Antigens of *Fusarium solani:* types and criteria for immunochemical characterization

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SUMMARY

Background: there are no established methods for the preparation and standardization of *Fusarium solani* antigens. This lack of standardization makes it difficult to use these antigens in allergenic diagnostic tests.

Objective: to obtain an appropriate standardized method for the preparation of the different antigen types of *F. solani*.

Methods: production of fungal extracts, followed by biochemical and immunological characterization.

Results: the somatic antigens presented the greatest protein content most of these proteins are common to the metabolic and hydrosoluble antigens, particularly those proteins at 35-39 kDa, 29-32 kDa and 15-16 kDa, as detected by electrophoresis and immunoblotting. The hydrosoluble antigens presented the highest protein diversity; these proteins were the most specific, showing minor determinants in common with the other antigens.

Conclusions: it is recommended that a mixture of the different antigen sources be used in order to obtain extracts which would cover the maximum number of diagnostic possibilities.

Key words: Fungi. *Fusarium solani.* Antigens characterization. Cross reactivity. Standardization.

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INTRODUCTION

Species of the *Fusarium* genus are major plant pathogens, and are frequently isolated from soil and vegetable material (1). *F. solani* and other species are considered important agents in emerging opportunistic mycosis, since they are responsible for serious systemic infections in immunodepressed patients (2, 3). *Fusarium* antigens can induce the development of hypersensitive respiratory processes, although their frequency (4, 5), epidemiological characteristics and clinical relevance are still unknown partly due to the insufficient knowledge of these antigens and the absence of any adequate standardization (6) for their use in diagnostic tests.

There are no established methods for the preparation and standardization of fungal antigens, even though they are commonly cultivated in a synthetic, liquid media, without macromolecules and with a controlled pH. Incubation time, temperature, humidity and aeration should be adequate for each cultivated species; in some cases it is convenient to shake the culture constantly to prevent sporulation, while other authors (7) prefer static cultures in order to promote sporulation by using agar-containing media incubated for long periods of time.

The requirements for optimum growth and controlled sporulation differ from one species to another. In order to obtain optimized antigens, which will provide reproducible results, it is crucial to apply a correct standardization of the employed methodology. This includes the culture media composition, the incubation time, shaking and

temperature, the method for separating the mould from its culture media, as well as the dialysis, concentration and purification methods. All of these parameters influence the presence and relative content of certain antigens which play a determinant role in the diagnostic performance of the resulting fungal extract.

The main objective of this study was to obtain an appropriate standardization for the preparation of the different antigen types of *F. solani,* as well as to acquire more data about their biochemical and immunological characteristics.

MATERIALS AND METHODS

F. solani strains

Eight strains of different origin were used: two from type collections (CBS 49063 and the Colección Española de Cultivos Tipo, CECT 2199) and the other five from human patients; four were isolated from systemically infected patients from Brazil, and the six, which was isolated in our laboratory, from an onichomycosis (MRL 95650).

Strains were cultured in potato-agar (Potato Dextrose agar, Merck) and cultivated for one week at 30 °C. Once the purity of the culture was assured, a mixture of mycelium and conidia was inoculated into each of the two culture media employed to prepare the antigens.

Culture media

A conventional Czapek-Dox (Difco) liquid medium containing 1% (wt:wt) yeast extract was modified by adding glucose and salts (39 g Cazpek-Dox, 20 g glucose, 1 g yeast extract, 0.01 g zinc sulphate and 0.005 g copper sulphate).

Antigen preparation

Mould spore suspensions were inoculated in one litre Erlenmeyer flasks containing 300 ml of culture medium and were incubated for 21 days at room temperature (25-27 °C) with constant stirring. Mycelia were then separated by vacuum-filtration through a Büchner porcelain funnel. This raw material was used to prepare three different antigens: metabolic, hydrosoluble and somatic (Figs. 1 and 2).

The metabolic antigen was obtained by first filtering the culture media through a 0.7 μm-pore filter (Millipore). It was then concentrated with

