Characterization of the mucilage sheaths of *Lemonniera aquatica* by lectin-gold labelling

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A pre-embedding lectin-gold labelling method was used to characterize the carbohydrate components in the mucilage of *Lemonniera aquatica*. A specific tissue processing protocol was developed, namely: a) primary fixation in 2% paraformaldehyde and 0.2% glutaraldehyde in PIPES buffer (pH 7.2) for 30 min; b) secondary fixation in 2% glutaraldehyde in the same buffer system for 1 h; c) post-fixation in 1% aqueous OsO_4 for 1 h; d) embedment in Möllenhaur's resin. The three gold conjugated lectins used were: concanavalin A, wheat germ agglutinin and *Limax flavus* agglutinin, allowing detection of their complementary saccharides, namely α-D-mannose/α-D-glucose, N-acetyl-D-glucosamine (GluNAc), and N-acetylneuraminic acid (NANA), respectively. N-Acetyl-D-glucosamine and NANA residues were the major components of germ tube mucilage with only a small amount of α-D-mannose/α-D-glucose. However, NANA was restricted to the mucilage in the region of germ tube emergence from the conidial arm. The abundance of GluNAc and NANA residues on hyphae and appressoria was less than that on the germ tube. Conversely, α-D-mannose/α-D-glucose was more abundant in the appressorial mucilage. Variability of mucilage composition was found to exist between different structures of the germinated conidium and also between different regions of the same structure. Further, the conidial cell wall of *L. aquatica* is not chitinous, and lacks NANA and α-D-mannose/α-D-glucose.

Key Words——appressorium; aquatic Hyphomycetes; germ tube; hyphal sheath; mucilage composition.

Conidial attachment of the saprobic aquatic fungus *Lemonniera aquatica* de Wild. (Hyphomycetes) has been previously studied by Read (1990) and Read et al. (1991, 1992a, b, c). Further studies confirmed that initial conidial attachment of *L. aquatica* is achieved by active release of mucilage as a result of a thigmotropic response to the substratum, and subsequent stages in attachment of germ tubes, hyphae and appressoria, are all by the production of extracellular mucilage (Au, 1993; Au et al., 1996). Studies using an indirect enzymatic method indicated that the mucilage of *L. aquatica* is a polysaccharide, comprising β-1,3-glucan, β-glucuronide and β-mannosyl residues; variability of mucilage composition exists between different species and also between different structures of the same species (Au, 1993).

In order to identify further the carbohydrate components in the extracellular mucilage of *L. aquatica*, a lectin-gold histochemical approach was used. Lectin-gold probes have been used to locate carbohydrate and glycoconjugates in pathogenic fungi of several different taxonomic and ecological groups, e.g. *Fusarium oxysporum* Schlechtendahl (Chamberland et al., 1985; Benhamou and Charest, 1986; Benhamou et al., 1988); *Verticillium albo-atrum* Reinke et Berth and *Ophiostoma ulmi* (Buism.) Nannf. (Benhamou, 1988); *Ascocalyx abietina* (Lagerberg) Schlaepfer-Bernhard (Benhamou and Ouellette, 1986); in mycorrhizal fungi, *Hymenoscyphus ericae*

(Read) Korf et Kernan (Bonfante-Fasolo et al., 1987) and *Laccaria bicolor* (Maire) Orton (Lei et al., 1991); and in lower fungi, *Saprolegnia parasitica* Coker and *S. diclina* Humphrey (Burr, 1991).

The effect of tissue processing for electron microscopy on lectin-gold histochemistry and colloidal gold cytochemistry has been previously reported (Armbruster et al., 1983; Roth, 1983; Bendayan et al., 1987; Kellenberger et al., 1987) and it is important to achieve a compromise between the retention of lectin-labelling activities and retention of cell micromorphology.

Pre-embedment labelling of tissue has the advantage of exposing lectin-labelling sites to lectins before they are affected by the organic solvents used in dehydration and embedment. Although the pre-embedment techniques give rise to penetration difficulties for large, conjugated molecules entering tissues (Roth, 1983; Newman et al., 1983), they have been successful in the localization of cell surface carbohydrates of fungi (Hardham, 1985; Burr, 1991).

Fixation involves the formation of intramolecular and intermolecular linkages between fixatives and tissue constituents; the quality of fixation and ultrastructural preservation being proportional to the number of cross-linkages. Conversely, the preservation of chemical specificities and antigenicities of tissue constituents is inversely proportional to the number and speed at which

these cross-linkages are introduced (Hayat, 1970). The conventional electron microscope fixation protocols used in the study of fungal spores have included potassium permanganate (1%, w/v), glutaraldehyde (4%, v/v) and osmium tetroxide (2%, w/v) (Hyde, 1985; Read, 1990; Stanley, 1991; Van Wyk et al., 1991; Van Wyk and Wingfield, 1994; Yusoff et al., 1994) either singly, in combination or successively. These fixation methods have been found to retain the micromorphology of many types of fungal spore. However, these techniques may be less suitable for lectin-gold histochemistry and immunocytochemistry.

For resin embedment, the resin components and the embedment conditions have potential for reducing immunoreactivity of a cell component by conformational or chemical alteration. Correspondingly, consideration should involve not only the effect of resin types on image contrast, stability under the electron beam, image granularity, and sizes of cells and organelles, but also on characteristics such as hydrophobicity and electrical charge on the sections (Aldrich and Möllenhauer, 1986). Möllenhauer's resin (Möllenhauer, 1964) has proved useful in electron microscope studies of several types of aquatic fungal spore (Stanley, 1991; Manimohan et al., 1993; Hyde et al., 1994; Yusoff et al., 1994) and was satisfactory for the conidia of aquatic Hyphomycetes (Read, 1990; Au, 1993; Au et al., 1996). However, there are no reports of the application of Möllenhauer's resin to immunocytochemistry or lectin-gold histochemistry.

In the present study, a specific fixation and resin embedment protocol was developed to determine the optimal conditions for lectin-gold labelling of L. aquatica and then this was used to investigate further the carbohydrate components in the extracellular mucilage of germinated conidia.

Materials and Methods

Conidial suspension A conidial suspension of L. aquatica was prepared by submerging two 9 mm diam discs from a 3-4 wk old 2% malt extract agar culture in 50 ml sterilized distilled water under aeration for 3-4 d at 20°C.

Conidial settlement and development Conidia in suspension were settled on to Thermanox coverslips (previously sterilized in 95% ethanol and then by UV radiation) for 2 h and 24 h (in sterilized moist chambers at 20°C) for germ tube and appressorium formation, respectively. Previous studies on germination and appressorium formation of L. aquatica when grown under the conditions used in study showed that 80% of the settled conidia germinated after 2 h, and 90% of the germinated conidia produced hyphae and appressoria after 24 h of settlement (Au, 1993; Au et al., 1996).

Fixation At the end of the settlement period, the Thermanox coverlips with settled conidia were primary fixed in 0.2% (v/v) glutaraldehyde and 2.0% (w/v) paraformaldehyde in 50 mM PIPES buffer (Burr, 1991). Fixation for 30 min and 1 h, at room temperature, was used to estab-

lish the minimum primary fixation time required. Both paraformaldedyde and PIPES buffer were prepared immediately before use or stored for not longer than overnight at 4°C. After primary fixation, the samples were washed twice (15 min) in 50 mM PIPES buffer followed by a wash in 50 mM PIPES buffer containing 75 mM glycine to remove all aldehyde groups introduced by the fixatives and then washed in phosphate buffer at pH 7.2 for 10 min before incubation in the lectin-gold conjugate. After incubation in the lectin-gold conjugate (see section on lectin-gold labelling), samples were washed (3 × 5 min) in phosphate buffer followed by PIPES buffer (1 × 5 min) before a second fixation in 2% (v/v) glutaraldehyde in 50 mM PIPES (pH 7.2) for 1 h. Fixed material was washed in 50 mM PIPES (3 × 10 min) and then in distilled water (1 × 10 min) to remove all the residual glutaraldehyde which would otherwise react with the osmium tetroxide used as the post-fixative. Washed material was then post-fixed in 1% (w/v) aqueous osmium tetroxide for 1 h at room temperature. Fixed material was dehydrated in a graded ethanol series: (10-90% in 10% steps, 95%, 100% (each for 10 min)) and then to acetone through a 3 : 1, 1 : 1 and 1 : 3 series (each for 15 min) followed by 3 changes in pure acetone (each for 20 min) prior to infiltration and embedment in resin.

