Characterisation of the mycelial glycoproteins of the "Ascochyta pea complex" (Ascochyta pisi Lib.,Mycosphaerella pinodes (Berk. and Blex.) and phoma medicaginis var. pinodella (Jones) Boerema)

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Original article

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Abstract – Mycelial glycoproteins of the three phytopathogenic fungi causing *Ascochyta* blight on pea (*Ascochyta pisi*, *Mycosphaerella pinodes* (anamorph *Ascochyta pinodes*) and *Phoma medicaginis* var. *pinodella*) were investigated using affinity chromatography. Three types of functional structures were present in the three pathogens, N-glycosylated mannose structures, α-D-glucosyl residues and terminal α-D-galactosamine residues. The antigenic properties of the different glycoprotein fractions extracted from the three species were tested by ACP-ELISA using an antiserum produced against *Mycosphaerella pinodes* soluble mycelial extract. For all three fungi, the fraction containing a high mannose structure was preferentially recognised, whereas the fraction with glycosyl residues showed a weak reaction. The fraction containing α-D-galactosamine residues was not recognised. The most antigenic (mannose-rich) glycoprotein fraction of *Mycosphaerella pinodes* was used as immunogen to produce a polyclonal antiserum, in order to evaluate its specificity. The response (by ACP-ELISA) to this serum is similar for the three fungi. (© Inra/Elsevier, Paris.)

Pisum sativum / pea / *Ascochyta* / functional glycoprotein / mannose structure / affinity

Résumé – **Caractérisation des glycoprotéines mycéliennes des *Ascochyta* du pois (*Ascochyta pisi* Lib., *Mycosphaerella pinodes* (Berk. et Blox.) et *Phoma medicaginis* var. *pinodella* (Jones) Boerema).** Les glycoprotéines du mycélium de trois champignons phytopathogènes apparentés responsables de l'anthracnose du pois (*Ascochyta pisi, Mycosphaerella pinodes* (anamorphe *Ascochyta pinodes)* et *Phoma medicaginis* var. *pinodella*) ont été étudiées. La nature de ces glycoprotéines fonctionnelles a été déterminée en se basant sur leur affinité pour différentes lectines. Trois types de structures ont pu être mises en évidence chez les trois agents pathogènes, des structures N-glycosylées riches en mannose, des résidus α-D-glucosyle, et des résidus α-D-galactosamine terminaux. Les propriétés antigéniques des dif-

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férentes fractions glycoprotéiques des trois parasites ont été comparées par une méthode immunoenzymatique (ACP-Elisa) à l'aide d'un sérum dirigé contre les extraits mycéliens solubles de *Mycosphaerella pinodes*. Pour chacune des trois espèces étudiées, une forte réaction est observée pour la fraction glycoprotéique riche en mannose, tandis que celle de la fraction présentant des résidus glucosyle est plus faible. La réponse de la fraction caractérisée par des résidus α-D-galactosamine terminaux n'est pas significativement différente de celle du témoin. La fraction glycoprotéique de *Mycosphaerella pinodes* la plus antigénique (riche en mannose) a été utilisée pour produire un sérum polyclonal, dans le but d'évaluer sa spécificité. La réponse (en Elisa indirect) vis-à-vis de ce sérum est similaire pour les trois champignons. (© Inra/Elsevier, Paris.)

***Pisum sativum* / pois / *Ascochyta* / glycoprotéine fonctionnelle / structure mannose / affinité**

1. INTRODUCTION

Three related fungi, namely *Ascochyta pisi* Lib., *Mycosphaerella pinodes* (Berk. and Blox.) (anamorph *Ascochyta pinodes*) and *Phoma medicaginis* var. *pinodella* (Jones) Boerema, can cause important yield losses in the pea (*Pisum sativum*) crop. Infected seeds are often symptomless. The sexual form is only known for *Mycosphaerella pinodes*, which is also considered to be the most damaging of the three [24].

Their identification on the basis of symptoms or morphological criteria is lengthy because sporulation is needed. Neither soluble mycelial protein electrophoresis nor serology could differentiate the three pathogens clearly [3, 10]. Only recently biochemical and molecular tools have given promising results [9].