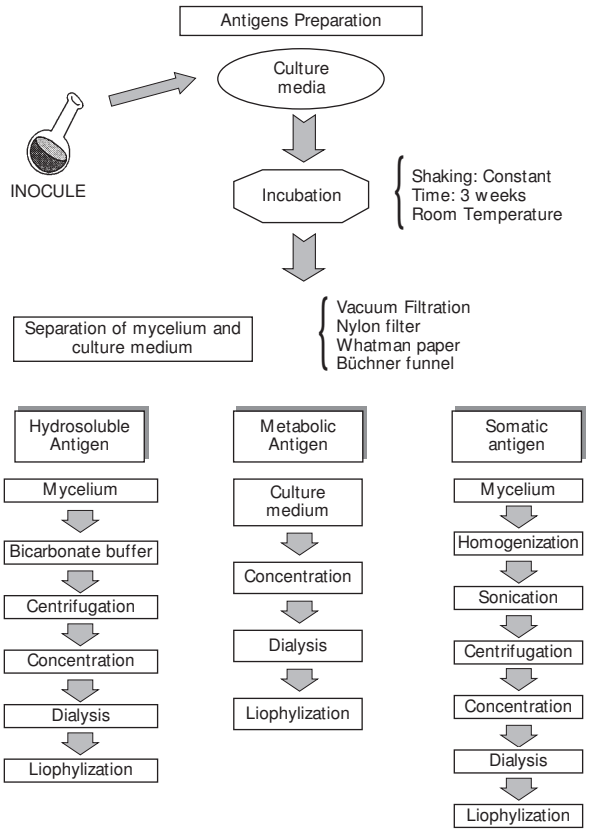

Figure 1.—Schematic diagrams of antigen production.

polyethylene glycol 6000 (PEG) at 4 °C and dialyzed against running tap water followed by distilled water (20 volumes) overnight. The resulting product was freeze-dried and stored.

The hydrosoluble antigen was obtained by extracting the mycelia with ammonium bicarbonate (0.1 g/ml) overnight. The resulting solution was

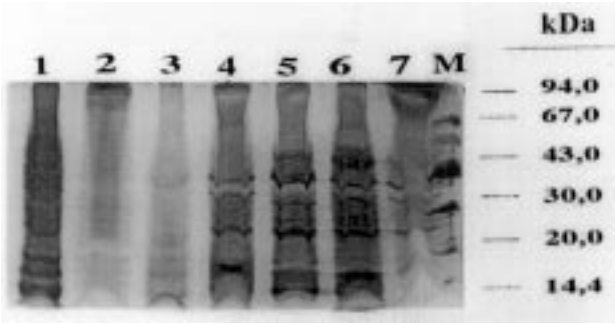

Figure 2.—Electrophoresis in SDS-PAGE of the different hydrosoluble antigens after silver staining. Lane number corresponds to the following *F. solani* strains: 1: 4578; 2: JM (modified media); 3: CBS (modified media); 4: CECT 2199 (modified media); 5: *F. solani* JM (Czapek); 6: CBS (Czapek); 7: CECT (Czapek).

Table I

Protein (Prot) carbohydrate (Carb) concentrations in antigens of *Fusarium solani.* In micrograms per milligram of dry weight of antigen according to the culture media and strain used

Fusarium	Hydrosoluble		Metabolic		Somatic	
Strains	Prot	Carb	Prot	Carb	Prot	Carb
Conventional Czapek						
CECT	40.38	162.66	61.825	217.66	112.90	129.57
CBS	93.93	70.66	32.525	122.14	59.23	133.42
JM	48.4	65.5	33.5	176.17	73.74	131.77
Modified Czapek						
CECT2199	57.53	111.61	38.06	122.9	66.23	123.27
CBS	ND	ND	33.11	186.34	48.08	37.113
JM	59.97	43.15	37.35	193.72	29.722	80.60
4578	23.83	27.18	21.64	26.23	106.29	50.87
5233	28.72	40	38.02	168	23.21	49.67
4558	11.55	116.1	49.27	219.86	81.60	137.47
5747	55.00	251.23	15.50	184.23	135.30	191.35
11020	25.10	38.32	36.30	237.36	51.71	91.82

ND: Not done.

centrifuged and concentrated with polyethylene glycol 6000, followed by dialysis and lyophilization.

The somatic antigen was obtained after cool homogenization of the mycelia at 20.000 rpm for 10 min. The homogenized mass was sonicated for 10 min, followed by concentration, dialysis and lyophilization.

Biochemical characterization

Protein and carbohydrate contents were determined for each of the extracts using the Bradford (9) and Dubois (10) methods, respectively. Vertical electrophoresis was carried out on each extract under denaturing conditions, in polyacrylamide gels (12.5% acrylamide) with sodium dodecyl sulphate (11) staining with Coomassie or silver solution (12).

Immunological evaluation

Rabbit antisera were obtained from the metabolic, hydrosoluble and somatic extracts. Weekly immunization was performed by intramuscular and subcutaneous routes for 2 months (13). A mixture of the same type of antigen obtained was used for immunization.

The agarose gel double-diffusion technique (14) was used to assay the different antigens and to detect cross-reactions.

