

A newly isolated lectin from the plant pathogenic fungus *Sclerotium rolfsii*: purification, characterization and role in mycoparasitism

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A novel lectin was isolated and purified from the culture filtrate of the soilborne plant pathogenic fungus *Sclerotium rolfsii* by anion-exchange chromatography using a DEAE-Sephrose column. The lectin came through the column with the flow-through, whereas all the non-agglutinating proteins present in the crude preparation remained bound to the column until elution in a NaCl gradient. SDS-PAGE analysis of the agglutinating fraction revealed a single band corresponding to a protein with a molecular mass of approximately 45 kDa. Agglutination of *Escherichia coli* cells by the purified lectin was not inhibited by any of the mono- or disaccharides tested, whereas the glycoproteins mucin and asialomucin did inhibit agglutination. Proteases, as well as 1,3- β -glucanase, were found to be totally destructive to agglutination activity, indicating that both protein and 1,3- β -glucan are necessary for agglutination. Using a biomimetic system based on binding of the lectin to the surface of inert nylon fibres revealed that the presence of the purified agglutinin on the surface of the fibres specifically induced mycoparasitic behaviour in *Trichoderma harzianum*. *Trichoderma* formed tightly adhering coils, which were significantly more frequent with the purified agglutinin-treated fibres than with untreated ones or with those treated with non-agglutinating extracellular proteins from *S. rolfsii*. Other mycoparasite-related structures, such as appressorium-like bodies and hyphal loops, were only observed in the interaction between *T. harzianum* and the purified agglutinin-treated fibres.

Keywords: *Sclerotium rolfsii*, lectin, *Trichoderma harzianum*, mycoparasitism

INTRODUCTION

Mycoparasitism appears to be a complex process made up from several successive steps (Chet, 1987, 1990; Tunlid *et al.*, 1992). It may be initiated by a remote sense of the host which stimulates directed growth of the parasite towards that host (Chet *et al.*, 1981). Subsequently, contact is made between the fungal parasite and the host surface. This step provides the specific recognition event, which may be physical, as in thigmotropism, or chemical (chemotropism). The latter involves hydrophobic interactions or interactions between complementary molecules present on both the host and the parasite (e.g. lectin–carbohydrate

interactions). This crucial event leads to a differentiation process which results in the formation of infection structures such as appressoria, hyphal coils and hook-like bodies. Eventually, extracellular hydrolytic enzymes are secreted and penetration of the host takes place (Chet, 1987, 1990; Manocha & Chen, 1990; Tunlid *et al.*, 1992).

The destructive parasitic mode in *Trichoderma*, a natural antagonist to other fungi and well-known biocontrol agent of plant pathogenic fungi, appears to consist of all the events described above (Chet, 1990; Sivan & Chet, 1992). Upon contact with the host, *Trichoderma* coils around or grows along the host hyphae and forms hook-like structures, presumably appressoria, that aid in penetration of the host's hyphal cell wall (Chet *et al.*, 1981; Elad *et al.*, 1983b). In *Trichoderma*, this reaction has been

Abbreviation: SEM, scanning electron microscope.

found to be rather specific, as it attacks only a few fungi. Moreover, Dennis & Webster (1971), using plastic threads of a diameter similar to that of *Pythium ultimum* hyphae, concluded that the coiling of *Trichoderma* is not merely a thigmotropic response. The *Trichoderma* hyphae did not coil around the threads but rather grew over or followed them in a straight course. This led to the idea that there is a molecular basis for this specificity. Indeed, lectins have been found to be produced by some soilborne plant pathogenic fungi, such as *Rhizoctonia solani* and *Sclerotium rolfii* (Barak *et al.*, 1985; Barak & Chet, 1990; Elad *et al.*, 1983a) and by different members of the *Sclerotiniaceae* (Kellens *et al.*, 1992). The ability of different isolates of mycoparasitic *Trichoderma* to attack *S. rolfii* has been correlated with the agglutination of *Trichoderma* conidia by *S. rolfii* agglutinin (Barak *et al.*, 1985).