Several authors suggest that glycoprotein antigens can improve serological diagnostic tests [1, 2, 18, 29]. Wycoff and Ayers [27] have shown that monoclonal antibodies raised against *Phytophthora megasperma* antigens recognise carbohydrate epitops.

The importance of pathogen glycoproteins in host–pathogen interaction (especially at the adhesion and recognition levels) has been shown [5, 7, 12, 14, 15, 17, 19, 22]. Chrispeels and Raikhel [6] proposed that the carbohydrate binding domains of plant vacuolar lectins have become incorporated into families of proteins that play important roles in different plant defence mechanisms, as the chitin binding domain found in several proteins inhibitory to fungi. Wade and Albersheim [26] isolated, from

culture filtrates of a non-virulent race of *Phytophthora megasperma,* a glycoprotein capable of protecting the host (soybean) from the attack of a virulent race.

In this study different lectins were used to determine and compare the functional glycan containing structures of the three pea pathogens. The objectives of this research work were: i) the characterisation of the mycelial glycosylated proteins of the three related pathogens by affinity chromatography using different plant lectins; ii) the comparison of their different mycelial glycosylated fractions, particularly their antigenic properties by antigen-coated plate ELISA; iii) the determination of the specificity of a polyclonal serum raised against the most antigenic fraction of *Mycosphaerella pinodes*.

2. MATERIAL AND METHODS

2.1. Fungal cultures

The following fungal isolates were used: *Ascochyta pisi* (Ap-001Fra), *Mycosphaerella pinodes* (Mp-001Fra) and *Phoma medicaginis* var. *pinodella* (Pmp-001Fra). Faris-Mokaiesh et al. [9] described previously the origin and culture of fungal isolates.

2.2 Methods

2.2.1. *Extraction of soluble mycelial proteins*

A sample (100 mg) of freeze-dried mycelium was ground in liquid nitrogen using a mortar and pestle, and

suspended in 1.0 mL of extraction buffer (PBS – phosphate buffered saline: 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 2.9 g Na_2HPO_4, 12 H_2O, H_2O q.s.q. 1 000 mL, 2 mM EDTA, 10^{-4} mM pepstatine A and 1 mM phenyl methyl sulfonyl fluoride (PMSF), pH 7.4). Suspensions were chilled in an ice bath for 1–3 h and centrifuged at 20 000 g for 30 min at 4 °C. The supernatant was then collected, divided into 100 µL aliquots and stored at –80 °C for later use [10].

2.2.2. Extraction of lithium chloride soluble glycoproteins

Several authors [16, 23, 26] have shown that cell wall glycoproteins from different micro-organisms can be extracted with lithium chloride.

A sample (1 g) of freeze-dried mycelium was ground in liquid nitrogen with a mortar and pestle, suspended for 30 min in 10 mL of a solution containing 1 M lithium chloride, 20 mM sodium phosphate, pH 7, and 0.1 M PMSF, and centrifuged at 20 000 g for 20 min at 4 °C. The supernatant was then collected, dialysed against a solution containing 20 mM Tris HCl, pH 7.4, 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, filtered successively on a Whatman GF/B 1.0 µm and a Millex-HV Millipore 0.45 µm filters, and desalted on a column of Sephadex G25 (PD 10, Pharmacia).

2.2.3. Extraction of lithium chloride insoluble glycoproteins

Sodium deoxycholate was used in order to extract the glycoproteins that were not extracted by lithium chloride. The insoluble fraction was ground in liquid nitrogen with a mortar and pestle, and suspended for 30 min in 1 mL of a solution of Tris/HCl – 0.1 M Tris, pH 8.0, containing 7 % sodium deoxycholate. The supernatant was then collected, dialysed against a solution containing 20 mM Tris HCl pH 7.4, 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, filtered successively on a Whatman GF/B 1.0 µm and a Millex-HV Millipore 0.45 µm filters and desalted on a column of Sephadex G25 (PD 10, Pharmacia). Hayman et al. [13] have shown that this method preserves the functional properties (binding capacity, immunogenic, enzymatic) of the extracted glycoproteins.