Resin embedment The effect of Möllenhauer's resin (Möllenhauer, 1964) and Spurr's resin (Spurr, 1969) on ConA-Au_{20} labelling was compared. Infiltration of material with Möllenhauer's resin was carried out in steps of 20%, 40%, 60%, 80% resin in acetone and finally 100% resin over 2 d. Embedded material was kept in a desiccator at room temperature overnight and then transferred to a 60°C oven for 3-5 d to polymerize. For embedment in Spurr's resin, propylene oxide was used as the intermediary dehydrant between absolute ethanol and the resin. Infiltration through a graded propylene oxide-resin series was completed in 1 d. Embedded material was stabilized at room temperature for 3-4 h before polymerization at 70°C for 2 d.

Ultrathin sections were stained with Reynolds' lead citrate (Reynolds, 1963) followed by a saturated solution of uranyl acetate in 50% ethanol (40 min each) before examination with a JEOL 100S transmission electron microscope at 60 kV.

Lectin-gold labelling Three lectin-gold conjugates: concanavalin A (Con A)-$Au_{20\,nm}$ (Sigma L3642) for α-D-mannose/α-D-glucose, wheat-germ agglutinin (WGA)-$Au_{10\,nm}$ (Sigma L1894) for N-acetyl-D-glucosamine oligomer and Limax flavus (LFA)-$Au_{10\,nm}$ (Polyscience 11758) for sialic acids were used.

Phosphate buffer with 0.5% bovine serum albumin (Sigma A7638) was used for dilution of the lectin-gold conjugate; the bovine serum albumin rendered inactive the non-specific "sticky" proteins on tissues. In the Con A-gold experiments, additional 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$ were included in the phosphate buffer preparation. In order to determine the optimal labelling conditions for each lectin-gold conjugate, different concentrations of lectin-gold and incubation times were studied (Table 1). Incubation was carried out at 20°C in the

Table 1. Concentration of lectin-gold conjugates, incubation time and the corresponding control experiments studied for the pre-embedment lectin-gold labelling of *Lemonniera aquatica* conidia.

Lectin-gold	Concentration (dilution)	Incubation time	Control experiment[a]
Con A-Au$_{20}$:	1 : 5, 1 : 10, 1 : 40, 1 : 80,		
	1 : 200	1 h	0.1 M α-methyl-D-mannopyranoside
	1 : 5, 1 : 10	3 h	1 mg/ml unlabelled Con A[b]
	1 : 5	6 h	
	1 : 5	12 h	
WGA-Au$_{10}$:	1 : 5, 1 : 10, 1 : 20	3 h	1 mg/ml *N, N'N''*-triacetyl-chitotriose
	1 : 5, 1 : 10, 1 : 20	12h	0.1 M *N*-acetyl-D-glucosamine
LFA-Au$_{10}$:	1 : 5, 1 : 10, 1 : 20	12 h	1 mg/ml *N*-acetyl-neuraminic acid

a) A stock solution of 0.2 M or 0.2 mg/ml inhibitor was prepared in phosphate buffer (pH 7.2) and added to the diluted lectin-gold conjugate (at 1 : 1 ratio) for 30 min prior to incubation in the primary fixed conidia.

b) Unlabelled lectin was added to the primary fixed conidia for 30 min before incubation in the diluted lectin-gold conjugate.

dark.

Control tests using inhibitor or unlabelled lectin are required for all the lectin-gold labelling experiments in order to assess the specificity of the lectin-gold complexes (Table 1). Inhibitory saccharide is used to block the binding sites of lectin-gold conjugates and unlabelled lectin is used to pre-occupy the lectin binding sites on the material.

Results

Fixation After primary fixation for 30 min (Fig. 1) and 60 min (Fig. 2), appressoria showed well-preserved micromorphology although the 60 min fixed material showed marginally better retention of membranes and membrane-bounded structures. The electron-dense mucilaginous sheath occurred in aggregates that varied in size and shape.

Resin embedment Comparisons were made of germ hyphae embedded in Möllenhauer's and Spurr's resins after incubation overnight in 1 : 5 diluted Con A-Au$_{20}$. The pattern and the density of gold labelling on the hyphae embedded in Möllenhauer's resin (Fig. 3) were similar to those embedded in Spurr's resin (Fig. 4). In addition, the image contrast, image granularity and dimensions of organelles showed no differences between the two resins.

Concanavalin A-Au$_{20}$ labelling Table 2 summarizes the distribution and abundance of Con A-Au$_{20}$, WGA-Au$_{10}$ and LFA-Au$_{10}$ labelling on different structures of *L. aquatica* conidia after settlement on Thermanox coverslips for up to 24 h.

Sparse or no gold labelling was found on germinated conidia when the Con A-Au$_{20}$ conjugate used for incubation was diluted greater than 1 : 20 times and/or when the lectin-gold incubation time was shorter than 6 h at 20°C. No Con A-Au$_{20}$ labelling was found on conidial

Table 2. Distribution and density of Con A-Au$_{20}$, WGA-Au$_{10}$ and LFA-Au$_{10}$ labelling on different structures of the conidium of *Lemonniera aquatica* after settlement on Thermanox coverslips for up to 24 h.

Structures	Con A-Au$_{20}$[a]	WGA-Au$_{10}$[b]	LFA-Au$_{10}$[c]
1. Conidial arm	—	—	—
2. Germ tube			
a. apex	+	****	#
b. distal region	+ +	***	###
3. Germ hypha	+ +	***	##
4. Lateral hypha	N.I.	—	##
			(—: if mucilage was fibrillar)
5. Appressorium/subtending hypha	+ + +	**	##