Based on the above observations, a role for lectins in the recognition and specificity of attachment between *Trichoderma* and its host fungi has been suggested (Barak & Chet, 1990; Nordbring-Hertz & Chet, 1986). In an attempt to test this hypothesis, we recently developed a biomimetic system based on the binding of lectins to the surface of nylon fibres. This system simulates the host hyphae and enabled us to examine the role of lectins in mycoparasitism (Inbar & Chet, 1992). The first direct evidence for this role was provided by the mycoparasitic biocontrol fungus *Trichoderma harzianum*, which, when allowed to grow on nylon fibres treated with concanavalin A or crude *S. rolfii* agglutinin, coiled around the nylon fibres and produced hooks in a pattern similar to that observed with the real host hyphae. The incidence of interaction was significantly higher with agglutinin-treated fibres than with controls (Inbar & Chet, 1992).

In this study we describe the isolation and characterization of a novel lectin from *S. rolfii*. The ability of this purified lectin to induce mycoparasitic behaviour and formation of infection-related structures in *T. harzianum* was examined.

METHODS

Culture of organisms. *Sclerotium rolfii* Sacc. type A, ATCC 26325, was maintained on synthetic medium (SM) (Okon *et al.*, 1973). For lectin production and purification, *S. rolfii* was grown in liquid SM supplemented with 5×10^{-6} M polyoxin D (Barak & Chet, 1990). After 5 d, the culture filtrate (50 ml) was collected and dialysed for 24 h against 20 mM Tris/HCl buffer (pH 7.0; 2×5 l) (crude agglutinin). *Trichoderma harzianum* Rifai, T-Y, was grown on potato dextrose agar (PDA; Difco). *Escherichia coli* was grown in LB medium at 37 °C for 24 h in a rotary shaker at 250 r.p.m.

Ion-exchange chromatography. The crude agglutinin was applied to a DEAE-Sepharose fast-flow anion-exchange column (Pharmacia; 1.5×2.5 cm) which had been equilibrated with 20 mM Tris/HCl buffer (pH 7.0). After washing the column with 6 ml of the same buffer, bound proteins were eluted using a stepwise NaCl gradient to 0.2 M (flow rate 0.5 ml min^{-1}). Eluted proteins were collected, pooled and dialysed for 24 h against distilled water (5×5 l).

Agglutination assay. The titre of *S. rolfii* lectin was determined by agglutination assay with *E. coli* cells as previously described (Barak *et al.*, 1985). Each experiment was repeated at least three

times. The titre was defined as the reciprocal of the highest lectin dilution that agglutinated the cells.

Gel electrophoresis. SDS-PAGE was performed in 10% (w/v) polyacrylamide vertical slab gels using the Mini-Protein II system (Bio-Rad) according to the manufacturer's instructions. Electrophoresis was carried out under reducing conditions as described by Laemmli (1970). Following electrophoresis, the gels were stained for protein with Coomassie brilliant blue (Sigma). The electrophoretic procedure was repeated at least four times.

Agglutination inhibition assay. Various sugars or glycoproteins were serially diluted twofold with PBS (NaCl, 8 g l^{-1} ; KH_2PO_4 , 0.3 g l^{-1} ; K_2HPO_4 , 1.3 g l^{-1} ; pH 7.4). To 20 μl of each dilution, an equal volume of the anion exchange purified lectin solution was added and incubated for 1 h at room temperature. Purified lectin solution diluted with PBS alone (1:1), and sugar or glycoprotein solutions without lectin, were tested as well. After a 1 h preincubation, agglutination of *E. coli* cells was examined as described above. The minimal sugar or glycoprotein concentration needed for 90% inhibition was determined by microscopic examination of the number of aggregates formed, in 10 different microscopic fields, in the agglutinin and sugar or glycoprotein solutions as compared to agglutinin plus PBS. All experiments included three replicates and were repeated at least three times.

Enzymic treatments. Crude and purified lectins were treated with various enzymes by incubating 250 μl of enzyme in the appropriate buffer with 250 μl of agglutinin at the appropriate conditions and assayed for agglutination activity. 1,3- β -Glucanase ($4.5 \text{ units ml}^{-1}$) from *Penicillium* spp. (Makor Chemicals), and chitinase (Sigma; 64 units ml^{-1}), both in 0.1 M phosphate buffer (pH 5.5), were incubated for 3 h at 37 °C. Protease from *Streptomyces griseus* (Sigma; 4 units ml^{-1}), trypsin and chymotrypsin (Sigma; each at a concentration of 1%, w/v) were mixed in 0.1 M Tris/HCl buffer (pH 7.6) and incubated for 1 h at 37 °C. Lysing enzymes from *T. harzianum* (Sigma), containing cellulase, protease and chitinase activities, and β -glucuronidase (Sigma), both in 0.1 M Tris/HCl buffer (pH 7.0), were incubated for 3 h at 37 °C. All reactions were stopped by moving the test-tubes to an ice-bath and adding the protease inhibitor PMSF to the tubes containing proteolytic activity. Control samples were treated with the corresponding buffer only. In all of these experiments, inhibition of lectin activity was determined by agglutination assay with *E. coli* cells as described above. The experiments were repeated at least three times.

