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Visualization of Surface Sugar Residues in Mycorrhizal Ericoid Fungi by Fluorescein Conjugated Lectins

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Abstract

This research is based on the hypothesis that an adhesion process may be the first event occurring between an ericoid mycorrhizal fungus and a host root for the establishment of the symbiosis. Since surface glycoconjugates are considered the key molecules in many adhesion processes, we localized the surface sugar residues in 8 different strains of ericoid mycorrhizal fungi, which have different symbiotic characteristics. Sugar residues were localized by using labeled or unlabeled lectins and by following direct and indirect techniques.

Results show that only Con A and WGA receptors can be localized: while Con A receptors are regularly scattered on the longitudinal walls of all the strains, two different patterns are recognized after WGA treatment. WGA binding sites are present at the tip and on the longitudinal walls of the strain Duclos XXII, while in the other strains they become evident on the longitudinal walls only after a long pronase digestion, therefore indicating an inner location of chitin.

The comparison between these results and the ultrastructural features of the different strains suggests that the symbiotic establishment is related to the presence of an extracellular substance, which covers the structural polysaccharides of the cell wall and is rich in pronase susceptible glycoconjugates.

Keywords: adhesion, glycoconjugates, ericoid fungi, lectins, mycorrhizae

Abbreviations: Con A = Concanavalin A, LEA = *Lycopersicon esculentum* agglutinin; PWM = Pokeweed mitogen; SBA = Soybean agglutinin; RCA 120 = *Ricinus communis* agglutinin; UEA = *Ulex europaeus* agglutinin; WGA = Wheat germ agglutinin; FITC = fluorescein isothiocyanate; GlcNAc = N-acetylglucosamine

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1. Introduction

Mycorrhizae are symbiotic associations between higher plants and soil fungi. They display a wide range of morphological expressions that reflect the taxonomic diversities of the involved partners. At the cellular level all these diversities are expressed by a great variety of cellular relationships between fungus and host (Scannerini and Bonfante-Fasolo, 1983). However, the mechanism whereby mycorrhizal plants interact with their fungal symbionts and form a highly integrated mycorrhizal association is not known. Two preliminary events seem to be required for the establishment of the symbiosis: (a) growth and development of the mycorrhizal fungus in the rhizosphere of the appropriate plant, a fact which depends on complex organism-environment interactions and (b) contact and adhesion between the cell surfaces of both partners. Only when the latter condition is satisfied, other more complex events (the so called recognition phenomena) may occur and control mycorrhiza formation.

In animal tissues, adhesion between cell surfaces is mediated by the cell coat or glycocalix, i.e. the carbohydrate-rich peripheral zone at the cell surface or by a complex network of extracellular macromolecules referred to as extracellular matrix (Alberts et al., 1983). Among the different macromolecules of these surface areas, glycoconjugates (proteoglycans or glycoproteins) are considered the key molecules. Recently glycoproteins known as cell adhesion molecules (CAMs) have been studied in great detail (Edelman, 1984) and molecules with similar features have been suggested to be involved in partner adhesion during the lichen symbiosis (Bubrick et al., 1985). The aim of this work was to localize the surface sugar residues of some mycorrhizal fungi (using differently labeled lectins) in order to find out whether CAMs are also involved in the initial events of mycorrhizal establishment. The ericoid mycorrhizal fungi were chosen, because they are easily grown in pure culture, and we are well acquainted with their host specificity and infection process (Bonfante-Fasolo and Gianinazzi-Pearson, 1979, 1982; Bonfante-Fasolo et al., 1984).

2. Materials and Methods

Fungal strains

Different fungi isolated from ericoid mycorrhizae and related to the species *Pezizella ericae* Read were used in this study (Table 1). The strains were cultured on 2% malt agar extract and the colonies were then subcultured on the following media for lectin binding tests: (a) a liquid medium containing

Table 1.

Isolate Name	Species	Host Plant	Origin
Duclos VI	—	*Rhododendron* sp.	Duclos & Fortin, 1983
Duclos VIII	—	*Vaccinium corymbosum*	Duclos & Fortin, 1983
Duclos IX	—	*Erica vagans*	Duclos & Fortin, 1983
Duclos XV	—	*Vaccinium corymbosum*	Duclos & Fortin, 1983
Duclos XXII	—	*Erica carnea*	Duclos & Fortin, 1983
—	*P. ericae*	*Calluna vulgaris*	Read, 1974
Strain A	—	*Calluna vulgaris*	Pearson, 1971
Rhododendron	*P. ericae*	*Rhododendron* sp.	Vegh et al., 1979

2% malt extract, and (b) soil/water agar medium, according to Pearson and Read (1973). Fungal colonies were used 2–8 weeks after inoculation.

Chemicals

Lectins

Different lectins specific for different sugar residues were used: Con A, RCA 120, SBA, UEA, WGA, PWM, LEA in unlabeled or fluorescein isothiocyanate (FITC) labeled form and were purchased from Vector Laboratories, Inc. (Burlingame, CA), Sigma Laboratories (St. Louis, MO), or Polysciences, Inc. (Warrington, PA). In addition, all the listed lectins (with the exception of the last two) were purchased in the biotinylated form from Vector Laboratories (Burlingame, CA), together with FITC labeled Avidin. FITC alone was purchased from Polysciences.

Antibodies

Goat antibodies against WGA as well as FITC labeled antigoat antibodies (IgG) were purchased from the Vector Laboratories and Miles Scientific respectively.

Monosaccharides and oligosaccharides

α-methyl-D-mannoside, L-fucose; D-galactose; N-acetyl-D-galactosamine; N-acetyl-D-glucosamine; N,N'-diacetylchitobiose; N,N',N"-triacetyl-chitotriose and ovomucoid were used for the inhibition of lectin binding. They were purchased from Sigma.

Pronase obtained from *Streptomyces* (Koch-Light Laboratories) was used in order to reveal other than surface lectin-binding sites.

Table 2.

Buffer		WGA (Sigma)	LEA	PWM	CONA	WGA (Vector)	SBA	UEA	RCA120	CONA (Polysciences)	WGA (Polysciences)
Tris-HCl pH 7 Ca/Mn	50 mM 10 mM									■	
	pH 7.1							■			
HEPES	pH 7.5						■				
10 mM	pH 8				■	■					
	pH 8.5								■		
PBS	pH 6.8	■	■	■							
10 mM	pH 7.4										■

Calcofluor, used to reveal fibrillar structures, was purchased from Polysciences, Inc, Warrington.