a) Germinated conidia were incubated for 12 h in 1 : 5 diluted Con A-Au$_{20}$ conjugate.
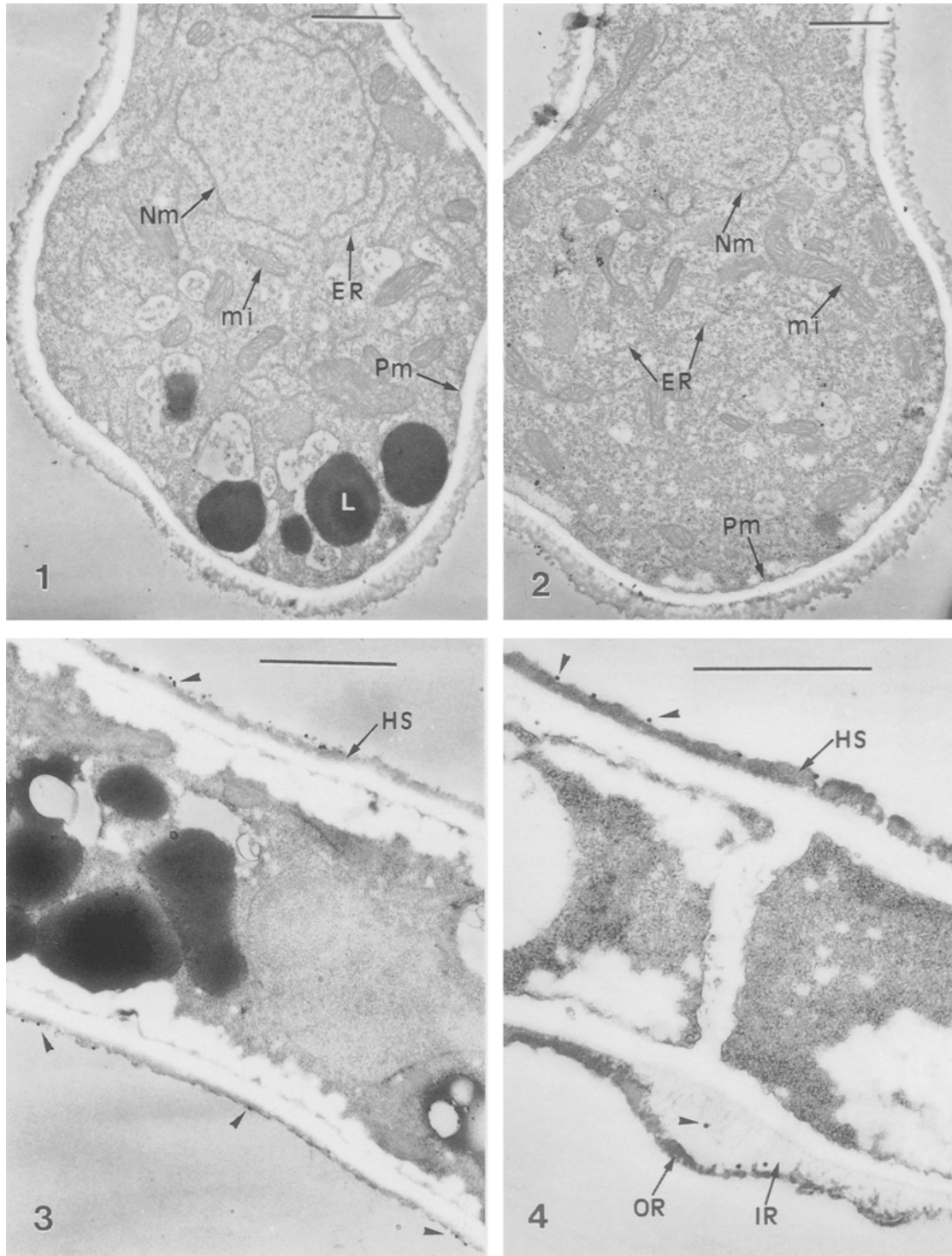b) Germinated conidia were incubated for 3 h in 1 : 20 diluted WGA-Au$_{10}$ conjugate.
c) Germinated conidia were incubated for 12 h in 1 :5 diluted LFA-Au$_{10}$ conjugate.
—: No gold labelling.
+, *, #: Gold conjugate labelling, increase in the number of symbols indicates increase in the density of gold labelling.
N.I.: Not investigated.