Binding of proteins to nylon fibres. Purified lectin was concentrated by ultrafiltration (C-0.5 membrane, cut-off 500 Da; Amicon). The filtrate ($< 500 \text{ Da}$) contained no agglutination activity. Non-agglutinating fractions eluted from the anion-exchange column were pooled, dialysed against distilled water and lyophilized. Proteins were redissolved in 0.1 M sodium borate (pH 8.5) as a coupling buffer. Concentrated lectin or non-agglutinating fractions at a concentration of $40 \mu\text{g protein ml}^{-1}$ were coupled to glutaraldehyde-activated nylon 66 fibres (approx. $13 \mu\text{m}$ in diameter; kindly supplied by Nilit, Migdal Haemek, Israel) according to Inbar & Chet (1992).

Biomimetic system and electron microscopy. The biomimetic system to examine the induction of infection-related structures by the various nylon fibres was designed as previously described (Inbar & Chet, 1992). After 24 h in the dark (to prevent conidiation), fibres were removed aseptically and vapour-fixed (Barak *et al.*, 1986). The samples were then dried for 24 h, coated with gold in a Polaron E-5100 sputter coater (Polaron Equipment) and observed under a scanning electron microscope (SEM) (JEOL JSM 35C).

Table 1. Purification of agglutinin from *S. rolfssii*

Values are means of four purifications with SD $\leq 1\%$.

Fraction	Volume (ml)	Total agglutination activity (titre ml ⁻¹)	Protein (mg)	Specific activity (titre mg ⁻¹ ml ⁻¹)*	Recovery (%)	Purification (-fold)
Crude filtrate	50	200	0.3	666.7	100	1
Purified agglutinin	50	192	0.048	4000	96	6

* Reciprocal of the highest lectin dilution that agglutinated *E. coli* cells.

Evaluation of the incidence of interaction between *T. barzianum* and the various nylon fibres was carried out as previously described by Inbar & Chet (1992).

RESULTS AND DISCUSSION

Lectin purification and characterization

Agglutination activity could be detected in the DEAE-Sephrose column flow-through, indicating that, under these conditions, the lectin did not bind to the column. The flow-through fraction contained 0.95 µg protein ml⁻¹ (16% of the total). Its specific activity (titre mg⁻¹ ml⁻¹) increased from 666.7 to 4000 and purification was sixfold with 96% recovery (Table 1). No agglutination activity could be detected in the NaCl-eluted fractions. SDS-PAGE analysis of the purified lectin (Fig. 1, lane 2) revealed a single, well-defined band with an estimated molecular mass of 45 kDa. The same results were obtained

Table 2. Sugar specificity of *S. rolfssii* agglutinin

Inhibition was estimated by microscopic examination of the number of aggregates formed in the agglutinin and sugar or glycoprotein solution as compared to agglutinin plus PBS. These concentrations were obtained in at least three independent experiments. Agglutination activity was not inhibited by any of the mono- or disaccharides tested (D-glucose, D-mannose, methyl- α -D-glucoside, methyl- α -D-mannoside, maltose, sucrose, lactose, fructose, L-arabinose, D- or L-fucose, D- or L-galactose, L-fucosylamine, D-glucosamine, N-acetyl D-glucosamine and rhamnose), up to concentrations of 200 mM.