Western blotting (15) was carried out by electrical protein transfer onto 0.45 μm Immobilon-P membranes (Millipore). Membranes were then incubated two hours with a 1:1000 dilution of rabbit polyclonal antiserum. Anti-rabbit IgG conjugated to peroxidase (1:3000 dilution) and diaminobenzidine were used for immunochemical staining according to the manufacturer's instructions (Sigma, St Louis, USA). The resulting immunoblotting band pattern was analyzed using the software package (1998) Bio-Rad Diversity Database. A phylogenetic tree was constructed based on UPGAMA with the Jaccar coefficient to detect antigen similarities.

RESULTS

From the three *F. solani* collection strains, nine antigens (metabolic, hydrosoluble and somatic) were prepared by culturing the antigens in conventional Czapek media. The same antigens were also prepared in modified Czapek media. Another 15 antigens were prepared from patient-isolated strains, cultivated exclusively in modified Czapek media. The antigen yield was quite variable, having no relation to the composition of the culture media nor to the strain used in the production.

It was observed that somatic antigens presented a relatively higher protein content than the other antigens. Moreover, protein content in the somatic antigens was higher when prepared with the conventional Czapek media (table I).

Table II

Bands detected by vertical SDS-PAGE electrophoresis (IEF) and immunoblotting (IB) of the different pools of antigens of *Fusarium solani* incubated with the homologous antisera

	Hydrosoluble		Metabolic		Somatic	
	EF	IB	EF	IB	EF	IB
161				*		
99		*				
90			*			
83-80			*		81-79	
74			*			
69-66	*	67	69-71			
61	*					
60-57			*		60	
53					*	
48		*	*			
44-43	*		*		*	
43-42			*			
41				*		*
39-36	*	36		36	38	39
35			*			
34-32	*	33		32		
29		*	*			*
28				*	*	
27	*					
25		*				
24-23			*	24	*	
22	*	*				
17			*			
16-15	*	*			*	
14-13			*	14		
11		*	*			
10				*		
8	*			*		

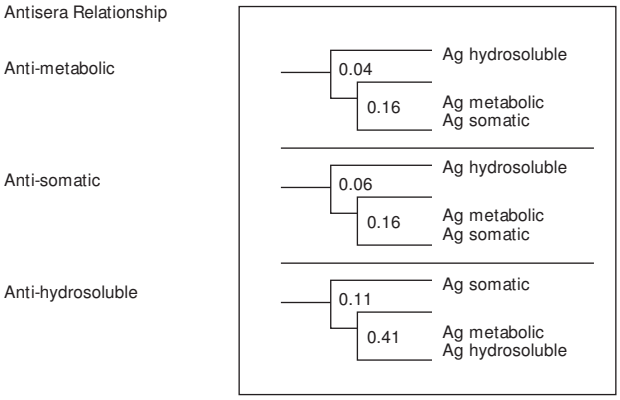

Figure 3.—Dendograms showing the relationship between the different pools of antigens of *Fusarium solani* and their antibodies. Abbreviations used: Ac: Antibody. Ag: Antigen. Hydro: Hydrosoluble. Met: metabolic. Som: Somatic.

Antigens prepared from strains belonging to CBS and CECT yielded higher protein contents, as well as a greater number of electrophoretic bands (Fig. 2).

The hydrosoluble antigens presented the highest number of protein bands, whilst the somatic antigens presented the lowest (table II).

Common bands were found in all extracts, and were distributed among 15; 16-17; 23-25; 36-38; 44-46 and 73-77 kDa molecular weight. Antigens obtained from patient-isolated strains presented the higher molecular weight bands in common (116 and 97 kDa). In some extracts a broad and intense protein band could be observed at 53-64 kDa, which could consist of several fractions. Somatic antigen electrophoresis presented two bands at 60 and 53 kDa, while the metabolic antigens exhibited just one band of 57-60 kDa, and the hydrosoluble antigens showed a band of 61-62 kDa. The results obtained by immunoblotting of antigens and their corresponding homologous antisera are shown in table II. Somatic antigens presented the smallest number of protein bands both in electrophoresis and in immunoblotting.

The combined analysis of electrophoretic results obtained with the three antigens pools (metabolic, hydrosoluble and somatic) confirmed the existence of the following common bands: 64, 61-60, 53, 43, 30-27, 24-22 and 15-14 kDa. When evaluating the electrophoresis and immunoblottings, three immunogenic bands were common for the three types of antigens were detected at 39-35 kDa, 29-32 kDa and 15-16 kDa, respectively.

The graphic representation of these results as dendograms showed very little similarity between the different antigens (Fig. 3), although the metabolic antigen presented the highest similarity independently of the serum employed. The highest similarity was found between the hydrosoluble and metabolic antigens.

The double-diffusion and immunoblotting confirmed several cross-reactions among the antigens. Metabolic antigens showed the highest degree of cross-reactivity, whilst the hydrosoluble antigens showed the lowest cross-reactivity despite presenting the highest number of bands in electrophoresis and immunoblotting.