Figs. 1-4. *Lemonniera aquatica*. Transmission electron micrographs.
Figs. 1, 2. Appressorium. 1. 30 min in primary fixative. Micromorphology is well-preserved; appearance of the endoplasmic reticulum (ER), lipid (L), mitochondria (mi), nuclear membrane (Nm) and plasmamembrane (Pm) is consistent with good fixation. 2. 60 min in primary fixative. The retention of membranes and membrane-bounded structures is marginally better than 30 min fixation (Fig. 1). ER=endoplasmic reticulum, mi=mitochondria, Nm=nuclear membrane, Pm=plasma membrane. Figs. 3, 4. Germ hyphae incubated overnight in 1 : 5 diluted Con A-Au$_{20}$ after settlement for 24 h. 3. Embedment in Möllenhauer's resin. The Con A-Au$_{20}$ particles (darts) are electron-opaque, circular and 20 nm in diam. HS=hyphal sheath. 4. Embedment in Spurr's resin. The hyphal sheath (HS) comprises a fibrillar inner layer (IR) and an electron-dense outer layer (OR) with a discrete margin. Con A-Au$_{20}$ labelling (darts) is associated mostly with the outer electron-dense layer of the sheath but some labelling is present in the inner layer. Scale bars = 1 μm.
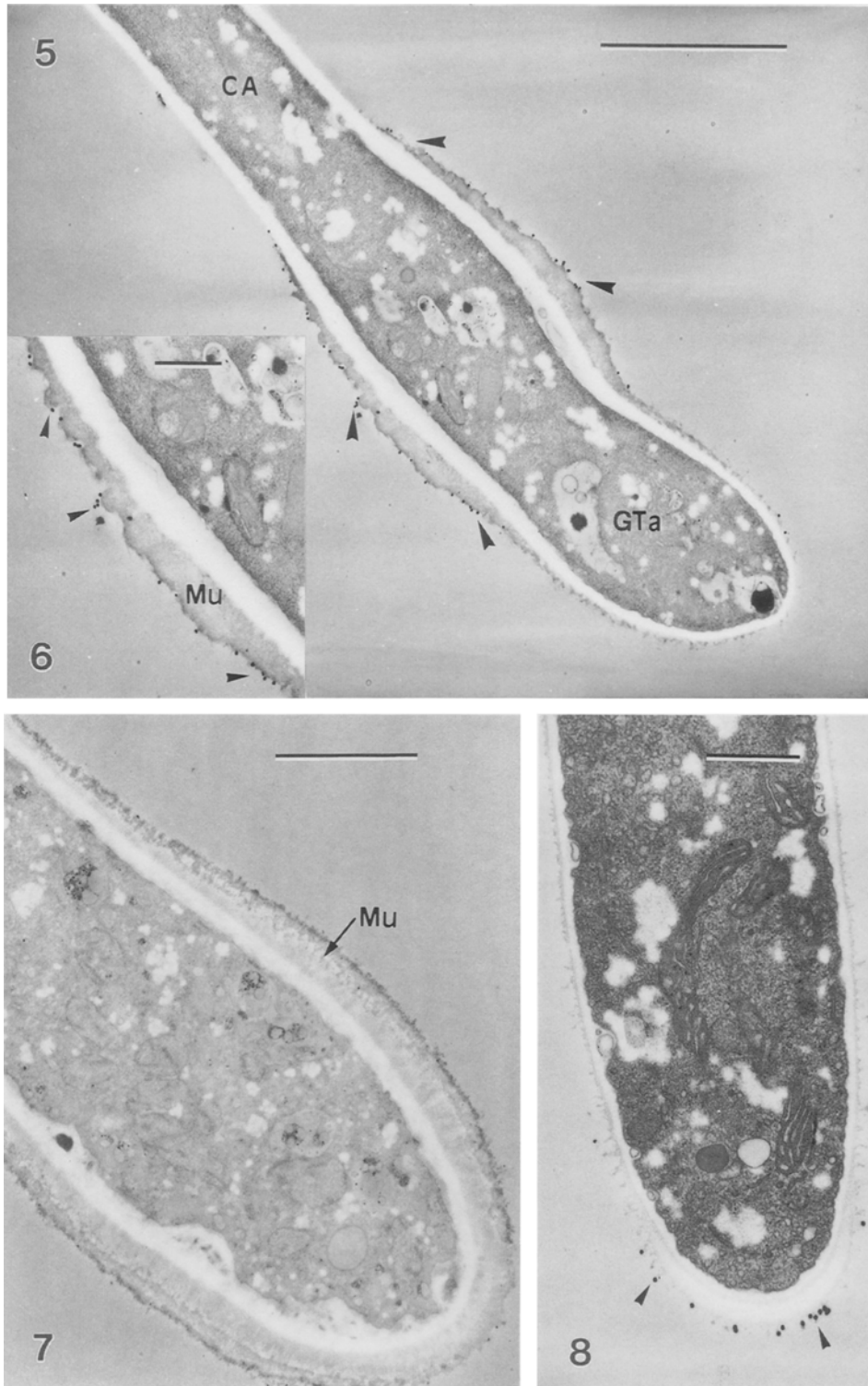
Figs. 5-8. *Lemonniera aquatica.* Transmission electron micrographs.
    Figs. 5-7. Germ tubes incubated overnight in 1 : 5 diluted Con A-Au$_{20}$ conjugate after settlement for 2 h. 5. The Con A-Au$_{20}$ labelling (darts) is located predominantly on the electron-dense mucilaginous sheath with less on the apex of the germ tube (GTa) and none on the conidial arm (CA). 6. Higher magnification of the mucilaginous sheath (Mu) in Fig. 5 showing the gold labelling (darts). 7. Control. Incubated together with 1 mg/ml Con A showing an absence of gold labelling on the germ tube mucilage (Mu). 8. Germ hypha incubated overnight in 1 : 5 diluted Con A-Au$_{20}$ conjugate after settlement for 24 h. Gold labelling (darts) is more abundant on the hyphal apex than subapically (Fig. 4). Scale bars: Fig. 5 = 5 μm; Figs. 6,8 = 0.5 μm; Fig. 7 = 1 μm.
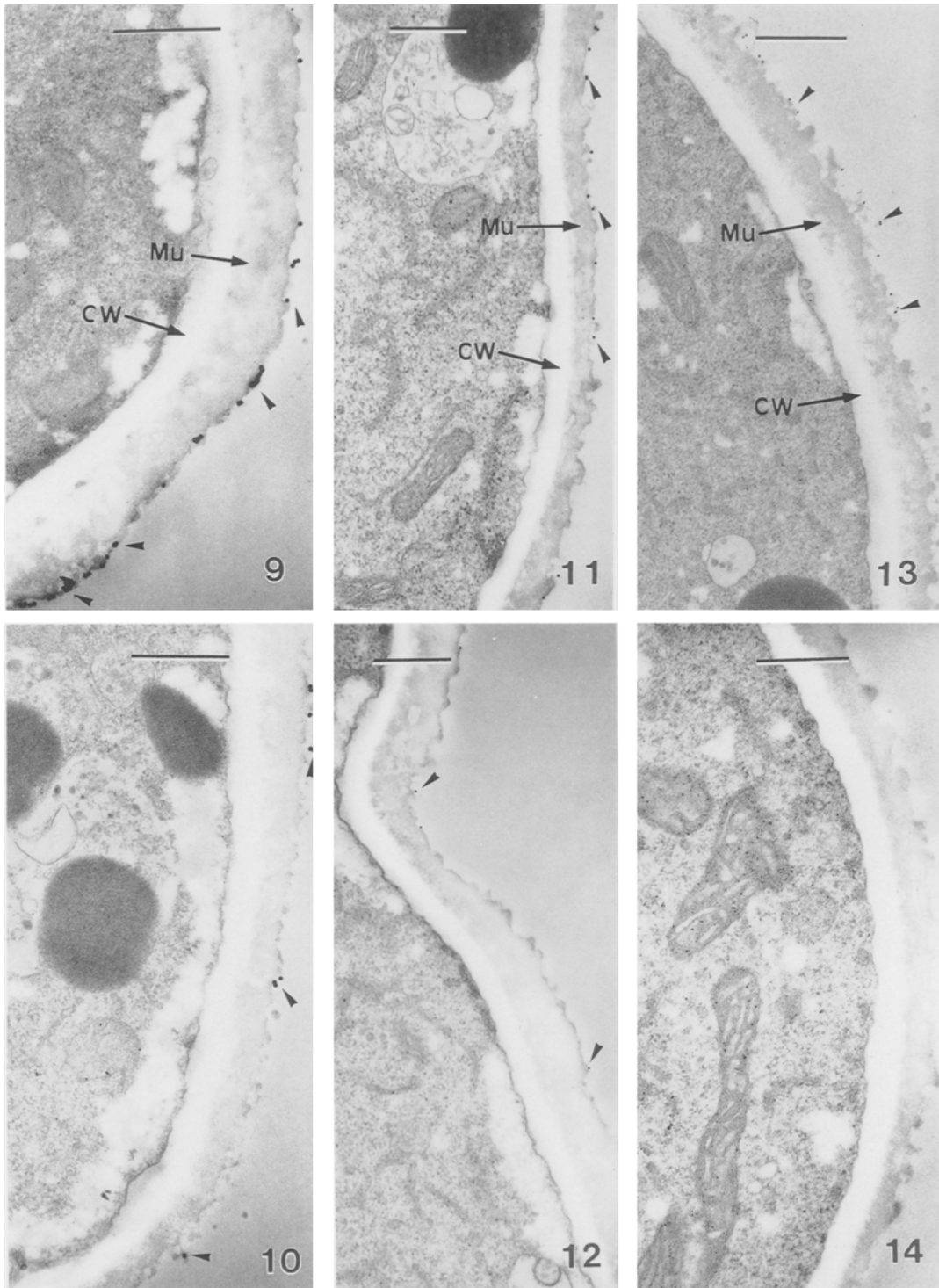
Figs. 9–14. *Lemonniera aquatica*. Appressoria incubated in lectin-gold conjugates after settlement for 24 h. Transmission electron micrographs.

Figs. 9, 10. Incubated in 1 : 5 diluted Con A-Au$_{20}$ conjugates for 12 h. 9. Gold labelling (darts) is abundant and distributed evenly on the periphery of the mucilaginous sheath (Mu). CW=cell wall. 10. Control. Incubated together with 0.1 M methyl-α-D-mannopyranoside. The level of gold labelling (darts) is low compared to noncontrol material (Fig. 9). Figs. 11, 12. Incubated in 1 : 20 diluted WGA-Au$_{10}$ for 3 h. 11. Gold labelling (darts) are on the surface of the mucilage (Mu). 12. Control. Incubated together with 1 mg/ml *N, N'N''*-triacetyl-chitotriose. Note the low level of gold labelling (darts) on the mucilage surface compared to Fig. 11. Figs. 13, 14. Incubated in 1 : 5 diluted LFA-Au$_{10}$ for 12 h. 13. The level of gold labelling (darts) is constant over the surface of the mucilaginous sheath (Mu). 14. Control. Incubated together with 1 mg/ml *N*-acetylneuraminic acid. No gold labelling is present on the appressorial wall and mucilage. Scale bars=0.5 μm.

Table 3. Abundance and distribution of sugars detected by WGA-, LFA- and Con A-gold conjugates in the extracellular mucilage on different structures of *Lemonniera aquatica*.

Lectin[a] -gold	Sugar specificity	Germ tube	Germ hypha	Lateral hypha	Appressorium	Conidial arm
WGA -Au$_{10}$	GluNAc oligomer	A[b]: ++++ D[c]: +++	++	−	+	−
LFA -Au$_{10}$	NANA	A: +/− D: +++	++	++[d]	+	−
Con A -Au$_{20}$	α-D-Mannose/ α-D-Glucose	+	+	−	++	−

a) At 1 : 5 dilution and 12 h incubation at 20°C.
b) "A"—apex of germ tube.
c) "D"—near the region of germination.
d) labelling occurred if mucilage was less fibrillar.
++++ extremely strong, +++ very strong, ++ strong, + moderate, − nil, based on the density of gold labelling.

arms even at the optimal conditions of 1 : 5 dilution and 12 h incubation (Fig. 5).