	Concn (µg ml ⁻¹) needed for 90% inhibition
Glycoproteins	
Mucin	0.48
Asialomucin	0.96
Fetuin	125
Asialofetuin	62.5

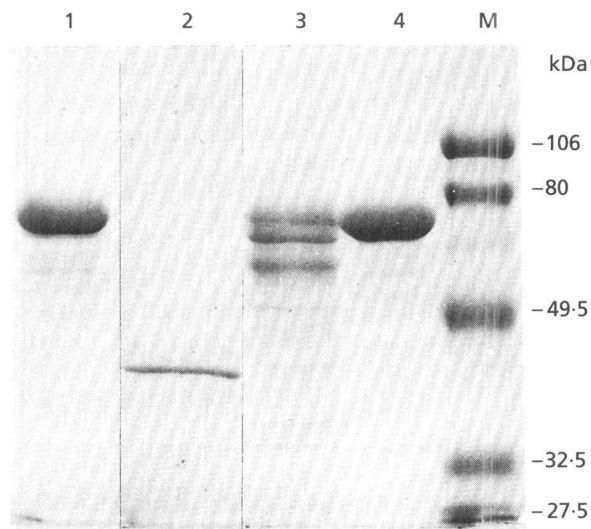


Fig. 1. SDS-PAGE of *S. rolfssii* proteins. Lanes: 1, crude cultured filtrate; 2, purified agglutinin appearing with the flow-through from a DEAE-Sephrose column; 3 and 4, non-agglutinating extracellular proteins from *S. rolfssii* eluted from the anion-exchange column by NaCl at concentrations of 100 (lane 3) and 200 (lane 4) mM in 20 mM Tris/HCl buffer (pH 7.0). M, molecular mass markers.

when the gel was silver-stained (data not shown). Non-agglutinating fractions (Fig. 1, lanes 3 and 4) contained all of the major proteins present in the crude preparation (Fig. 1, lane 1). The purified lectin agglutinated *E. coli* cells. Agglutination activity was not inhibited by any of the mono- or disaccharides tested, up to concentrations of 200 mM (Table 2). However, the glycoproteins mucin (from porcine stomach) and asialomucin (from bovine submaxillary gland) inhibited *E. coli* cell agglutination at concentrations as low as 0.48 and 0.96 µg ml⁻¹, respectively. Fetuin and asialofetuin (both from foetal calf serum) were much less effective at inhibiting agglutination (needing 125 and 62.5 µg ml⁻¹, respectively).

This lectin, therefore, differs from that previously reported from *S. rolfssii*, which contained two major bands corresponding to proteins with molecular masses of 55 and 60 kDa (Barak & Chet, 1990). Although the latter also agglutinated *E. coli* cells, it exhibited specificity towards D-glucose, D-mannose and several of their derivatives (Barak *et al.*, 1985). In the present study, purification was performed without first lyophilizing and

Table 3. Effect of various enzymes on *S. rolf sii* agglutinin activity

Inhibition was estimated by microscopic examination of the number of aggregates formed in the agglutinin after incubation with either boiled or native enzyme as compared to agglutinin plus the corresponding buffer only. These results were obtained in at least three independent experiments.

Enzyme	Inhibition (%)	
	Crude filtrate	Purified agglutinin
Trypsin (1%)	100	100
Chymotrypsin (1%)	100	100
Protease from <i>S. griseus</i> (4 units ml ⁻¹)	70	70
1,3- β -Glucanase from <i>Penicillium</i> sp. (4.5 units ml ⁻¹)	100	100
Chitinase (64 units ml ⁻¹)	0	0
β -Glucuronidase	0	0
Lysing enzymes from <i>T. harzianum</i> (containing chitinase, cellulase and protease activity)	100	100

concentrating the culture filtrate. After dialysis against the appropriate buffer, the filtrate was purified by anion-exchange chromatography on a DEAE-Sepharose column. Barak & Chet (1990) used a different approach, i.e. they lyophilized the culture filtrate and separated it by gel-filtration on Sephadex-G75. Hence, it appears that the use of different purification procedures gives different products (i.e. agglutinins). Lectins with different molecular masses and carbohydrate specificities are produced by the same fungus in some other cases as well (Kellens *et al.*, 1992). For example, lectin activity in *Rhizoctonia solani*, another major plant pathogenic fungus, was demonstrated by the attachment of O but not A or B erythrocytes to the fungal hyphae. This attachment was prevented by galactose and fucose (Elad *et al.*, 1983a). However, Vranken *et al.* (1987) isolated another lectin (RSA) from the mycelium of *R. solani* by affinity chromatography on gum-arabic-Sepharose which exhibited specificity towards *N*-acetylgalactosamine and preferentially agglutinated human type A over type B and O erythrocytes. Kellens *et al.* (1989) isolated two lectins from mycelia of *Rhizoctonia crocorum* and *Athelia rolf sii* by affinity chromatography on mucin-Sepharose. In contrast to RSA, the *R. crocorum* lectin and the *A. rolf sii* lectin had complex specificities. They were both specifically inhibited by fetuin and asialofetuin, whereas none of the mono- or disaccharides tested inhibited their activities (Kellens *et al.*, 1989).