DISCUSSION

F. solani is considered the most pathogenic species of the *Fusarium* genus. The scarcity of reports existing on antigens from *Fusarium* refer mainly to this species, which is also considered the most allergenic and capable of causing

hypersensitivity in atopic subjects exposed to this mould.

Allergies due to *Fusarium* are not frequently reported in epidemiological studies. This could be due to the lack of systematic studies that include extracts from this genus, mainly because of the absence of standardized and chemically and immunologically characterized antigens (16).

Qualitative differences were found according to the culture media used, as the antigens obtained after culturing in conventional Czapek showed a higher diversity in protein composition. Thus, it is recommended that conventional Czapek media be used for the culture of *F. Solani.* Modified Czapek media was found to be more appropriate for other moulds such as *Aspergillus* (8).

It has been demonstrated here that the collection strains, although stored for long periods of time, were adequate for the preparation of *F. solani* antigens. Such storage times however would not be adequate for the preparation of *A. fumigatus* antigens (17).

In allergological diagnoses, the extract considered as optimum is that which contains the most potent and common allergens (18). In order to prove this fact, IgE antibodies purified by affinity columns have been used (19, 20). The low affinity of the antibodies found for some antigens could be attributed to an insufficient amount of protein and/or to an excessive carbohydrate content. Nevertheless, Baer (20) suggests that proteins bound to carbohydrates could also be allergenic; therefore, a high carbohydrate content, such as that found in *Fusarium* antigens, does not necessarily affect their quality.

Verna, et al (7, 21) produced metabolic antigens of *F. solani* with 11-day cultures, based on the maximum protein content and number of bands resolved by isoelectric-focusing. The methodology for antigen preparation used by these authors differs from that used in the present work, since the composition of the culture media was different. The hydrosoluble extract was obtained from mycelia cultivated in solid media where a maximum sporulation is produced. In the present study, cultivation took place in liquid media, so the extraction of the hydrosoluble antigen was obtained from mycelia with a small number of spores. Generally, the carbohydrate content in extracts prepared from spores and mycelia predominantly showed a protein/carbohydrate ratio of around 1:2. For metabolic extracts a 1:4 ratio was obtained, which is much higher than the published mean ratio of 1:1 (21). These proportions remained constant, regardless of the culture medium used.

As previously described (22), the results obtained by electrophoresis were complemented by the immunoblotting techniques, which allowed the detection of some proteins that probably, due to their low concentration, are only visible by an antigen-antibody reaction. In order to simplify the present study, mixtures of each type of antigen in the same proportions, were pooled. This method could, however, mask some protein fractions, which may be present in individual extracts.

Á protein band of 64 kDa common to the three *F. solani* extracts was described (23). In the present study, such a band was not detected in any of the different pools of antigens, but it was identified in some of the individual antigens. Somatic antigens presented a lower diversity of bands while at the same time had the highest total protein content. In contrast, the hydrosoluble antigens with a low protein content showed the highest electrophoretic diversity. A higher protein content does not necessarily assure an adequate diversity and better quality of antigens. This divergence between protein content and diversity indicates the need for a systematic analysis of extracts.

Three common bands of 15-16, 24-25 and 27-30 kDa were detected in all antigens, independently of the method of preparation and the strain used. Two of these proteins, those detected at 15-16 and 27-30 kDa, were also detected by others (7, 21, 23). The greatest diversity of proteins with antigenic activity was provided by the hydrosoluble and metabolic extracts, and considering their high yields and ease of production, these latter antigens would be the most suitable for diagnostic purposes.

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RESUMEN

No existen métodos estandarizados para la preparación de antígenos de *Fusarium solani.* La falta de estandarización hace más difícil el uso de éstos antígenos en las pruebas de diagnóstico.

Objetivo: obtener un método estandarizado apropiado para la preparación de diferentes tipos de antígenos de *F. solani.*

Resultados: los antígenos somáticos presentan el mayor contenido proteico, muchas de estas proteínas también se encuentran en los antígenos hidrosolubles y metabólicos, particularmente las proteínas de 35-39 kDa, 29-32 kDa y 15-16 kDa, detectadas por electroforesis e immunobloting. Los antígenos hidrosolubles presentan la mayor diversidad proteica incluyendo más específicas que muestran menos determinantes comunes con los otros tipos de antígenos.

Conclusiones: debido a su heterogeneidad es recomendable emplear una mezcla de diferentes tipos de antígenos para incrementar las posibilidades diagnósticas.

Palabras clave: Hongo. *Fusarium solani.* Caracterización antigénica. Reactividad cruzada. Estandarización.

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