On the germ tube, a high level of gold labelling was located on the region with a well-developed mucilaginous sheath (Figs. 5, 6). Gold labelling was less concentrated at the apex of the germ tube where the extracellular mucilage was less extensive (Figs. 5, 6). In control treatments with the inhibitor, 0.1 M methyl-α-D-mannopyranoside or 1 mg/ml unconjugated Con A (Fig. 7), no Con A-Au$_{20}$ labelling on the germ tube occurred. Conversely, the mucilaginous sheath at the apex of the germ hypha was dispersed and Con A-Au$_{20}$ labelling occurred (Fig. 8). At the sub-apical region of the germ hypha (Fig. 4), gold labelling was distributed evenly on the outer margin of the electron-dense outer layer of mucilage with a low density of labelling in the inner region of the sheath.

The density of gold labelling on the appressorial mucilaginous sheath (Fig. 9) was higher than that on the germ hypha (Figs. 4, 8). In the presence of the inhibitory saccharide methyl-α-D-mannopyranoside, the intensity of gold labelling on the appressorium was reduced (Fig. 10) and labelling was absent after treatment with the unconjugated Con A.

Wheat germ agglutinin-Au$_{10}$ labelling Regardless of the concentration of WGA-Au$_{10}$ conjugate used and the time of incubation, no gold labelling was found on the conidial arms which lacked mucilaginous sheaths (Fig. 15).

After 3 h incubation, the high density of WGA-Au$_{10}$ labelling on germ tubes incubated in 1 : 5, 1 : 10 and 1 : 20 diluted WGA-Au$_{10}$ conjugate was similar. The apex of a germ tube shown in Fig. 16 comprises a fibrillar component interspersed with electron-dense granular regions, a high density of WGA-Au$_{10}$ labelling was evenly distributed over the surface of the mucilaginous layer. On the distal region of the same germ tube (Fig. 17), a similar pattern of labelling, although less dense than that at the apex, was observed. Germ tubes incubated overnight in 1 : 10 diluted WGA-Au$_{10}$ (Fig. 18) showed a similar density of labelling to those incubated in more diluted conjugate (1 : 20) for a shorter time (3 h) (Figs. 16, 17). Gold labelling was also observed in the sub-surface region of the outer layer of mucilage (Fig. 18). In the

presence of inhibitor : N,N'N''-triacetyl-chitotriose or N-acetyl-D-glucosamine (Fig. 19), the density of WGA-Au$_{10}$ labelling on germ tube mucilage was much reduced. N,N'N''-Triacetyl-chitotriose showed a greater inhibition in WGA-Au$_{10}$ labelling than the control incubated in N-acetyl-D-glucosamine.

Wheat germ agglutinin-Au$_{10}$ labelling was found only on the condensed mucilaginous sheath of the germ hypha (Fig. 20) but not on the lateral hypha (Fig. 21) which possessed a more dispersed fibrillar extracellular mucilage. The density of gold labelling on the germ hypha was similar to or slightly less than that on the germ tube. On the appressorium, gold labelling was found on the surface of the mucilaginous sheath (Fig. 11) but the labelling intensity was lower than that on the germ tube (Figs. 16-18) and the germ hypha (Fig. 20). In the presence of N,N'N''-triacetyl-chitotriose or N-acetyl-D-glucosamine inhibitor, the density of gold labelling on appressorial mucilage was almost absent (Fig. 12).

Limax flavus agglutinin-Au$_{10}$ labelling Gold labelling was sparse on germ tubes incubated in the 1 : 20 diluted LFA-Au$_{10}$ conjugate. At 1 : 10 dilution, the density of gold labelling was low on the germ tube apex but was high on the basal region of the germ tube. At a 1 : 5 dilution (Fig. 24) a greater density of labelling was obtained subapically but was absent on the germ tube apex (Fig. 25). At the region of germination (Fig. 26) LFA-Au$_{10}$ labelled not only the surface of the mucilaginous sheath but also the inner surface of the mucilage outer layer which had become recurved from the inner layer of mucilage and exposed to the LFA-Au$_{10}$. In the presence of 1 mg/ml N-acetylneuraminic acid, LFA-Au$_{10}$ labelling on the germ tube mucilage was inhibited (Fig. 27) with little labelling on the mucilage near the region of germination. No LFA-Au$_{10}$ labelling was found on conidial arms which had been incubated overnight at the highest concentration (1 : 5) of conjugate (Figs. 24, 26).

Limax flavus-Au$_{10}$ labelling was distributed evenly on the surface of the germ hyphal sheath (Fig. 22) but was absent on young lateral hyphae which possessed a fibrillar sheath. Conversely, the mucilage on mature lateral hyphae (Fig. 23) was abundant, less fibrillar, showed

Table 4. Occurrence of Con A-, WGA- and LFA-gold conjugate labelling on the extracellular mucilage, cell wall and cytoplasm of fungi from different taxa.

Lectin	Sugar specificity	Extracellular mucilage	Cell wall	Cytoplasm
Con A	α-D-man	Ascomycotina	Ascomycotina	
	α-D-glu	*Magnaporthe grisea*[a]	*Ascocalyx abietina*[i]	
		Pezizella ericae[b]		
		Ophiostoma ulmi[e]		
		Candida albicans[h]		
		Basidiomycotina	Basidiomycotina	Basidiomycotina
		Hebeloma crustuliniforme[c]	*H. crustuliniforme*[c]	*H. crustuliniforme*[c]
		Laccaria bicolor[c]	*P. tinctorius*[c]	*L. bicolor*[c]
		L. laccata[c]		*L. laccata*[c]
		Paxillus involutus[c]		*P. involutus*[c]
		P. tinctorius[c]		*P. tinctorius*[c]
		Suillus granulatus[c]		*S. granulatus*[c]
		Deuteromycotina		
		Lemonniera aquatica[d]		
		Mastigomycotina		
		Phytophthora cinnamomi[f]		
		P. palmivora[f]		
		Saprolegnia diclina[g]		
		S. parasitica[g]		
LFA	NANA	Ascomycotina		Ascomycotina
		Ascocalyx abietina[i]		*A. abietina*[i]
				O. ulmi[e]
		Deuteromycotina	Deuteromycotina	Deuteromycotina
		L. aquatica[d]	*Paracoccidioides brasiliensis*[q]	*F. oxysporum*[l]
WGA	GluNAc oligomer > NANA	Deuteromycotina	Ascomycotina	
		L. aquatica[d]	*A. abietina*[i]	
			P. ericae[b]	
			O. ulmi[e]	
			Saccharomyces cerevisiae[p]	
		Zygomycotina	Deuteromycotina	
		Choanephora cucurbitarum[i]	*Aspergillus niger*[k]	
		Mortierella candelabrum[i]	*A. flavus*[k]	
		M. pusilla[i]	*A. ochraceus*[k]	
		Phascolomyces articulosus[i]	*Fusarium moniliforme*[k]	
		Piptocephalis virginiana[i]	*F. oxysporum*[l]	
			Penicillium italicum[k]	
			P. expansum[k]	
			P. citrinum[k]	
			P. digitatum[k]	
			Trichoderma viride[m]	
			Verticillium albo-atrum[e,n]	
			Basidiomycotina	
			Puccinia graminis[o]	

a) Hamer et al., 1988; b) Bonfante-Fasolo et al., 1987; c) Lei et al., 1991; d) present investigation; e) Benhamou, 1988; f) Hardham, 1985, 1989; g) Burr, 1991; h) Tronchin et al., 1984; i) Benhamou and Ouellette, 1986; j) Manocha et al., 1990; k) Barkai-Golan et al., 1978; l) Chamberland et al., 1985; m) Mirelman et al., 1975; n) Benhamou and Charest, 1986; o) Harder et al., 1986; p) Erdos, 1986; q) Soares et al., 1993.