The effects of various enzymes on the agglutination activity of the purified lectin were examined. Controls for each enzyme experiment were: (a) lectin with boiled enzyme and (b) lectin with equal volumes of the corresponding buffer, without enzyme. All proteases inhibited agglutination activity (Table 3). Trypsin and

chymotrypsin effected total inhibition, whereas protease from *S. griseus* was less effective (70% inhibition). Treating the lectin for 3 h with 1,3- β -glucanase from *Penicillium* spp. also totally inhibited activity. This enzyme was protease-free, as determined by ninhydrin and hide powder azure reactions (Barak & Chet, 1990) and by fluorescein isothiocyanate-casein hydrolysis (Dr Th. Akermann, Makor Chemicals, Israel, personal communication). Incubation of the lectin with either chitinase or β -glucuronidase had no effect on agglutination activity. However, the lysing enzyme from *T. harzianum*, containing chitinase, cellulase and protease activities, totally inhibited agglutination activity, probably due to the protease activity. No inhibition was observed in either of the controls, indicating that the inhibition effect was due to enzyme activity. The same inhibition effects were observed when crude agglutinin was treated with the same enzymes (Table 3). The inhibition of *E. coli* agglutination in these experiments resulted from lectin inactivation, rather than from an effect of the enzymes on *E. coli* cells: agglutination of the bacteria appeared within seconds after mixing the cells with the lectin. Moreover, the reaction tubes were transferred to an ice-bath and protease inhibitor (PMSF) was added before agglutination tests were performed. Therefore it is unreasonable to assume that under such conditions and in such a short time the enzymes could degrade components of the bacterial cell wall.

It is apparent from these results that both protein and 1,3- β -glucan are necessary for agglutination activity of the lectin studied here. *S. rolf sii* is known to produce extracellular polysaccharides when grown in liquid media. Kritzman *et al.* (1979) isolated and analysed these polysaccharides, five sediments of which were extracted from *S. rolf sii*. The first and major sediment was found to contain 1,3- β -glucan (laminarin) which was similar to commercial laminarin, as evidenced by their respective IR spectra. Moreover, 1,3- β -glucanase extracted from *S. rolf sii* degraded this sediment, but none of the others. The sediment was also found to contain proteins. Acid hydrolysis and amino acid analysis of this glucan revealed 12 different amino acids, proline being the major one (approx. 33%; Kritzman *et al.*, 1979). The association between 1,3- β -glucan and protein may explain the fact that the agglutinin flowed through the anion-exchange column without interacting with it, while other extracellular proteins not associated with the polymer remained bound to the column.

Induction of infection-related structures in a biomimetic system

The idea that adhesion of fungal parasites to their host's surface is mediated by complexed extracellular polymers has been advanced in several electron microscopic studies (Hoch & Staples, 1987; Hamer *et al.*, 1988; Tunlid *et al.*, 1991). Tunlid *et al.* (1991) studied the adhesion mechanism in the nematophagous fungus *Arthrobotrys oligospora*. Contrary to previous suggestions (Nordbring-Hertz & Mattiasson, 1979), this mechanism was found to be a