Techniques

Lectin binding sites were demonstrated by direct and indirect methods.

Direct method

Small amounts of the fungal colonies were washed in buffer, a different one for each lectin, according to the manufacturer's instructions (Table 2), and incubated with the FITC lectins in the dark for 30 min (Fig. 1,a). The lectin concentration ranged from 10μg to 100μg/ml in the appropriate buffer. Other samples were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 for 15–30 min, washed in the same buffer and treated as described.

Indirect methods

Biotin-Avidin

A modification of the procedure by Farnum and Wilson (1984) was used. Fungal hyphae were washed in the appropriate buffer (HEPES 10 mM, pH 7), according to manufacturer's instructions and incubated in the lectin-biotin solution at room temperature in the dark for 30 min. The lectin concentration was 100μg/ml. After washing carefully in the same buffer for 30 min, the samples were treated with the Avidin/FITC solution for 15 min, washed again and observed directly (Fig. 1b).

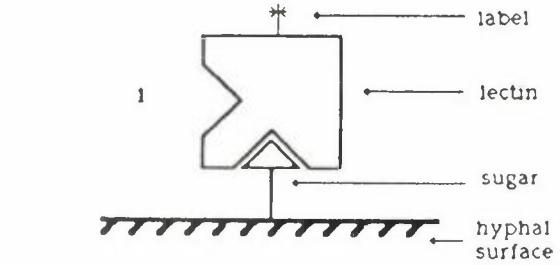
For the indirect demonstration of WGA, the fungal samples were treated according to the schedule by Fisher et al. (1984). Briefly, the samples were incubated with the unlabeled lectin (50–100μg/ml) for 30 min and after buffer washings (PBS 10 mM, pH 7.4) treated with goat anti-lectin antibodies (diluted 1:100 with PBS) for 30 min at room temperature. After three or more washes in the buffer they were incubated with the FITC labeled anti-goat antibodies (diluted 1:10 or 1:40 in the buffer) for 30 min at room temperature (Fig. 1,c).

Controls

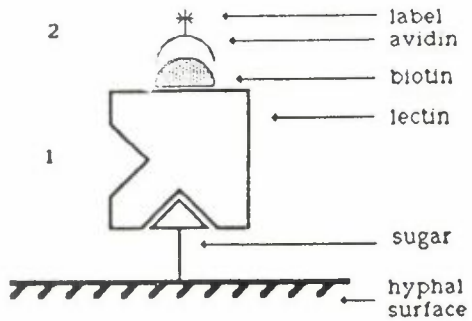
The following controls were carried out for direct labeling:

1. Lectin binding was tested in the presence of the inhibitory sugars listed under "chemicals". The sugars were added at 300 mM concentration to the labeled or unlabeled lectin before incubation. To inhibit the WGA reaction, ovomucoid was added to the WGA/FITC solution (15 mg/ml), as well as GlcNAc monomers and oligomers.
2. Samples were treated with FITC without lectins.
3. Samples were directly observed in the buffer to check their autofluorescence.
4. Fungal colonies were pretreated with a pronase solution for 22 hrs at room temperature and then treated with WGA/FITC as described above.

In the case of the indirect methods, inhibitory sugars were used as mentioned for the direct method and biotin-lectin was also omitted to check aspecific staining due to Avidin/FITC. In the antibody technique, either the unlabeled lectin (step one) or the lectin antibody (step two) was omit-



A



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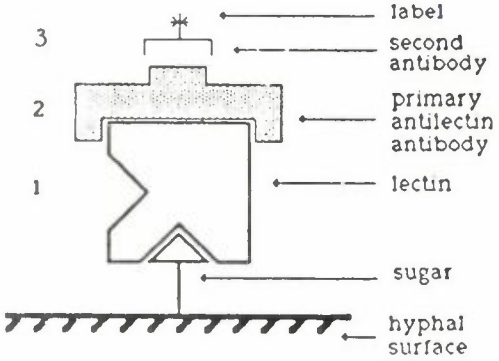


C

Figure 1. Scheme showing direct and indirect techniques to reveal sugar residues by using lectins: (A) Direct staining; (B) the avidin-biotin lectin method; (C) the two step antibody method.

ted (Fig. 1,c) to assay the presence of endogenous lectin-like substances or aspecific fluorescence caused by FITC/IgG.

Samples were immediately observed using a Zeiss Universal photomicroscope equipped for epi-illumination with a 485 excitation filter and a LP 520 barrier filter for all the FITC labeled chemicals: a 365 excitation filter and a LP 420 barrier filter for Calcofluor.

3. Results

Direct method

Reactions with FITC conjugated lectins: Con A, WGA, PWM and LEA bound to the hyphal walls. Con A treatment caused a continuous fluorescence along the longitudinal walls, sometimes more marked at the apex, in all the strains (Figs. 2,3,4). In some strains (Figs. 2,4), fluorescence was not limited to the wall, but it surrounded the hyphae as a thin halo. The inhibitory sugars (glucose and α-methyl-D-mannopyranoside) prevented the reaction altogether (Fig. 5). The response was constant with colonies of all ages. With WGA, specific for GlcNAc residues, different fluorescence patterns were observed. The first pattern was shown by the Duclos XXII strain: the hyphae appeared strongly labeled in the apical zones, when the colony was two weeks old (Fig. 6), but later a large part of them showed a strong fluorescence along the longitudinal walls (Fig. 7 and inset). The reaction was well inhibited by N,N'-diacetylchitobiose, by N,N',N"-triacetyl-chitotriose and by ovomucoid (Fig. 8), while GlcNAc did not prevent the reaction. WGA/FITC strongly bound to the hyphal walls also after pronase treatment (Fig. 9 and inset). The second pattern was shown by *Pezizella ericae* Read and strain A. Fluorescence was irregularly scattered on the longitudinal walls of some hyphae and lacking on others (Fig. 10). A similar pattern was observed in the strain VI (Fig. 11), also when it had globose cells. In the other strains (*P. ericae* from *Rhododendron*, Duclos VIII, IX, XV) fluorescence was irregularly spread on the longitudinal walls of the young hyphae, but at maturity fluorescence was strictly limited to septa appearing as ladder rungs (Figs. 12,13). Moreover, when the hyphae broke to form arthrospores, the fluorescent septa displayed a typical X shape, resulting from a double plate (Fig. 14). In these strains, oligomers of GlcNAc were effective in preventing the reaction, while ovomucoid enhanced fluorescence along all the longitudinal walls (Figs. 15, inset and 16). Furthermore, a strong increase of fluorescence on the longitudinal wall was observed after pronase treatment, followed by the WGA/FITC treatment (Fig. 17).