2.2.4. Protein concentration

Protein concentration was estimated according to the method of Bradford [4], based on a bovine serum albu-

min standard dilution curve (concentration ranges 0.01–0.1 mg mL^{-1}). The presence of protein compounds was also determined by measuring the absorbency at 280 nm.

2.2.5. Purification of the mycelial extracts (figure 1) in order to preserve functional properties

2.2.5.1. Affinity on concanavalin A

Concanavalin A (Con A) binds molecules containing mannose-rich N glycans. The binding sugar requires a structure containing the C-3, C-4 and C-5 hydroxyl groups to react with Con A.

The lithium chloride solubilised extract was applied to a Con A Sepharose (Pharmacia) column (automated Economo System BIO-RAD, φ 0.7 cm, L 10 cm) equilibrated with a solution containing 20 mM Tris HCl, pH 7.4 , 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$. A flow rate of 0.5 mL min^{-1} was applied. When absorbency at 280 nm increased slightly owing to the presence, in the eluate, of the first unbound proteins, the flow was stopped and the sample left in contact with Con A for 30 min. Fractions of 4 mL absorbing at 280 nm were automatically collected. A protein fraction unbound to Con A was first collected. Con A bound fractions were eluted at a flow rate of 0.1 mL min^{-1} by competition using sequentially a gradient of α D-methylglu-
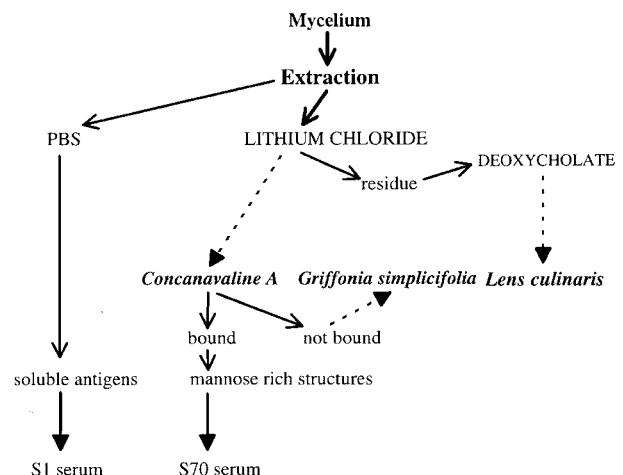

Figure 1. Extraction and characterisation of the mycelial proteins of the fungal pathogens of the pea 'Ascochyta complex'.

copyranoside (0–0.1 M), followed by a gradient of α D-methylmannopyranoside (0–0.5 M) at a flow rate of 0.2 mL min⁻¹. Three fractions were obtained: 1) an unbound fraction, without specificity to Con A; 2) a bound fraction, eluted with α D-methylglucopyranoside; 3) a bound fraction, eluted with α D-methymannopyranoside. Each fraction was desalted in a column of Sephadex G25 (PD 10, Pharmacia).

2.2.5.2. Affinity on Griffonia simplicifolia lectin

Griffonia simplicifolia GS-I lectin presents a major affinity for α D-galactosyl residues and a secondary affinity for N acetyl-α D galactosaminyl terminal residues.

A sample (3 mL) of the lithium chloride extract fraction unbound to Con A was put in contact with a bed volume of the lectin Griffonia simplicifolia (GS-I, Sigma) coupled to CNBr-activated Sepharose 4B (Pharmacia) in a beaker and left standing for 30 min. The mixture was then poured onto a column (BIO-RAD: φ 0.7 cm, L 10 cm, automated Economo System) equilibrated with 0.15 M of NaCl and 0.01 M Na$_2$HPO$_4$12 H$_2$O at a flow rate of 0.1 mL min⁻¹. The column was washed with at least ten volumes of starting buffer to eliminate unspecific bound proteins.