LFA-Au$_{10}$ labelling, and appeared similar to that on the germ hyphae (Fig. 22).

On the appressorium (Fig. 13), LFA-Au$_{10}$ labelling was found on the outer margin of the mucilaginous sheath but the labelling density was less than that on the germ tube (Fig. 24). In the presence of the inhibitor, N-
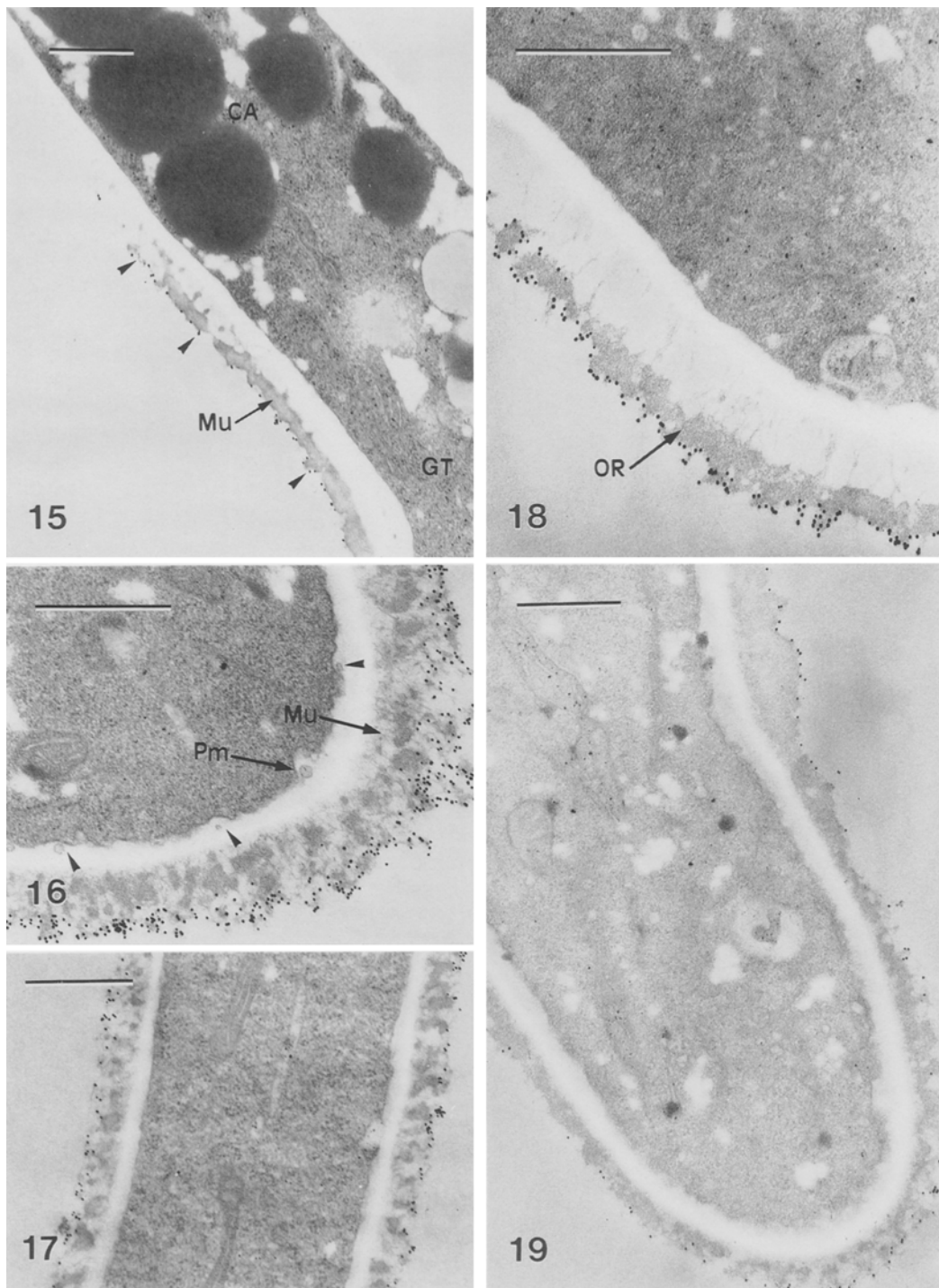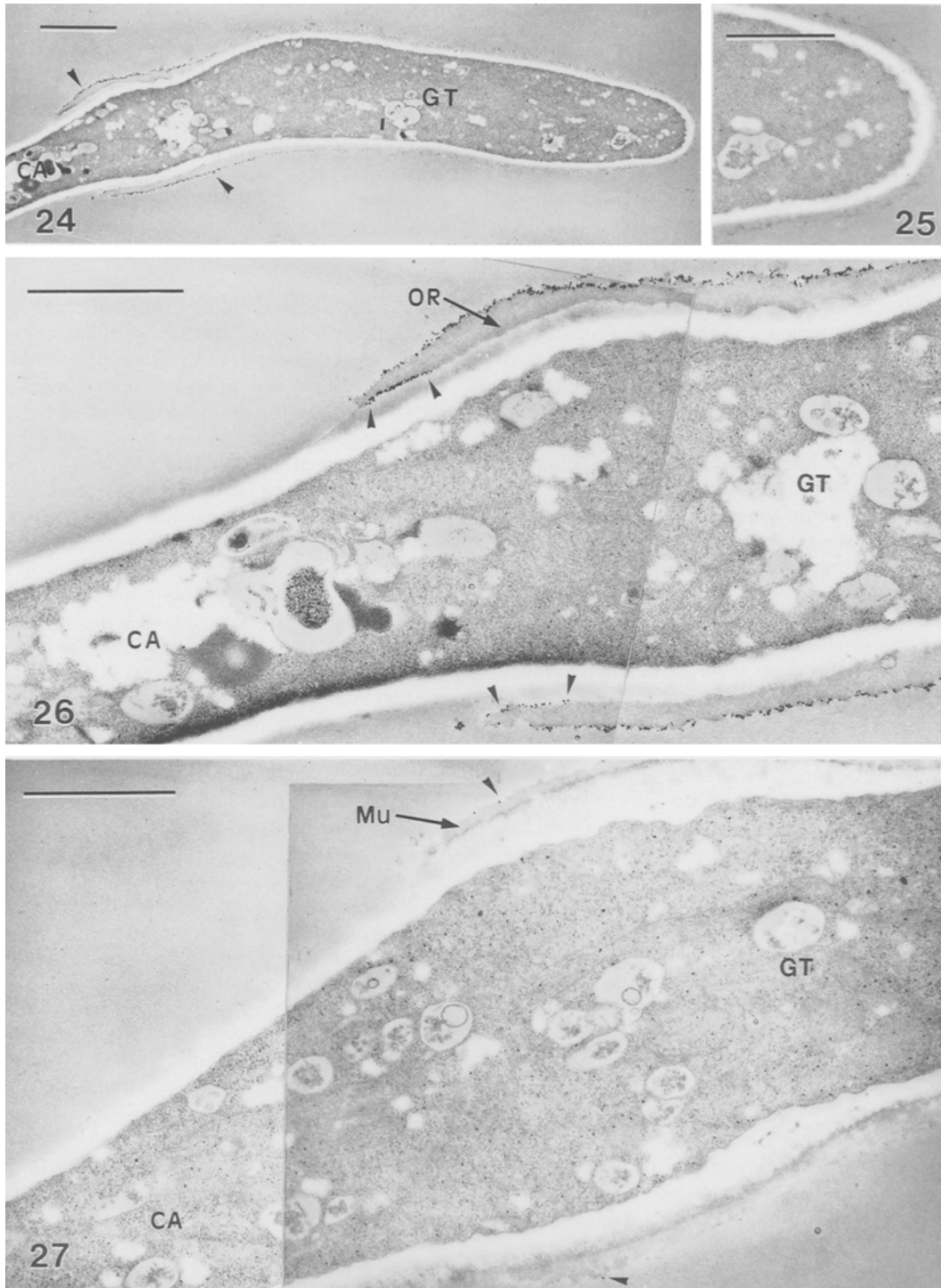
Figs. 15-19. *Lemonniera aquatica*. Germ tubes incubated in WGA-Au$_{10}$ after settlement for 2 h. Transmission electron micrographs.
Fig. 15. In 1 : 10 dilution for 12 h. Gold labelling (darts) is found only on the mucilaginous sheath (Mu) of the germ tube (GT). Neither mucilage nor gold labelling is present on the conidial arm (CA). Figs. 16, 17. In 1 : 20 dilution for 3 h. 16. *Apex of germ tube*. The extracellular mucilage (Mu) comprises fine fibrils and fibrillar masses; the level of gold labelling is high over the outer region of the mucilaginous sheath. Note the convoluted plasma membrane (Pm) associated with extraplasmamembrane vesicles (*darts*). 17. The pattern of gold labelling at the basal region of the germ tube is similar to but less than that at the apex (Fig. 16). Fig. 18. In 1 : 10 dilution for 12 h. Apex of germ tube. Gold labelling occurs mostly on the outer layer of mucilage (OR) but *less* in the subsuperficial region of OR. Fig. 19. Control. Incubated in 1 : 20 diluted WGA-Au$_{10}$ together with 0.1 M *N*-acetyl-D-glucosamine. The level of gold labelling is much lower than that of the non-control material (Figs. 15-18). *The electron-opaque particles in the cytoplasm are stain precipitates*. Scale bars = 0.5 μm.
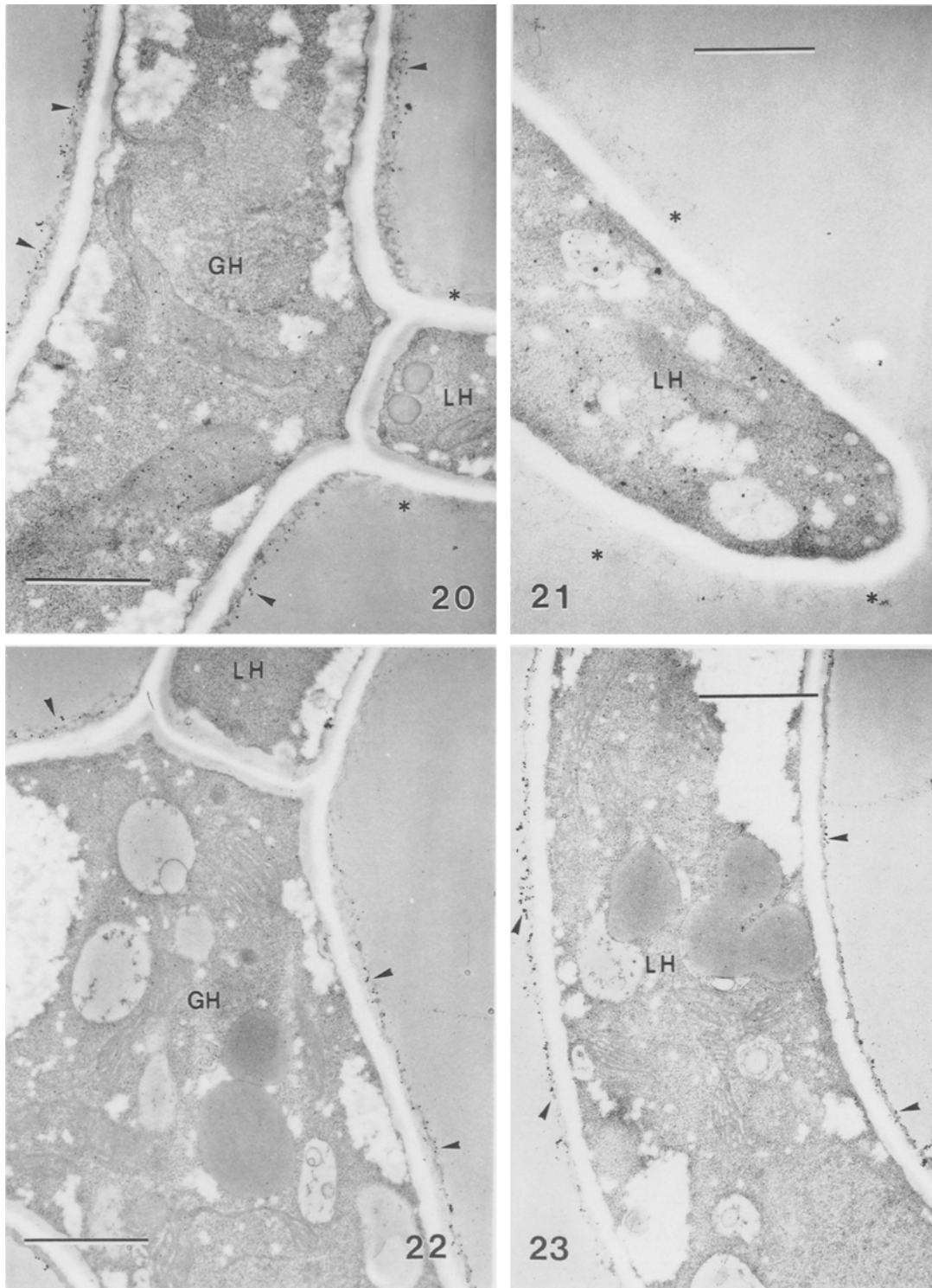
Figs. 20-23. *Lemonniera aquatica*. Transmission electron micrographs.
   Figs. 20-21. Incubated in 1 : 20 diluted WGA-Au$_{10}$ for 3 h after settlement for 24 h. 20. Germ hypha. Gold labelling (darts) is present on the mucilaginous sheath of germ hypha (GH) but absent on the more dispersed, fibrillar mucilaginous sheath (*) on the lateral hypha (LH). 21. Lateral hypha (LH) with a finely fibrillar mucilaginous sheath (*). Note lack of gold labelling. Figs. 22-23. Incubated in 1 : 5 diluted LFA-Au$_{10}$ overnight after settlement for 24 h. 22. Germ hypha (GH). Gold labelling (darts) distributed evenly on the surface of the germ hyphal sheath.   The lateral hypha (LH) has a dispersed, fibrillar mucilaginous sheath that lacks LFA-Au$_{10}$ labelling. 23. Lateral hypha (LH). The morphology, texture and the density and pattern of gold labelling (darts) of the hyphal sheath is similar to that on the germ hypha (Fig. 22). Scale bars = 1 μm.