Similar results were obtained: (a) by using the other two lectins specific for GlcNAc (PWM and LEA), though intensity of fluorescence was sometimes slightly different; (b) by fixing samples with paraformaldehyde and (c) by examining colonies grown on the soil-agar medium, even though in this case the hyphae sometimes appeared too compact to allow a good observation. No autofluorescence was observed in any studied strains and FITC did not cause any fluorescence on the walls. All the strains had a positive reaction to Calcofluor treatment, that labeled longitudinal walls and especially the septa (Fig. 18).

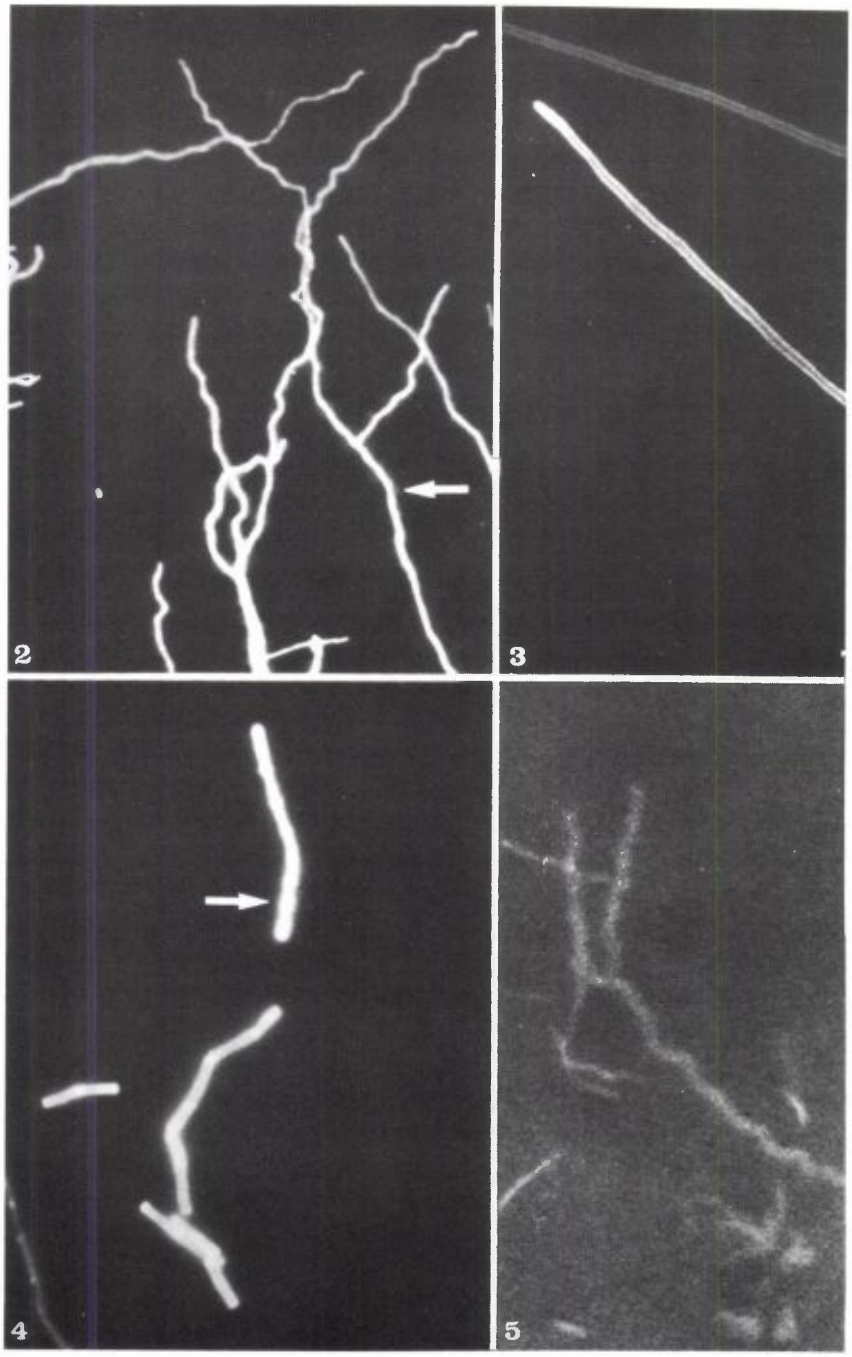
Indirect methods

The Biotin-Avidin technique

Hyphal wall staining resulted from exposure to biotinylated Con A and WGA, but not from exposure to other biotinylated lectins. After Biotin-Con A/Avidin-FITC treatment, all the strains were strongly fluorescent only on their longitudinal walls, mostly in the apical zones (Figs. 19,20,21,22). However, in controls with only Avidin-FITC, the reaction was prevented only in some strains (VI, IX, XXII) (Fig. 23); in the other strains (Duclos VIII, XV, *Pezizella ericae* Read and *P. ericae* from *Rhododendron*) fluorescence persisted on the longitudinal walls as defined patches (Fig. 24), while apical zones and branching points remained completely dark. Strains reacted differently with Biotin-WGA/Avidin-FITC: some of them (Duclos VI, IX) exclusively showed a strong fluorescence, localized on the septa (Fig. 25), while others (*Pezizella ericae* Read, from *Rhododendron*, strain A and Duclos VIII) showed a diffuse patch-shaped fluorescence along their longitudinal walls, as well as fluorescence of their septa (Fig. 27). Apical zones of Duclos XXII were strongly labeled (Fig. 26) and at maturity fluorescence occurred irregularly on the longitudinal walls of most hyphae. In this strain, the reaction was specifically inhibited by the addition of N,N'-diacetylchitobiose and N,N',N"-triacetyl-chitotriose. Avidin-FITC alone caused patterns as those already described (see Figs. 23 and 24).