The bound molecules were successively eluted with 0.1 M of N-acetyl-D-galactosamine, 0.5 M of D-galactose and 0.1 M de N-acetyl-D-glucosamine. The effluent absorbing at 280 nm was collected, purified on a column of Sephadex G25 (PD 10, Pharmacia), freeze-dried and maintained at –20 °C.

2.2.5.3. Affinity on lectin from Lens culinaris Sepharose 4B

Lentil lectin Sepharose 4B (Lens culinaris) presents an affinity similar to Con A. Sodium deoxycholate at a concentration of 1 % is used to disperse membranes because the soluble glycoprotein components still retain their ability to bind to lentil lectin [13].

The 1 % sodium deoxycholate extracts were applied to a column (BIO-RAD: φ 0.7 cm, L 10 cm) containing lentil lectin Sepharose 4B equilibrated with a 1 % (w/v) sodium deoxycholate solution in 10 mM Tris HCl, pH 8.2, 0.9 M NaCl at a flow rate of 0.5 mL min⁻¹. When the absorbency of the eluate at 280 nm started to increase, the flow was stopped and the sample left in contact with the lentil lectin for 30 min.

The flow was re-established and the column was washed with at least ten bed volumes of starting buffer to elute unbound proteins, followed by 2 % (w/v) of α-D-

methyl-glucopyranoside and 2 % (w/v) α-D-methyl-mannopyranoside in 1 % sodium deoxycholate.

2.2.6. Serology

2.2.6.1. Detection of sugars in glycoconjugates by an enzyme immunoassay (Boehringer, Mannheim)

The different fractions absorbing at 280 nm were blotted onto nitrocellulose. Dot blots of the different fractions are studied with the DIG Glycan Detection Kit (Boehringer, Mannheim). Hydroxyl groups in sugars of glycoconjugates are oxidised to aldehyde groups by periodate treatment. Digoxigenin is then attached to these aldehydes via a hydrazide group. Digoxigenin-labelled conjugates are detected by an enzyme immunoassay (digoxigenin-specific antibody conjugated with alkaline phosphatase).

Glycans were detected in all the extracts before affinity chromatography.

2.2.6.2. ELISA procedure

The antigen-coated plate ELISA (ACP-ELISA) was conducted. Polystyrene plates 'Nunc Immunoplate Maxisorp', Nunc A/S, Roskilde, Denmark) were used. All test volumes were 100 µL per well and three replicates were used for each sample. The wells were washed three times with PBS-T (PBS, containing 0.1 % v/v Tween 20, 0.02 % NaN$_3$) between each incubation step. Positive (homologous antigen) and negative (buffer) controls were included in each plate.

Microtitre plates were coated with antigens (mycelium extracts) that were adjusted to 0.5 or 1 µg of proteins mL⁻¹ in carbonate buffer (coating), and incubated for 2 h at 37 °C and overnight at 4 °C.

Antiserum diluted in PBS-T containing 0.2 % (w/v) BSA (Bovine serum albumin, Serva, Heidelberg, Germany), was added and incubated for 2 h at 37 °C.

The conjugate, alkaline phosphatase-goat anti-rabbit IgG (Sigma Chemical Co, St Louis, USA) was diluted 9 000 fold in the buffer described above, and incubated for 1 h 30 min at 37 °C.

Alkaline phosphatase substrate (P-nitrophenylphosphate substrate (Sigma)) was added at 1 mg mL⁻¹ of diethanolamine buffer (pH 9.8) and the reaction mixture incubated at room temperature.

Measurements of the absorbency at 405 nm were taken at 10 min interval for 1 h with a 'Emax' (Molecular

Devices, Palo Alto, CA, USA) connected to a computer. Slopes of A_{405} readings were computed and reported as $d(A_{405})/dt$. Slopes show the evolution of absorbency with time, thus allowing a better comparison of wells with different evolution rates, and of plates read at different times.