Figs. 24–27. *Lemonniera aquatica*. Germ tubes incubated in 1 : 5 diluted LFA-Au$_{10}$ for 12 h after settlement for 2 h. Transmission electron micrographs.

Fig. 24. Gold labelling (darts) is abundant in the region of conidium germination (CA) where the mucilage is most abundant. GT = germ tube. Fig. 25. Higher magnification of the apex of the germ tube in Fig. 24 showing absence of gold labelling. Fig. 26. Higher magnification of the germ tube in Fig. 24 showing the region of germination. The mucilaginous sheath on the germ tube (GT) contains an electron-dense outer layer (OR) with a discrete margin on which LFA-Au$_{10}$ labelling has occurred. Gold labelling (darts) is also found on the interface of the inner and outer mucilage layers where germination has occurred and the margin of the mucilage has recurved and become exposed to the LFA-Au$_{10}$. No mucilage and gold labelling are present on the conidial arm (CA). Fig. 27. Control. Incubated together with 1 mg/ml *N*-acetylneuraminic acid. The proximal part of the germ tube (GT) near the conidial arm (CA) shows a very low density of gold labelling (darts) on the mucilage (Mu). Scale bars: Fig. 24 = 2 μm; Figs. 25–27 = 1 μm.

acetylneuraminic acid, the density of LFA-Au$_{10}$ labelling was low or absent on the germ hypha, the lateral hypha and the appressorium (Fig. 14).