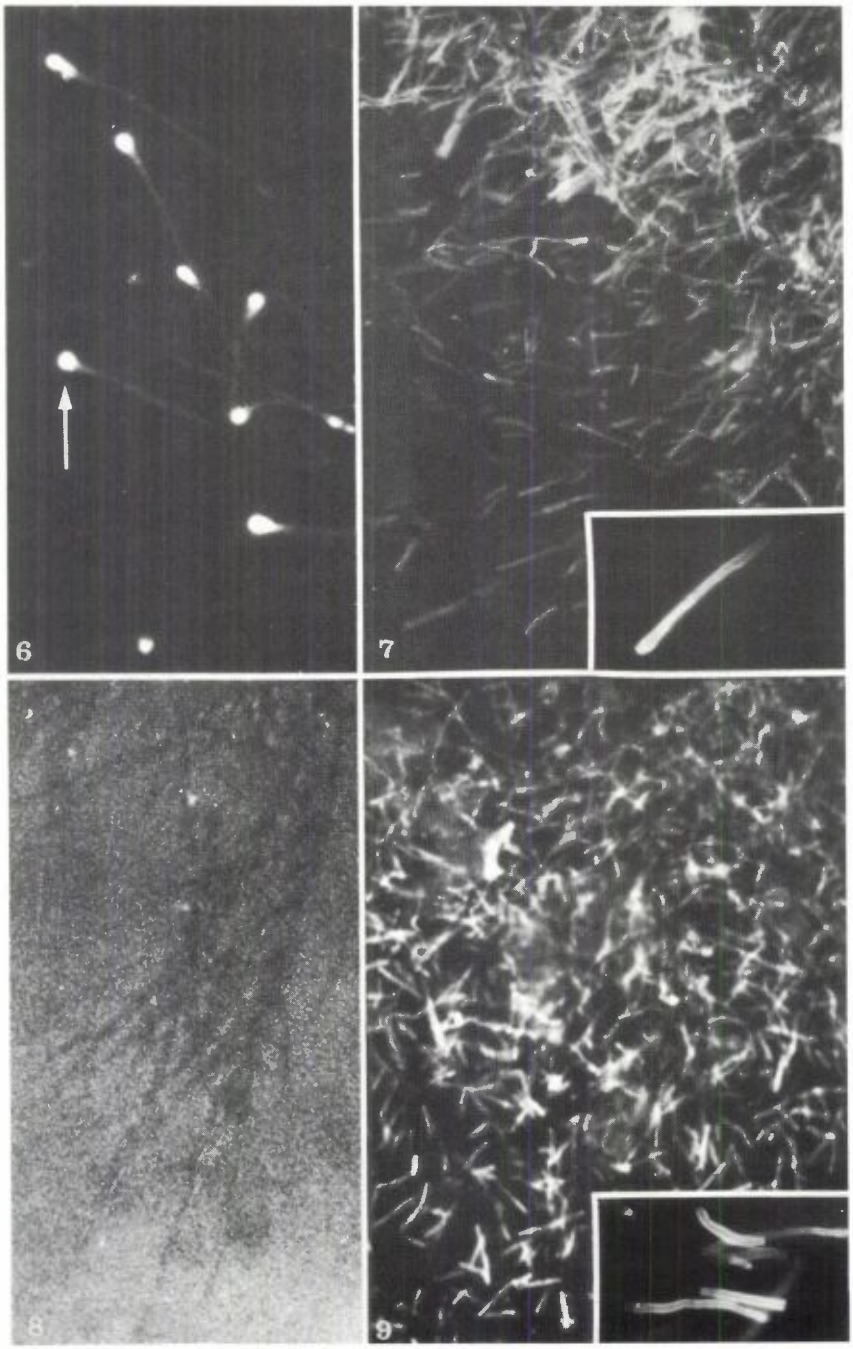
WGA — antilectin antibody — IgG/FITC

This indirect method gave results comparable to those obtained with the other techniques. Strong fluorescence was observed on the septa in many strains (*Rhododendron*, Duclos XV, IX, VIII, strain A) (Figs. 28 and 29), while in other strains fluorescence was diffuse (Duclos VI and *Pezizella ericae* Read). In the strain Duclos XXII, fluorescence was regularly spread on the longitudinal walls. However, during control experiments, new observations