2.2.6.3. Antisera production

Two male, 13-week-old, New Zealand white rabbits were bled and their pre-immune serum collected as a control. The animals were then immunised by intramuscular injections of 500 µL of mycelial extracts of *Mycosphaerella pinodes* emulsified with an equal volume of Freund's complete adjuvant (at a final concentration of 1 mg mL^{-1}). Booster injections of the same extracts with Freund's incomplete adjuvant were made 4 and 6 weeks later and the rabbits bled 10–14 days after each boost. After incubation at 37 °C for 1–2 h and at 4 °C overnight, antisera were collected, centrifuged at 800 *g* for 10 min and stored as 1 mL aliquots at –20 °C. Two antisera, collected after the last boost, had the highest titre and were selected for all subsequent experiments:

S1, anti-mycelial soluble extract [10];

S70, produced with the lithium chloride soluble fraction bound to Con A and eluted with α-D-methyl-mannopyranoside.

3. RESULTS

3.1. Presence of glycans in the separated fractions

The presence of glycan structures was detected in all extracts before affinity chromatography. Moreover, positive responses were obtained for the following fractions absorbing at 280 nm (*figure 2*): bound to Con A and a) eluted with α-D glucoside, b) eluted with α-D-mannoside, unbound to Con A, bound to *Griffonia simplicifolia* and eluted with N-acetyl-D-galactosamine and soluble in deoxycholate (weak response). The response was similar for the three species of fungi.

3.2. Characterisation of the glycoprotein structures

3.2.1. Concanavalin A

Three groups of substances absorbing at 280 nm were obtained. The first, and the most important, is
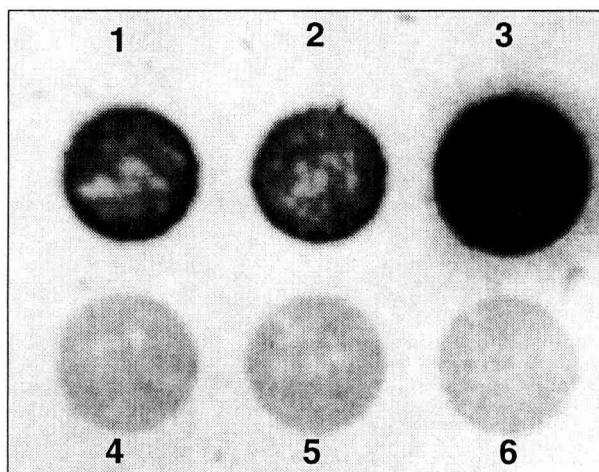

Figure 2. Detection of glycans (enzyme immunoassay, DIG Glycan Kit, Boehringer, Mannheim) in the fractions obtained from *Mycosphaerella pinodes* mycelial extract and blotted onto nitrocellulose: 1, soluble on lithium chloride; 2, eluted from concanavalin A with α-D-glucoside; 3, eluted from *concanavalin A with α-D-mannoside; 4, unbound to concanavalin A; 5, eluted from *Griffonia simplicifolia* with N-acetyl-D-galactosamine; 6, soluble in deoxycholate.

composed of the protein conjugates not bound to Con A (without mannose-rich N-glycans). The second is obtained by elution of the glycoproteins bound to Con A with α-D-glucopyranoside. The last group is obtained by elution of the bound molecules with α-D-methylmannopyranoside. Similar absorption profiles were obtained for the three species of fungi.

3.2.2. Griffonia simplicifolia

Two types of substances absorbing at 280 nm were obtained: unbound protein compounds and glycoproteins bound to *Griffonia simplicifolia* and eluted with N-acetyl-D-galactosamine solution.

Affinity chromatography with *Griffonia simplicifolia* produced similar absorption profiles at 280 nm for the three species of fungi.

3.2.3. Lens culinaris

Competitive elution from *Lens culinaris* lectin of the sodium deoxycholate extract did not produce

any substances with an absorbency at 280 nm. The sodium deoxycholate extract that was put in the column showed a positive but weak response using the DIG Glycan detection kit.

The unbound eluate, without specificity to *Lens culinaris* lectin, presented a weak absorption peak at 280 nm and a negative response to the DIG Glycan Kit.

