal tips of *B. sorokiniana* (Figs 24–26). The filaments (< 40 nm wide) were protruding approximately 3–5 μm from the surface of germlings. Based on their size and the technique needed to reveal these filaments, it was concluded that the observed structures were so-called ‘fungal fimbriae’ (Day & Gardiner, 1987). The highest resolution of the fimbriae was obtained using carbon-coated grids and uranyl acetate. Occasionally fimbrial bundles were observed radiating out over distances of 15 μm and contacting neighbouring hyphae or conidia (Fig. 24). Fimbriae were not detected on conidia.

DISCUSSION

Using cryo-SEM, we observed a fluid-like mucilage on *B. sorokiniana* conidia. To our knowledge, there are no earlier reports of conidial mucilage in *Bipolaris* spp. No mucilage was found when using LM, TEM, conventional SEM, or cryo-SEM to examine conidia inoculated in a drop of water. We, therefore, suggest that conidial mucilage is a water-soluble exudate. In addition, only ungerminated conidia were surrounded by mucilage, indicating that conidial mucilage is involved in establishing a substrate in which the conidium germinates, in a manner similar to that suggested for the conidia of *Erysiphe graminis* (Nicholson, 1996).



Figs 24–26. TEM of the surface fimbriae (arrow) of *Bipolaris sorokiniana* visualized using negative staining with uranyl acetate. The fungus was incubated for 3 h in 2.4% PDB on carbon-coated TEM grids. **Fig. 24.** Hyphal fimbriae extending to conidium. H = Hypha. C = Conidium. Bar = 5 μ m. **Fig. 25.** Detail of hyphae covered with surface fimbriae. Bar = 5 μ m. **Fig. 26.** High magnification of fimbriae. Bar = 1 μ m.

ECM surrounding germ tubes, germlings, hyphae and occasionally formed appressoria was detected irrespective of the method used. The appearance of the ECM was variable, however: LM and SEM revealed a net-like fibrillar structure, whereas the ECM appeared amorphous in cryo-SEM preparations. Chenu & Jaunet (1992) showed that microbial extracellular polysaccharides formed a network of fibrils when highly hydrated and that their appearance in cryo-SEM depended on the water content at the time of freezing: when desiccated the extracellular polysaccharides tended to be amorphous. Our histochemical study indicated that ECM contains a high proportion of polysaccharides. In the cryo-preparation the sample material might have become partly desiccated in connection with its application to the specimen holder and transfer to the nitrogen slush. These facts taken together suggest that the ultrastructure of the ECM of *B. sorokiniana* varies depending on its water content.

Irregular extensions of fibrillar ECM, most often observed between two closely lying hyphae, between hyphae and conidia, or seldomly between hyphae and foreign objects, were visualized in LM and SEM preparations. Cole, Dewey & Hawes (1996) also reported that ECM of *Botrytis cinerea* occasionally radiated out from the germ tube periphery. Similarly, in a study on ECM of *Cochliobolus heterostrophus*, a Con A-labelled ECM component able to spread over long distances was reported (Sugui *et al.*, 1999). The authors assumed that formation of extensions was a result of physical contact between ECM materials and adjacent hyphae. We observed the formation of ECM extensions between two hyphae as well as between hyphae and conidia that had never been in physical contact. In addition, the extensions often were formed along the surface, i.e., without contacting any other fungal structures. Fimbriae, the other extracellular surface structure of germlings of *B. sorokiniana*, frequently were directed towards other hyphae or conidia, in a manner similar to that of the observed fibrils. Directional growth of fimbriae between adjacent germlings of *Colletotrichum lindemuthianum* also has been observed by Pain *et al.* (1996). The strong resemblance between the extending organization of fimbriae and fibrils suggests that the fimbriae constitute a basic structural component of the ECM and serve as the aggregation centre for other ECM components.

To our knowledge, fimbriae never have been reported from *Bipolaris* spp., but they have been detected on the surfaces of other fungi (Day & Gardiner, 1987; Pain *et al.*, 1996). Fungal fimbriae are glycoprotein molecules that may arise from the interior surface of the plasma membrane and traverse the cell wall (Poon & Day, 1975). Celerin *et al.* (1996) claimed that fimbriae of *Microbotryum violaceum* were composed of fungal collagen. Fimbriae may function as the first interface in cell fusion processes such as mating and anastomosis (Castle, Stocco & Boulianne, 1996) and may also play an important role in pathogenesis, in particular as adhesion structures responsible for attaching the pathogen to its host (Rghei, Castle & Manocha, 1992).

The two-layered appearance of the ECM of *Bipolaris* spp. is well-known (Evans *et al.*, 1982; Braun & Howard, 1994*b*). Zhu *et al.* (1998) obtained a mutant of *Cochliobolus heterostrophus* that lacked the outer layer which showed that the two layers

can exist independently. Based on the staining procedures (LM and TEM) we concluded that the inner ECM layer was rich in proteins and that the outer contained mainly polysaccharides. The inner ECM was labelled with protein-specific stains, as was also found for *B. maydis*, *B. zeicola* and *B. turcicum* (Evans *et al.*, 1982; Braun & Howard, 1994*b*). The outer layer was labelled for acid polysaccharides and β -glucan polymers with RR and CFW, respectively (Luft, 1971; Handley *et al.*, 1988; Nicholas, Williams & Hunter, 1994). This is notable because previously only negative staining or immunofluorescence labelling procedures were known to completely visualise the copious outer ECM of *Bipolaris* (Evans *et al.*, 1981, 1982; Braun & Howard, 1994*b*). CFW is known to interact with cell wall chitin (Nicholas *et al.*, 1994), but neither Clay *et al.* (1994) nor we (present study) succeeded in localizing *N*-acetylglucosamine, a principal component of chitin, or other selected carbohydrates in the ECM of *B. sorokiniana* using lectin probes (Table 3).

We found that both the inner and outer ECM layers were stained with colloidal gold known to bind to proteins (Rohringer, 1989; Jones *et al.*, 1995; Kuo & Hoch, 1995; O'Connell *et al.*, 1996). Since other protein-specific stains labelled only the inner ECM but gold bound to both layers, we assume that gold-labelling to proteinaceous material was more sensitive than other staining techniques. It is also possible, however, that other macromolecules having positively charged groups and hydrophobic domains might have been labelled (Jones *et al.*, 1995).

LM studies using histochemical stains and colloidal gold showed that ECM of germ-tubes differed from that surrounding the germlings. Furthermore, the newly released ECM localized on germlings and hyphal tips showed affinity for microspheres carrying any type of surface properties while hyphal ECM had affinity only for negatively charged microspheres. The processes behind the development/aging of the ECM are not yet understood. Studies on these processes might provide new information on the importance of the ECM in fungal morphogenesis and in the establishment of the host-pathogen relationship.

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