Discussion

Chemical composition of the mucilage of *Lemonniera aquatica*

For *L. aquatica*, conidial attachment is established by the production of mucilage, together with the formation of germ tubes, development of germ hyphae and appressoria (Read, 1990; Read et al., 1991, 1992a, b, c; Au, 1993; Au et al., 1996). The lectin-gold studies described in this paper indicate that *N*-acetyl-D-glucosamine (GluNAc) residues, *N*-acetylneuraminic acid (NANA) and α-D-mannose/α-D-glucose residues, which generally occur as the carbohydrate components of acidic mucopolysaccharides and glycoconjugates on the surface of cells, are the components of mucilage on different structures of *L. aquatica*.

Composition of mucilage on the germ tube The GluNAc and NANA residues are the major components of germ tube mucilage with only a small amount of α-D-mannose/α-D-glucose residues present (Table 3). Both GluNAc and NANA residues were localized mainly on the outer layer of the mucilage; no gold labelling was observed on regions of the inner layer of mucilage which had been exposed to the gold conjugates. The distribution of GluNAc and NANA varied in different regions of a germ tube (Table 3). Wheat germ agglutinin labelling (specificity GluNAc≫NANA) was abundant over the germ tube especially on the germ tube apex. Whereas *Limax flavus* agglutinin labelling (for NANA) was restricted to the mucilage in the region of germ tube emergence from the conidial arm. These results indicate that GluNAc is a major component of the mucilage on the fast growing region of a germ tube whereas NANA was present on the "older" regions of the germ tube. It is postulated that *N*-acetylneuraminic acid is a component of the "initial mucilage," secreted immediately upon contact with a surface, and is part of the thigmotropic response of *L. aquatica* (Au et al., 1996). The secretion of NANA ceased or slowed down once contact had been made with the surface.

The presence of NANA on the mucilages of germ hyphae (which develop from a germ tube after septation) and lateral hyphae (which are branches of germ hyphae) indicates that NANA is also a component of "secondary mucilage" formed at a later stage of attachment.

Composition of mucilage on hyphae The mucilaginous sheath on the germ hyphae comprised both *N*-acetyl-D-glucosamine and *N*-acetylneuraminic residues (Table 3), but their abundance, based on the density of gold labelling, was less than that on the germ tube. Small amounts of α-D-mannose/α-D-glucose were also present on the hyphal sheath. The fibrillar mucilage at the apex of the lateral hypha exhibited no WGA labelling; LFA labelling was found only when the lateral hyphal sheath became similar to the germ hyphal sheath in morphology and texture. Based on these results, it is concluded that GluNAc and NANA are not the major components of the

mucilage on young lateral hyphae. However, the mucilaginous sheath on more mature lateral hyphae became similar to that on a germ hypha in chemical composition, morphology and texture.

Composition of mucilage on appressoria In contrast to the gold labelling affinities of mucilage on the germ tube and hypha, Con A-Au$_{20}$ labelling was more pronounced on the appressorial mucilage than that of either WGA or LFA (Table 3). This indicated that α-D-mannose/α-D-glucose is an abundant component of the appressorial mucilage.

Earlier ultrastructural studies and enzymatic studies (Au, 1993; Au et al., 1996) together with the present investigation demonstrate that variability of mucilage composition exists not only between fungal species and different structures of the same species but also between different regions of the same structure.

Composition of the conidial cell wall The conidial cell wall of *L. aquatica* lacked a mucilaginous sheath in conventional electron microscope studies (Read, 1990; Au, 1993; Au et al., 1996) and this was supported by the absence of Con A-, WGA-, LFA-gold labelling in the lectin-gold studies reported in this paper. The lack of WGA-Au$_{20}$ labelling on the conidium implies that the conidial wall of *L. aquatica* is not chitinous. The walls of the germ tube, hypha and appressorium are covered by a mucilaginous sheath which may prevent the WGA conjugates from penetrating the cell wall layers. However, WGA labelling was not observed on the walls of lateral hyphae which were covered only by a fine fibrillar mucilage. Enzymatic studies of *L. aquatica* (Au, 1993) showed that lyticase digested the cell walls of conidial arms, hyphae and appressoria indicating the presence of β-1,3-glucans. Changes in structure and/or composition of the walls associated with different structures of fungi have been reported for several rust fungi (Harder et al., 1986). The conidial cell wall of *L. aquatica* may be different to that of the hyphae. Additional information on wall composition, particularly inner layers, could be obtained by using a post-embedment labelling procedure.

Surface carbohydrate and fungal attachment Concanavalin A, WGA and LFA labelling sites have been found on the extracellular material, cell wall coats and surface components of several pathogenic fungi (Table 4). It has been postulated that these surface sugars play an important role in surface recognition, and subsequent attachment of fungi to their hosts.

The occurrence of NANA on the extracellular mucilage of fungi was first reported on the conifer pathogen *Ascocalyx abietina* (Lagerberg) Schlaepfer-Bernhard (Benhamou and Ouellette, 1986) and was claimed to play an active role during the infection process. A recent study by Soares et al. (1993) suggested that the NANA residues on the cell surface of hyphae and yeast forms of the human fungal pathogen *Paracoccidioides brasiliensis* (Splendore) Almeida may inhibit fungal phagocytosis during early infection. *N*-Acetylneuraminic acid, which has generally been reported on animal cell surfaces, has become frequently reported in fungi and other microorganisms, e.g. pathogenic bacteria, infective protozoa

(Soares et al., 1993).

High levels of *N*-acetyl-D-glucosamine have also been reported on the germ tubes of *Saprolegnia parastica* and *S. diclina* (Burr, 1991). In the mycoparasite *Piptocephalis virginina* Leadbeater et Mercer, GluNAc is involved in attachment to its host *Mortierella pusilla* Oudemans (Manocha, 1985; Manocha et al., 1990). Based on enzymatic studies (Au, 1993), extracellular mucilage of *L. aquatica* comprised β-glucuronide (βGlcUA) residues. Both the βGlcUA and GluNAc residues are components of glycosaminoglycans (GAGs) which occur widely in the extracellular matrix of mammalian cells (Lehninger et al., 1993). It is probable that hyaluronic acid, comprising βGlcUA-GluNAc, may be present in the extracellular mucilage of *L. aquatica*.

Concanavalin A labelling is common to most fungi so far studied (Table 4), e.g. the extracellular mucilage of five mycorrhizal fungi (Lei et al., 1991). However, α-D-mannose/α-D-glucose is not the major component in the mucilage of *L. aquatica*.

The presence of different mucilage types at different stages of germination and stages of conidial attachment may indicate a more complex mucilage system than has previously been speculated. It is probable that effective attachment of conidia of *L. aquatica* and their rapid colonization of natural substrata is mediated by sugars in the extracellular mucilage produced at different stages of conidial development. *N*-Acetyl-D-glucosamine and *N*-acetylneuraminic acid may constitute the principal components of the initial mucilage, secreted as a result of a thigmotropic response. While α-D-mannose/α-D-glucose on the appressorium may be responsible for further interaction and penetration into the substratum. The abundance of highly negatively charged *N*-acetylneuraminic acid on the cell surface of *L. aquatica* may affect the rigidity of the cell surface and/or biological behaviour, e.g. cell recognition, cell-substratum interaction.

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