3.3. Comparison between the three fungal pathogens referred as the 'Ascochyta complex'

This work revealed the presence of glycoprotein structures that could bind specifically to the lectins from concanavalin A and *Griffonia simplicifolia* in the mycelial extracts of the three species. A glycoprotein fraction extracted with lithium chloride contained mannose rich N-glyconjugate structures (bound to Con A, eluted with α-D-mannoside). Another fraction absorbing at 280 nm that specifically bound to Con A contains glucose-rich glycoprotein structures (eluted by α-D-glucoside). The fraction containing glycans but without affinity to Con A was characterised by the lectin from *Griffonia simplicifolia*. A portion bound to this lectin and was eluted by N-actyl-D-galactosamine, thus revealing the presence of galactosamine terminal residues. As competitive elution from *Lens culinaris* of the deoxycholate extract did not desorbed substances absorbing at 280 nm, the glycans detected in the deoxycholate fraction are not glycoproteins, but glycolipids or polysaccharides.

3.4. ELISA response of the fractions separated by chromatography

3.4.1. To the antiserum S1 raised to the soluble mycelial extract of Mycosphaerella pinodes (figure 3)

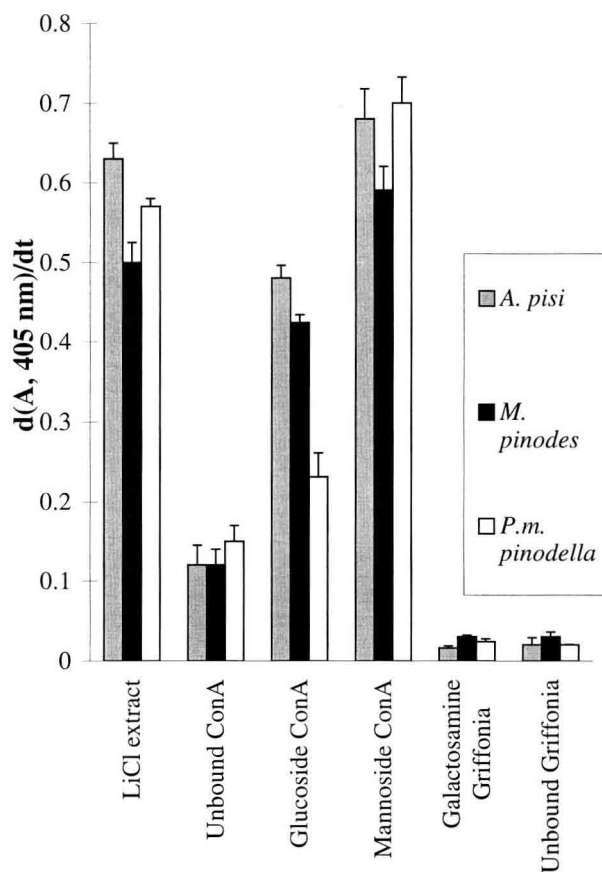ACP-ELISA responses to S1 antiserum for the three fungi (*A. pisi*, *M. pinodes* and *P. m.* var. *pin-*

Figure 3. ACP-ELISA response of the different fractions of *A. pisi*, *M. pinodes* and *P.m.* var. *pinodella* isolated by affinity chromatography to S1 serum raised against *Mycosphaerella pinodes* mycelial extract. Antigens were extracted from the mycelium by lithium chloride (LiCl), and separated on concanavalin A (Con A) and *Griffonia simplicifolia* (Griffonia) lectins by affinity chromatography. Bars indicate standard errors for the means of three replicates.

odella), were similar for the fractions presenting the same lectin specificity.

The response of the different fractions to the S1 serum is presented in *figure 3*. The strongest reaction was obtained with the fraction bound to concanavalin A and eluted with α-D-mannoside (N-glycosylated and mannose rich), followed by the fraction bound to concanavalin A and eluted with α-D-glucoside. The reaction of the fraction absorb-

ing at 280 nm without affinity (not bound) to concanavalin *A* was weak.

The reaction obtained with the whole lithium chloride extract before purification was weaker then with the fraction containing the mannose-rich structures. The two fractions separated with *Griffonia simplicifolia* lectin, unbound and eluted with N-acetyl-D-galactosamine, gave a similar response to the negative control.