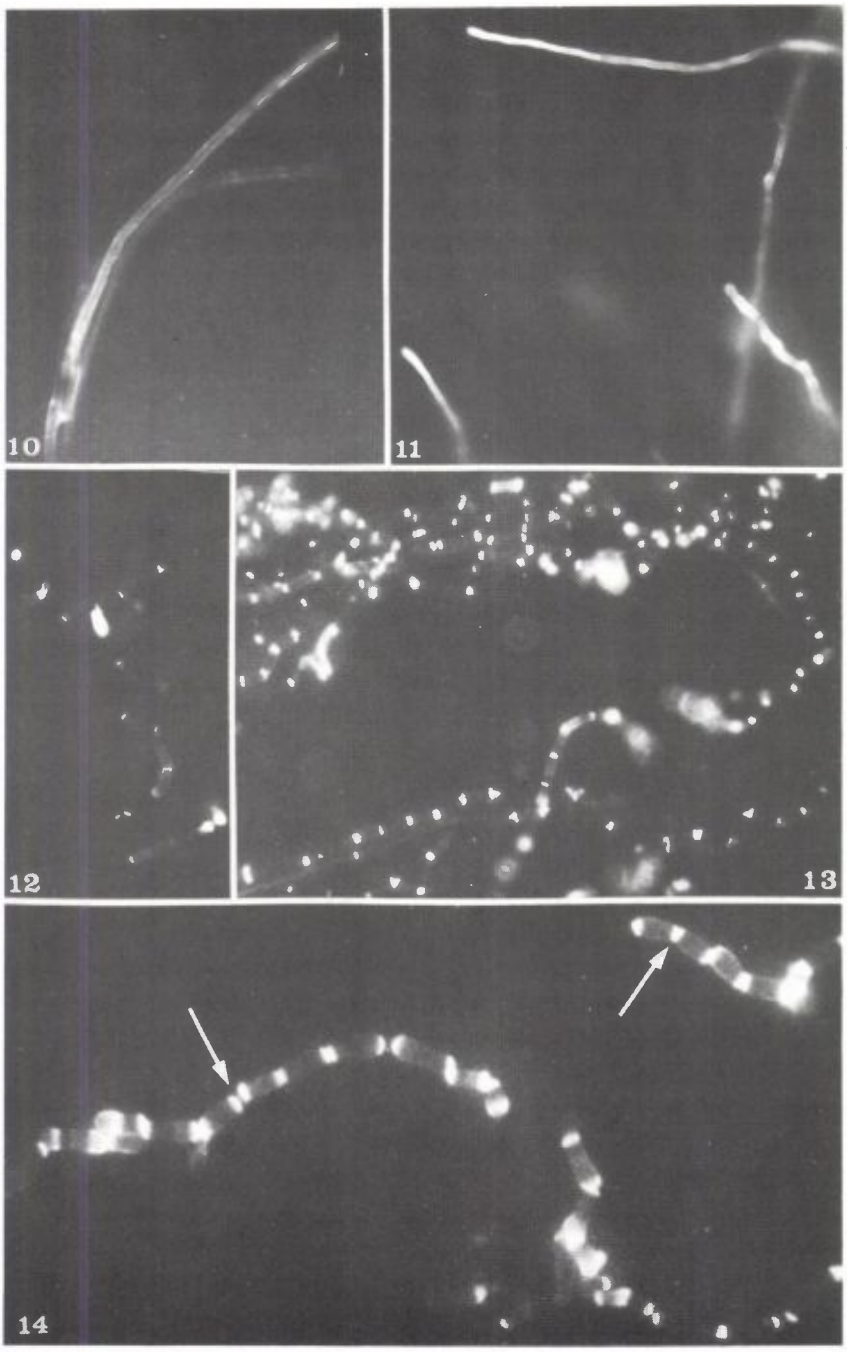
Figures 2–5. Direct labeling with Con A/FITC. ×675.

Fig. 2. Duclos VIII treated with Con A (50μg/ml) shows a diffuse fluorescence on all the longitudinal hyphal walls and around them (arrow), in the zone corresponding to the extracellular material. Fig. 3. Hypha of Duclos XXII showing a strong fluorescence on the longitudinal walls after Con A treatment (50μg/ml). Fig. 4. Short hyphal elements of *Pezizella ericae* from *Rhododendron* showing an intense fluorescence on the wall and the extracellular material (arrow). Fig. 5. *Pezizella ericae* treated with glucose and Con A/FITC. The sugar strongly decreases fluorescence.



Figures 6–9. Duclos XXII directly labeled with WGA/FITC (50μg/ml).

Fig. 6. Young fungal colonies showing strongly labeled apices (arrow), ×675. Fig. 7. At maturity fluorescence occurs on the longitudinal walls of many hyphae, ×235. In the inset a magnification of a hypha with fluorescent wall, ×675. Fig. 8. The reaction is inhibited by the addition of ovomucoid, a glycoprotein with a strong affinity for WGA, ×230. Fig. 9. After pronase treatment, the labeling occurs on many of the hyphae, exactly as in non-digested samples (see Fig. 7), ×230 and ×675.



Figures 10–14. Direct labeling with WGA/FITC (50μg/ml).

Fig. 10. Irregular labeling on the longitudinal walls of *Pezizella ericae* strain A, ×660. Fig. 11. Duclos VI shows strongly labeled apical zones, ×660. Figs. 12 and 13. Duclos IX and *P. ericae* from *Rhododendron* display a labeling localized almost exclusively on septa, ×675. Fig. 14. Higher magnification of the labeled septa of *P. ericae* from *Rhododendron*. A characteristic double structure is evident (arrows), ×1100.

