

Studies on *Fusarium solani*

Cross-reactivity among *Fusarium* species

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Allergenic cross-reactivity among three *Fusarium* species (*F. solani*, *F. equiseti*, and *F. moniliforme*) was determined by crossed immunoelectrophoresis (CIE), ELISA inhibition, and immunoprint-inhibition analysis. Inhibiting immunoprint was used to assess the cross-reactivity of individual allergenic proteins in culture filtrate (CF), separated on SDS–PAGE. Dose-response inhibition of band 14 kDa in immunoprints of *F. solani* CF by antigens of *F. solani* CF, *F. equiseti* CF, and *F. moniliforme* CF demonstrated significant cross-reactivity of allergenic proteins on analysis of densitometric scans. CIE and ELISA inhibition using hyperimmune sera raised in rabbits against *F. solani* CF antigen also demonstrated shared antigenicity among *Fusarium* species. Collectively, this study revealed the presence of shared as well as unique antigenic and allergenic determinants in *F. solani* CF, *F. equiseti* CF, and *F. moniliforme* CF antigens.

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The Fungi Imperfecti include a number of species that can cause IgE-mediated allergic reactions in man (2, 8, 14, 20). The genus *Fusarium* of class Deuteromycetes is thought to be among the genera of molds most responsible for respiratory allergic disorders (8, 17). Both upper and lower respiratory tract infections have been reported, but the actual prevalence of asthma or rhinitis resulting from inhalation of mold spores or mycelial fragments is not known (14). It has been shown that culture filtrate (CF), mycelium, and spores contain a number of allergenic proteins. These may be unique to one species or may show shared allergenicity with related species or genera. It has also been shown that the degree of IgE cross-reactivity between any two species depends on the number of individual allergens that cross-react (3).

Several reports on the cross-reactivity of molds have been published (1, 3, 5, 15, 18). Cross-reactions among different species of *Aspergillus* (*Asp. fumigatus*, *Asp. niger*, and *Asp. flavus*), and shared allergenic determinants between *Alternaria solani* and *A. tenuis* have been observed (10, 11, 23). An antigenic relationship among the different *Cladosporium* species has also been demonstrated by Honbo et al. (9). It has been shown that *Fusarium* cross-reacts with *Alternaria*, *Penicillium*, *Aspergillus*, *Cladospo-*

rium, and *Stemphylium* (7, 17), but shared antigenic and allergenic activity among the different species of the genus *Fusarium* has not been studied.

In the present study, shared allergenic/antigenic determinants in CF antigens have been demonstrated among the three most common species of *Fusarium* (*F. solani*, *F. equiseti*, and *F. moniliforme*) by immunoprint inhibition assay, crossed immunoelectrophoresis (CIE), and ELISA-inhibition experiments.

Material and methods

Strains and cultures

F. solani (3596), *F. equiseti* (3896), and *F. moniliforme* (3534) obtained from the Indian Agricultural Research Institute, Delhi, India, were used for the culture. They were grown at 30°C in synthetic medium (13) under constant agitation (125 rpm) to prevent sporulation.

Preparation of antigenic extracts

Cultures of *F. solani*, *F. equiseti*, and *F. moniliforme* were grown in synthetic medium. After days 5, 7, 9, 11, 16, 21, and 26, samples were withdrawn aseptically for measuring pH and protein. Mycelium was

obtained by centrifuging the cultures at 10 000 rpm for 30 min at 4°C and was frozen at -70°C until use. A small quantity of mycelium was dried, and dry weight was noted. The CF supernatants obtained after centrifugation were saturated by treatment with 90% ammonium sulfate. After centrifugation, pellets were dissolved in a minimum quantity of water and dialyzed against water (Spectrapor® membrane, mol. wt. cutoff 3500). The dialyzed extracts were passed through a 0.45-µm millipore filter and lyophilized. Protein contents in CF extracts were determined. On the basis of maximum protein content and number of bands on IEF pI (3–9), day 11 cultures of *F. solani* and *F. equiseti* and day 13 culture of *F. moniliforme* were selected for preparation of antigenic extracts.

Biochemical analysis

Protein content was estimated according to the method of Lowry et al. (16). Total carbohydrate content in all three CF antigens of *Fusarium* species was estimated by the phenol-sulfuric acid method (6).