3.4.2. To the antiserum S70 raised to the mycelial fraction of Mycosphaerella pinodes eluted with α-D-mannoside (figure 4)

A very high ACP-ELISA response (*figure 4*) to the anti-sera S70 was obtained for the mycelial glycoproteins containing mannose-rich structures (extracted with lithium chloride, bound to Con A and eluted with α-D-mannoside), not only extracted from *Mycosphaerella pinodes* (homologous antigen), but also for the ones extracted from *Ascochyta pisi* and *Phoma medicaginis* var. *pinodella*.

The results obtained for the three species were similar (*figure 4*). The serum specificity was not improved.

4. DISCUSSION

Our work revealed that similar structures with affinity to the lectins of concanavalin A and *Griffonia simplicifolia* were present in the mycelial extracts of the three fungal pathogens referred as the 'Ascochyta pea complex'. Gandon [11] isolated from the culture filtrate and the mycelial wall of a carnation fungal pathogen (*Fusarium oxysporum*) a polysaccharide fraction eliciting phytoalexin synthesis in carnation and containing mannose, glucose and galactose. Matsubara and Kuroda [20] found mannosyl and glucosyl residues in a glycoprotein of the O-glycoside type secreted from conidia of *Mycosphaerella pinodes* during germination. Our work detected the presence of mannose-rich structures of the N-glycoside type (presenting affinity to Con A) in the mycelial lithium chloride extract of
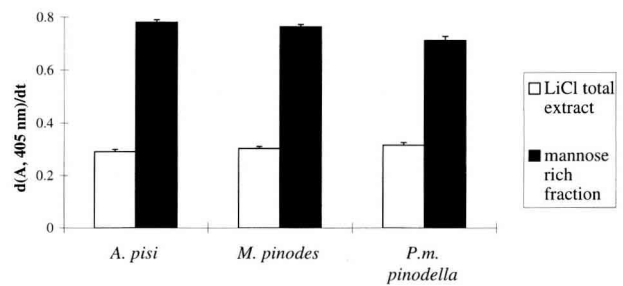

Figure 4. ACP-ELISA response of lithium chloride extracts and mannose-rich structures from the mycelium of A. *pisi*, M. *pinodes* and P.m. var. *pinodella* to S70 serum raised against the M. *pinodes* glycoproteins. Bars represents standard errors for the means of three replicates.

the three fungal pathogens referred to as the 'Ascochyta complex'. Ding et al. [8] showed that the adhesion of *Phytophthora megasperma* to the host (soybean) is due to glycoproteins presenting affinity to Con A and found that D-mannose could inhibit this adhesion. Peberty [21] suggested that O-glycosylation of a protein is essential for secretion and N-glycosylation is important for stability (protection against proteolyse, pH variations).

The affinity properties of the glycoconjugates extracted from the mycelium of the three species revealed not only the presence of mannose, glucose and galactose, but also of binding structures containing these sugars. The serological properties of those glycoconjugated structures extracted with lithium chloride coincide with the affinity chromatography properties. Similar glycan structure of the three fungi (thus presenting the same affinity) could not be differentiated serologically with the S1 serum.

The three fractions presenting different affinities isolated from the mycelium of all species (eluted from Con A with α-D-glucoside and α-D-mannoside, or eluted from *Griffonia simplicifolia*), showed different immunoenzyme responses (ELISA) to the S1serum.

This study proved that, among the mycelial glycoproteins, the fraction recognised the most strongly by the S1 serum was the fraction containing the mannose-rich glycan structures. This result lead to the production of the S70 serum raised against this

fraction, but the specificity was not improved. The mannose-rich glycoproteins are the most antigenic but could not lead to the production of a serum distinguishing the three species.

In conclusion, the results proved the presence in the three fungi of the pea 'Ascochyta complex' of mannose-rich N-glycans structures that were strongly antigenic and non-specific.

It would be interesting to study the role of these mycelium structures in the plant–pathogen interaction, in order to provide a better understanding and control of these pathogens.

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