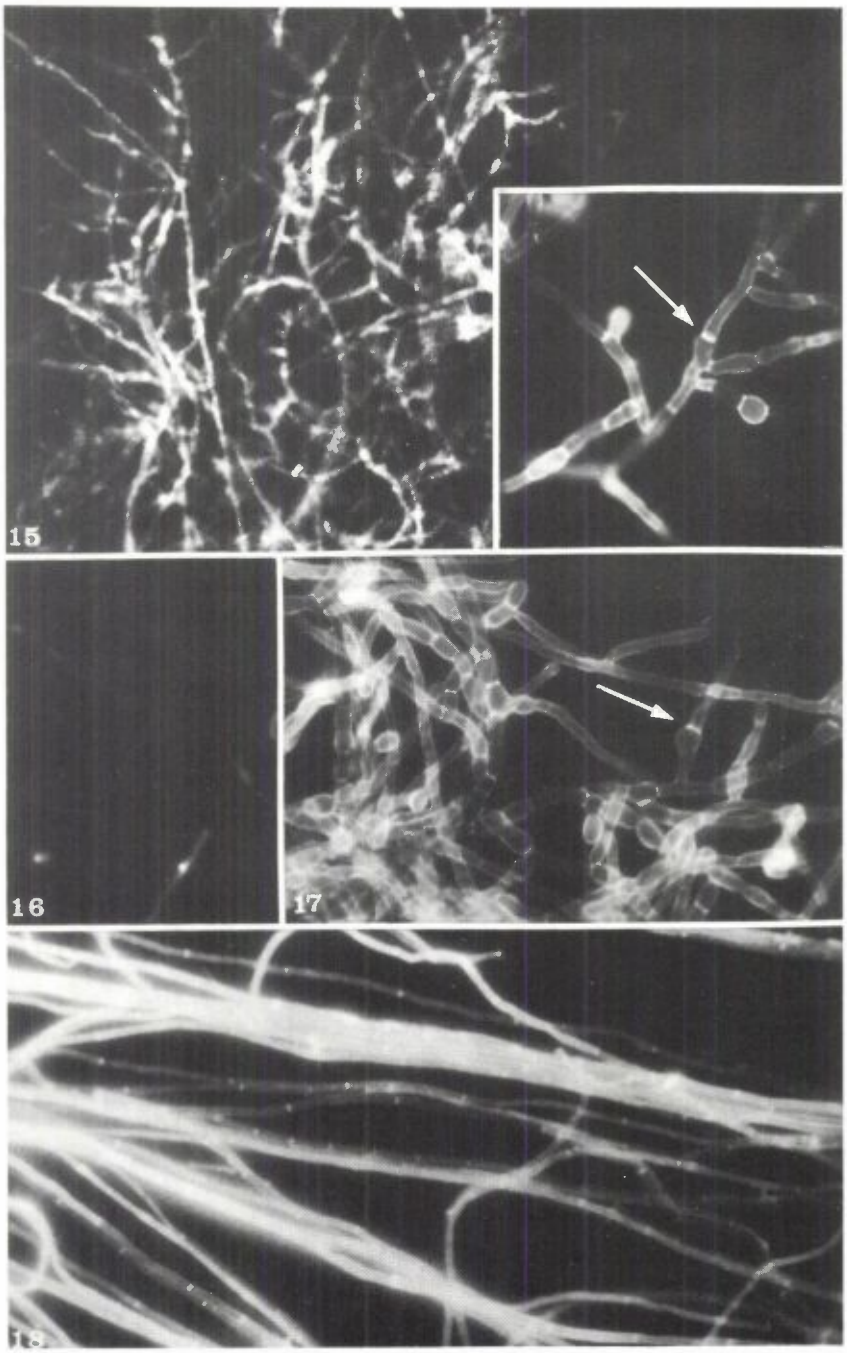
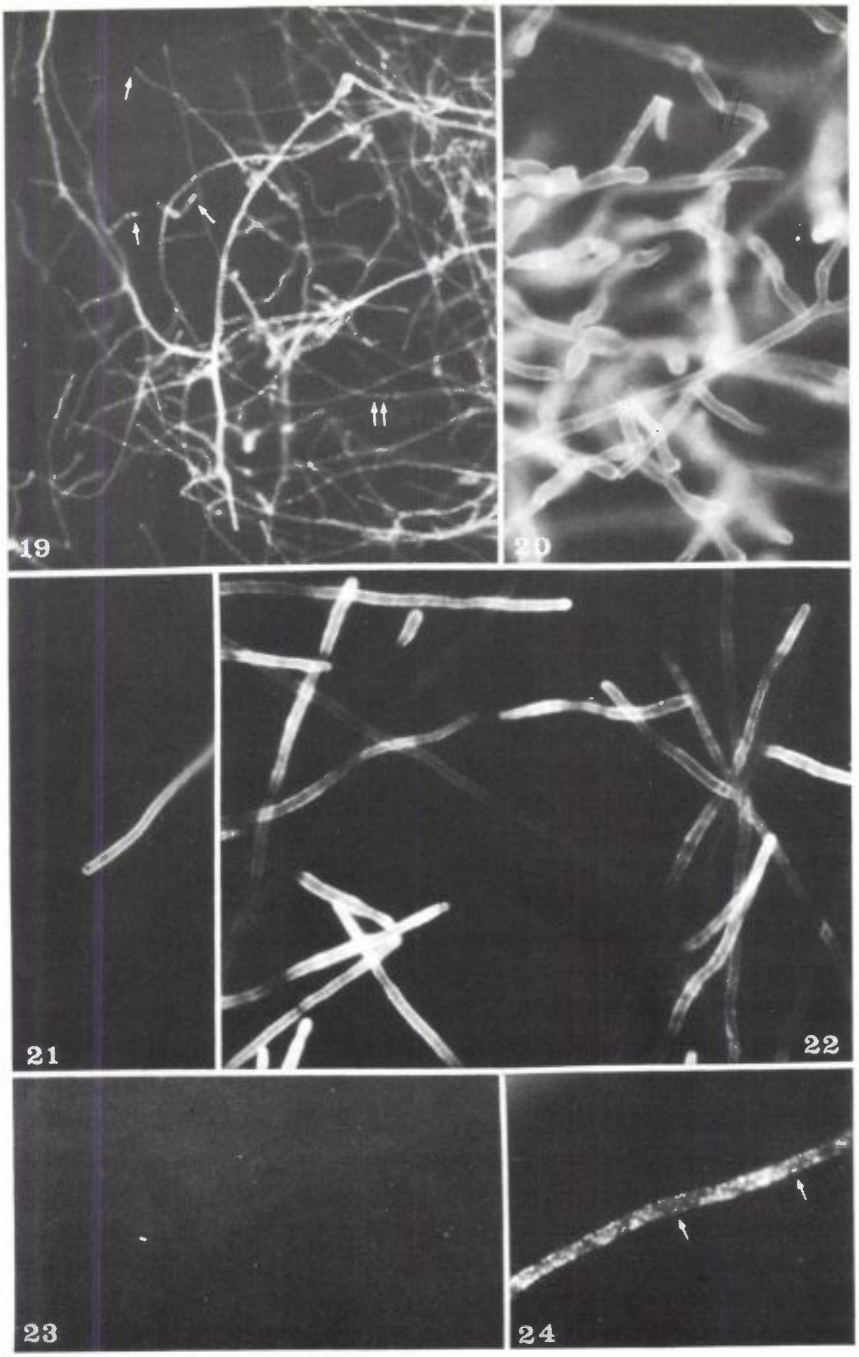


Figure 15. *P. ericae* from *Rhododendron*, where ovomucoid is added to WGA/FITC solution. There is a strong fluorescence on almost all the longitudinal walls, easily visible in the inset, at higher magnification. Septa are well labeled (arrow), ×230 and ×660. Fig. 16. The same fungus where N,N'- diacetylchitobiose is added to WGA/FITC solution. The reaction is much weaker, ×660. Fig. 17. The same fungus after pronase treatment and WGA/FITC labeling. An intense fluorescence is regularly present on the longitudinal walls, as well as on the septa (arrow), ×675. Fig. 18. Hyphae of *Pezizella ericae* treated with Calcofluor, septa are strongly fluorescent as well as the longitudinal walls, ×660.



Figures 19–24. Biotin/Con A-Avidin/FITC.