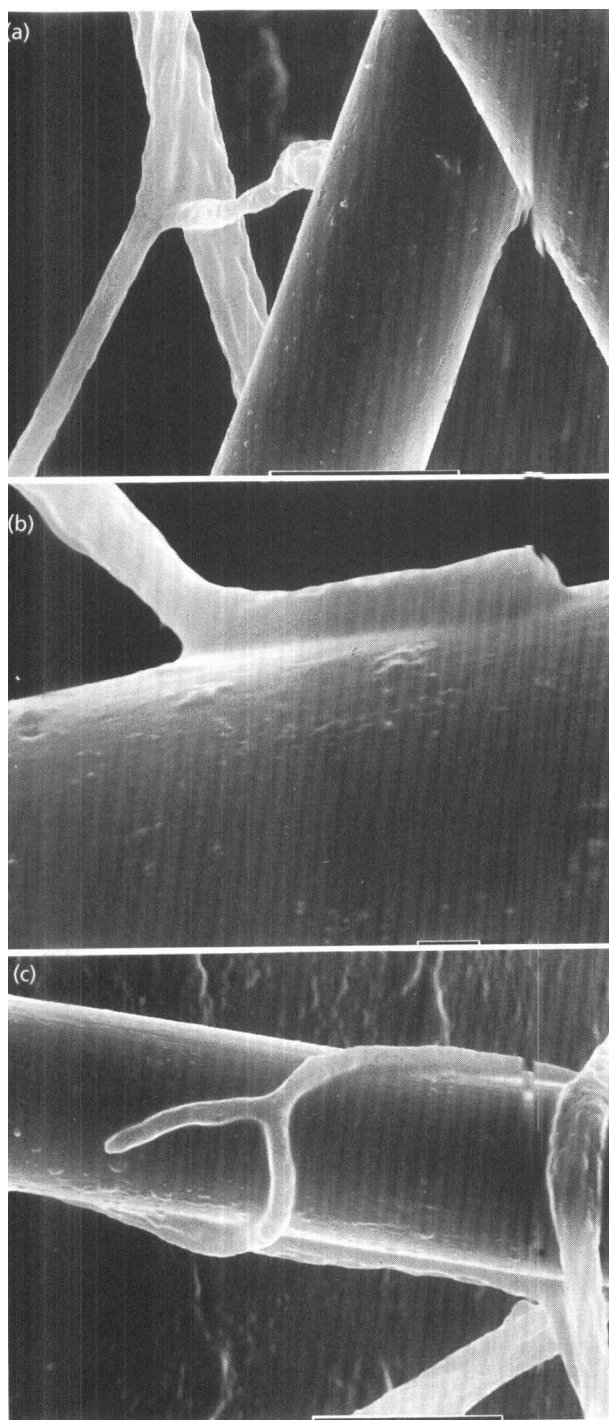


Fig. 2. Scanning electron micrographs demonstrating the different stages in the interaction between *T. harzianum* and the purified lectin-treated nylon fibres. (a) Atypical branching of the *Trichoderma* towards the fibres and contact of the branch tip with the fibre surface (bar, 10 µm); (b) subsequent elongation of the firmly attached tip along the fibre surface (bar, 1 µm); (c) *Trichoderma* hyphae coiled around the fibre, producing additional branches. The hyphal coils and branches are tightly adhered to the fibre surface (bar, 10 µm).

complex process. By examining the capture of nematodes by *A. oligospora* traps in which the layer of surface polymers had been modified, removed or enzymically treated, Tunlid *et al.* (1991) showed that both protein and carbohydrate-containing surface polymers are involved in the adhesion process.

Electron micrographs have revealed that the hyphal cell walls of *S. rolfii* are surrounded by a thick layer of polysaccharides (Kritzman *et al.*, 1979; Barak *et al.*, 1985). A similar mechanism to that suggested for *A. oligospora* may therefore operate, in that the complexed extracellular layer surrounding the cell walls of *S. rolfii* mediates the recognition and attachment of its mycoparasite, *Trichoderma*.

The presence of the purified agglutinin on the surface of inert nylon fibres specifically induced mycoparasitic behaviour in *T. harzianum*. SEM analysis of the interaction revealed several of the steps previously reported for *S. rolfii*-*Trichoderma* interactions (Elad *et al.*, 1983b). Initially the *Trichoderma* branched in an atypical way, approached the fibres and first contact took place (Fig. 2a). Subsequently, the attached hyphae adhered to the lectin-coated fibre surface and grew in very tight association with it (Fig. 2b). Eventually, coils and branches were formed (Figs 2c and 3b). *Trichoderma* spp. have been reported to sometimes produce appressorium-like structures (Chet, 1987, 1990). In the biomimetic system, formation of appressorium-like bodies could be observed at the tips of short hyphal branches (Fig. 3a) in the presence of purified lectin-treated fibres. These bodies adhered tightly to the surface of the fibres, possibly due to adhesive material produced by *Trichoderma*. The formation of hyphal loops, another unique mycoparasite-related structure, was also observed (Fig. 3b, arrows). These phenomena were not observed with the non-agglutinating protein-treated or untreated fibres.