Fig. 19. After the treatment, *Pezizella ericae* from *Rhododendron* shows an intense fluorescence on the apical zones (arrows) and a patched-shape fluorescence on the longitudinal walls (double arrow), ×230. Fig. 20. Duclos IX displays a strong and homogeneous fluorescence on apical and longitudinal walls, ×660. Figs. 21 and 22. Strongly labeled hyphae of Duclos VIII and XXII. ×660. Fig. 23. The lack of Biotin/Con A, using just Avidin/FITC completely prevents the reaction in Duclos XXII, ×660. Fig. 24. Reaction caused by using just Avidin FITC on *Pezizella ericae* from *Rhododendron*. A patched shape fluorescence is visible on the longitudinal walls, ×660.

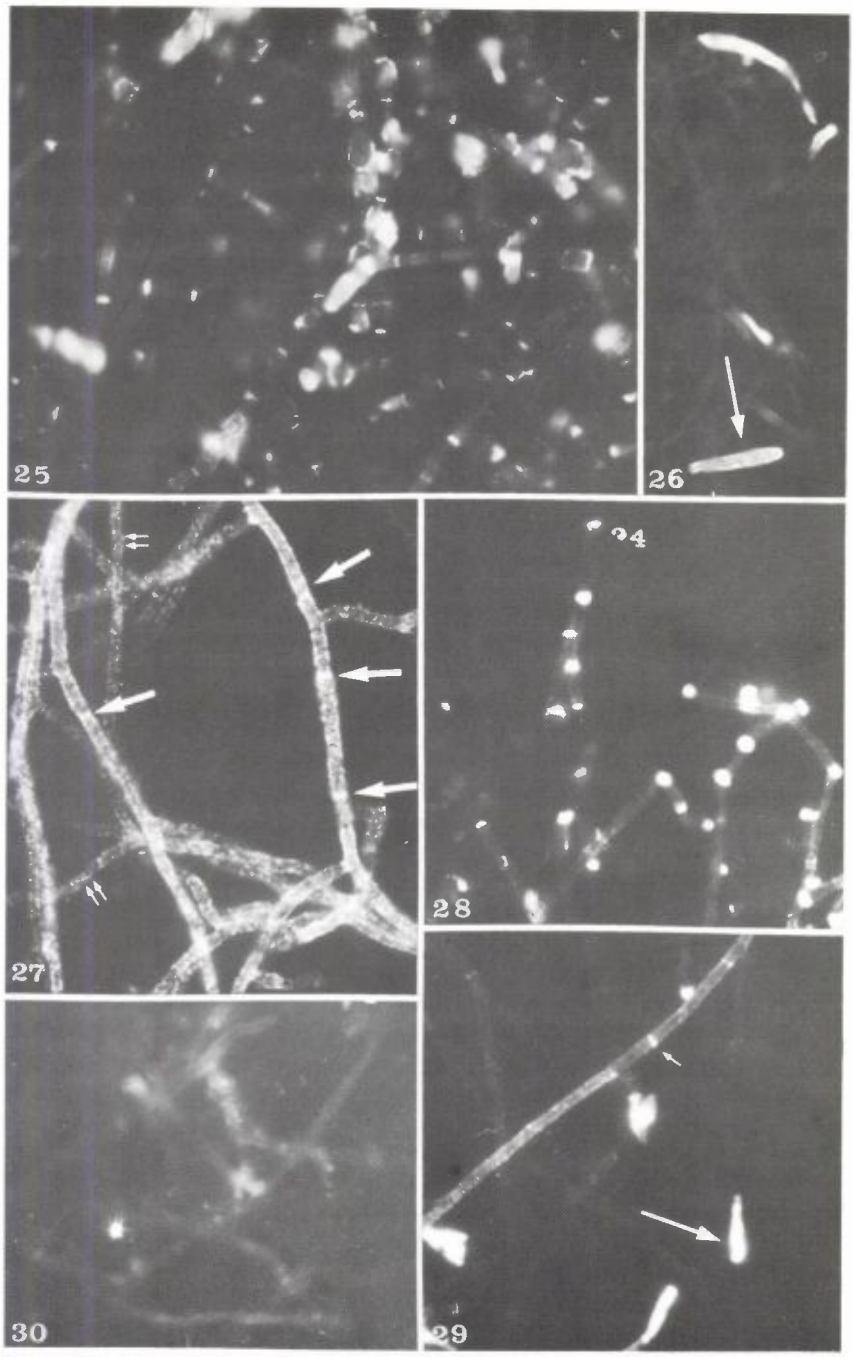
emerged: the reaction was 100% negative when WGA was omitted in strains XXII and VI, though a constant but weak fluorescence was present on the longitudinal walls of the other strains (Fig. 30). Reaction to IgG/FITC were from an irregular and weak fluorescence to a complete negative one, depending on the dilutions. Fluorescence was completely inhibited when N,N'-diacetylchitobiose and N,N',N"-triacetyl-chitotriose were added to WGA before the treatment.

4. Discussion

Lectins are non-enzymatic proteins and glycoproteins that bind to mono- and oligosaccharides in specific linkages (Sharon and Lis, 1981) with an antibody like affinity. They have been extensively used in cell surface studies as valuable tools to localize sugar residues in oligosaccharide units of glycoconjugates.

In the present study, the use of differently labeled lectins demonstrates that binding sites for Con A, WGA, PWM and LEA constantly occur on the surface of the symbiotic fungal isolates, but their distribution patterns greatly differ, suggesting a different glycoconjugate distribution, as it is summarized in Fig. 31.

Con A binding sites, specific for glucose and mannose sugar residues, are regularly spread on the fungal surfaces, and in some strains they can be localized as an extracellular material as already described at the ultrastructural level (Perotto and Bonfante-Fasolo, 1985). Binding sites for the other lectins, specific for GlcNAc residues, show three distinct localizations: (a) tips of young hyphae, (b) septa and, (c) as primary wall components along the hyphae. The specific binding of WGA to fungal tips is well known and has already been described in other symbiotic fungi by Galun et al. (1976), demonstrating the occurrence of chitin synthesis in the apical zone. In Ascomycetes to which *Pezizella ericae* belongs, the septum is considered a chitin-rich structure (Hunsley and Gooday, 1974; Molano et al., 1980; Galun et al., 1981). Ultrastructural observations (Bonfante-Fasolo and Gianinazzi Pearson, 1982) showed that the septa of *Pezizella ericae* are formed by an electron transparent layer, not reactive to the silver test for polysaccharides, characteristic for chitin. Moreover, our unpublished results performed by using a chitinase/colloidal gold complex clearly show a gold distribution on the septum zone as well as on the inner wall layer. Even if so far no cell wall biochemical analyses are available for the group of fungi here investigated, we suggest that chitin is an important component of the septa. We assume that the septum zone is a very dynamic one where longitudinal walls are discontinuous (Fig. 31b,c) and therefore the septa are accessible to labeled



Figures 25,26,27. Biotin/WGA-Avidin/FITC.