The incidence and frequency of the interaction between *T. harzianum* and the variously treated nylon fibres were expressed as the number of coils produced by *Trichoderma* per mm of nylon fibre in 20 different SEM fields (200 × 150 µm). The rate of interaction between *Trichoderma* and the purified lectin-treated fibres was sixfold and threefold higher than with the untreated fibres or fibres treated with non-agglutinating proteins from *S. rolfii*, respectively (number of coils per mm of nylon fibre 6.15 ± 0.49 , 2.20 ± 0.47 and 0.95 ± 0.14 , respectively). These findings indicate a specificity of interaction and attachment.

We therefore suggest that the recognition and attachment of the mycoparasitic fungus *Trichoderma* to the *S. rolfii* cell wall surface is indeed mediated by a complexed agglutinating polymer which surrounds the host hyphae, thereby initiating a sequence of events which eventually lead to the destruction of the host.

Models proposed to explain the interaction mechanism in fungal parasitism have suggested several putative events following recognition and attachment (Chet, 1987, 1990; Tunlid *et al.*, 1992). Tunlid *et al.* (1992) suggested that as

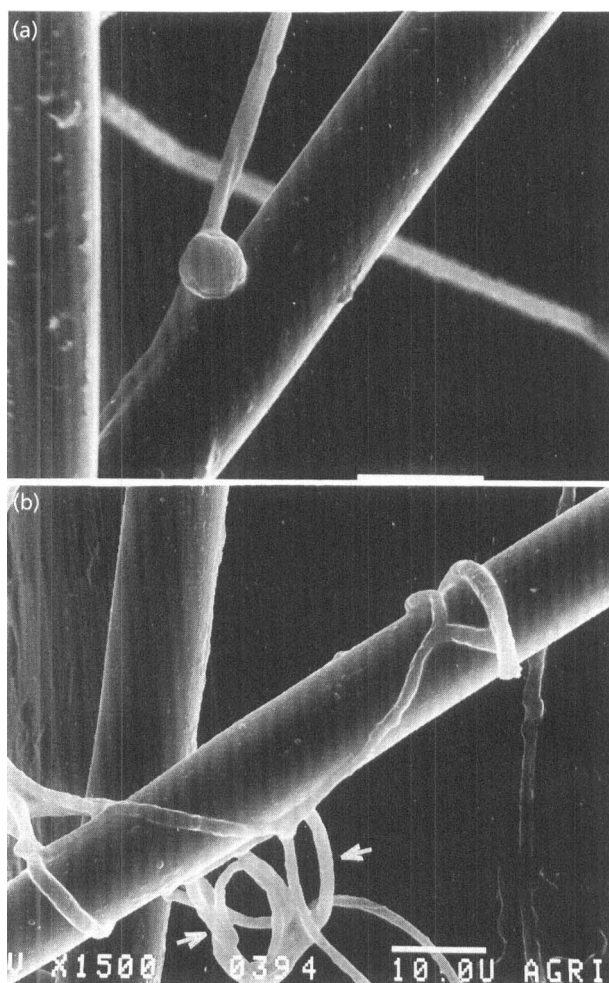


Fig. 3. Formation of mycoparasite-related structures by *Trichoderma* when interacting with the purified lectin-treated fibres. (a) Appressorium-like body at the tip of a short branch; (b) hyphal loops (arrows). Bars, 10 μ m.

a consequence of these first steps, differentiation processes take place leading to the formation of various infection structures and are accompanied by nuclear division, cytoskeletal rearrangement and synthesis of differentiation-related proteins. Simultaneously, adhesins are secreted to consolidate the attachment, together with extracellular enzymes, resulting in penetration of the host. Our results partially confirm this model: when the purified surface agglutinin from the host fungus *S. rolfii* was bound to nylon fibres, differentiation processes in *T. harzianum* leading to formation of infection structures were observed. The production of adhesive material(s) aiding in establishing this interaction was also observed. Neither synthesis of differentiation-related proteins nor secretion of extracellular enzymes was examined here. However, secretion of cell wall degrading enzymes and penetration of the host cell wall by *Trichoderma* have already been demonstrated (Elad *et al.*, 1983b; Benhamou & Chet, 1993). It is not yet known whether this event is derived directly from the recognition signal or if it

requires other components from the host cell wall. The biomimetic system used in this work could also serve to examine this question, as well as to detect other differentiation-related processes.

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