Fig. 25. Only septa are well labeled in the hyphae of Duclos IX. ×660. Fig. 26. Apical zones are well labeled in the young colonies of Duclos XXII. ×660. Fig. 27. *Pezizella ericae* from *Rhododendron* shows a double labeling: on septa (arrows) and on longitudinal walls as discontinuous patches (double arrows). ×660. Figures 28,29,30. The two step antibody method. Fig. 28. Septa of Duclos IX are wrongly labeled. ×660. Fig. 29. Septa and apical zones of Duclos VIII are well labeled. ×660. Fig. 30. A weak fluorescence persistes on the hyphae of Duclos VIII after omitting WGA. ×660.

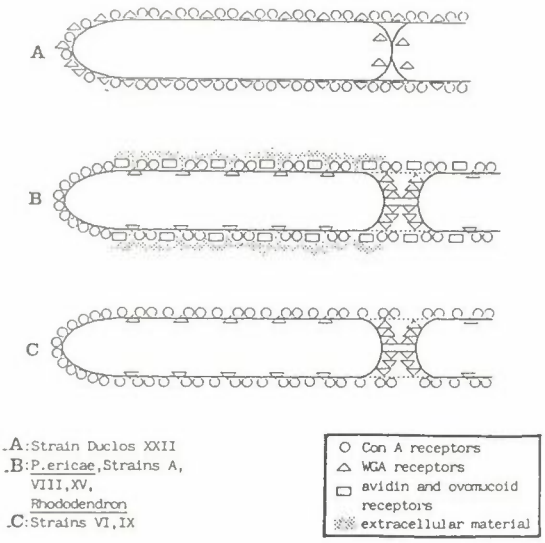


Figure 31. In the scheme the results are summarized, plotting three different types of glycoconjugate and sugar residue distribution on the surface of the fungal isolates.

WGA. Similar septa labeling was observed on the *Diplodia natalensis* hyphae (Galun et al., 1981) and in different filamentous fungi (Sengbusch et al., 1983). It is interesting that the X pattern shown by the septa when the hyphae break forming arthrospores, is comparable to that already described by Gooday and Gow (1983) in *Candida albicans* using different techniques.

The irregular visualization of WGA binding sites on the longitudinal walls of numerous strains or the regular presence of these sites on the longitudinal walls of the Duclos XXII strain suggest the presence of macromolecules rich in GlcNAc groups, that can be identified with chitin. In fact our previous ultrastructural observations demonstrate that in all strains, young hyphae are characterized by a completely electron transparent wall, corresponding to a chitin layer (Bonfante-Fasolo and Gianinazzi-Pearson, 1982; Perotto and Bonfante-Fasolo, 1985), but this pattern changes at maturity. The Duclos XXII strain develops a thick electron opaque wall, while the other strains develop an irregular sheath of extracellular material, well recognizable only after cationic dye binding techniques (Bonfante-Fasolo, in preparation). Results show that the strains lacking WGA binding sites on their

longitudinal wall (or with a reduced number of these sites) possess extracellular material, that probably prevents the lectin binding to the chitin located inside the wall as structural component (see Fig. 31).

Indirect methods gave interesting results not only as regards WGA and Con A binding site locations, since results were highly comparable to those obtained with the direct method. They show that some strains display a slight reaction with Avidin-FITC, suggesting the presence of endogenous receptors for this chemical. Avidin is a basic glycoprotein extracted from egg that contains glucosamine residues (Green, 1975). The strains reacting to Avidin are the same strains that enhanced their fluorescence after the ovomucoid-WGA treatment. Ovomucoid is a glycoprotein, rich in GlcNAc groups and with high affinity for WGA (Sharon, 1977): it is used in the cytochemical tests to bridge WGA to colloidal gold (Roth, 1983). In our experiments, ovomucoid inhibited WGA/FITC reaction in the Duclos XXII strain, but enhanced the fluorescence along all the longitudinal walls of the strains showing extracellular material. This unexpected pattern can be explained by the fact that in the complex ovomucoid — WGA/FITC, ovomucoid probably displays free GlcNAc groups. Thanks to these groups the complex binds to molecules located on/inside the extracellular substance and acting as ovomucoid binding sites. So one might suggest that such molecules, probably proteinaceous in nature (endogenous lectins?), able to react with GlcNAc groups, occur at the surface of the strains *Rhododendron*, VIII, XV and, to a lesser extent, on the surface of *Pezizella ericae* and strain A. This hypothesis is confirmed by the fact that a long pronase digestion is able to enhance the direct / or indirect WGA binding.

The characterization of surface macromolecules that react differently to lectins and to glycoproteins such as Avidin and ovomucoid also suggests a functional hypothesis. The strains studied in the present work show in fact different symbiotic capabilities: some of them are highly infective: *Pezizella ericae* from *Rhododendron*, strain A, Duclos VIII; Duclos VI has low infectivity; and Duclos XXII seems to have lost its infectivity (Gianinazzi-Pearson and Bonfante-Fasolo, 1985). These different symbiotic expressions closely correspond with the different production of extracellular material in pure culture and with the different patterns shown by the WGA receptors. In fact the strains lacking WGA surface receptors and showing extracellular material are the most infective and vice versa, the weak or not infective strains, lack the extracellular substance and bind WGA to their surface.

In conclusion, by using different lectins on some ericoid fungi, we were able to demonstrate a different pattern of surface sugar residues in infective and non-infective strains. In addition to GlcNAc residues of the septa and

the primary cell walls in both types, some glycoconjugates characterize the infective strains. These glycoconjugates cover the structural polysaccharides of the cell wall, are pronase-susceptible and possess glucose and mannose residues. Some of these molecules may be able to link GlcNAc residues. Therefore, we suggest that they may play an important role in the adhesion process between fungus and plant.

Acknowledgement

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