Isoelectric focusing

IEF was performed on ready-made gel (Pharmacia) of pI range (3–9). An amount of 3 µg protein of each of the three extracts of *Fusarium* species was resolved with the Phast system. The resolved proteins were stained with a silver-staining kit (Pharmacia).

Immunologic analysis

Fifteen patients' sera were collected on the basis of clinical history and 2 + -4 + grading in RAST (Pharmacia Diagnostics). Normal human serum showing negative skin test and RAST for *F. solani* was also included in the study as a negative control.

Rabbit antisera were obtained by immunizing two healthy male rabbits with crude CF extract of *F. solani* over a period of 6 months. The dose schedule was 0.5 mg *F. solani* protein in Freund's complete adjuvant (Difco) injected intradermally twice a week at 10–12 sites for 2 weeks, after which 2 mg protein in Freund's complete adjuvant was administered by intramuscular injection every month. The animals were bled from marginal ear veins at different intervals, and equal volumes of the serum from both the immunized animals were pooled for use in CIE.

Crossed immunoelectrophoresis (CIE)

CIE was performed by the method of Axelson et al. (4). An amount of 150 µg protein was analyzed by

CIE in the first dimension on 1% agarose (Sigma) in mol/l of veronal buffer (pH 8.6). The second dimension was performed against rabbit anti-*F. solani* CF antibodies containing gel on both cathodic and anodic sides. Precipitin bands were stained with Coomassie brilliant blue R-250.

Enzyme-linked immunosorbent assay – inhibition

An amount of 5 µg protein of CF extract of *F. solani* was coated on the wells of polystyrene microtiter plate (Dynatech) in carbonate buffer containing 0.01 mol/l of sodium carbonate and 0.01 mol/l of sodium bicarbonate, pH 9.6 (22). The plate was incubated overnight at 4°C and washed three times with rinse buffer, PBS-Tween. The plate was incubated at 37°C for 1 h with 1% BSA to block the free sites. It was washed with PBS-Tween and then incubated overnight at 4°C with 50 µl (1:100 v/v) rabbit serum mixed with 50 µl CF antigen of each of *F. solani*, *F. equiseti*, and *F. moniliforme* species in different concentrations (0.2–10 µg/50 µl). After washing with PBS-Tween, bound IgG was detected by incubating the plate at 37°C for 3 h with 100 µl (1:1000 v/v) diluted alkaline phosphatase conjugated antirabbit IgG (Sigma). After washing the plate, color was developed by incubating with the substrate *p*-nitrophenyl (1 mg/ml) in glycine buffer (pH 9.6) at 37°C for 45 min. Reaction in the plate was stopped with 50 µl 3 mol/l NaOH, and OD was measured on an ELISA reader (Nunc GmbH Roskilde, Denmark) at a wavelength of 405 nm.

Immunoprinting

SDS-PAGE of CF extracts of *F. solani*, *F. equiseti*, and *F. moniliforme* was performed on 10% gel. Amounts of 250 µg protein of each extract were loaded on separate lanes. Resolved proteins of CF extracts of three species of *Fusarium* were blotted electrophoretically (21) onto a nitrocellulose membrane (Sigma) with dry blot apparatus (Pharmacia). After blotting, the membrane was air-dried, and free sites were blocked by incubation in blocking buffer (PBS with 1% BSA) for 1 h at 37°C. After washing with rinse buffer (PBS containing 0.1% Tween), the membrane was incubated overnight at 4°C with (1:10 v/v of PBS-Tween) diluted patient serum. The membrane was washed with rinse buffer and incubated with (1:1000) diluted horseradish peroxidase conjugated antihuman IgE (Sigma) for 4 h at 37°C. The membrane was washed three times with rinse buffer, and color was developed with diaminobenzidine and hydrogen peroxide, dissolved in sodium acetate buffer (pH 5) in the dark (19). The reaction was stopped by washing the membrane with distilled water.

Immunoprint inhibition

Immunoprint inhibition was used to study the inhibition of binding of *F. solani* CF to patient serum IgE by CF proteins of the other two species of *Fusarium*. Proteins of *F. solani* CF, resolved on SDS-PAGE gel in 10 separate lanes, were blotted on a nitrocellulose membrane. Individual lanes were cut out, and each *F. solani* CF blotted strip was incubated at 4°C with 500 µl (1:5 v/v) diluted patient serum mixed with 500 µl CF antigens of each of *F. solani*, *F. equiseti*, and *F. moniliforme* species in different concentrations (5–500 µg/500 µl). For the positive control, an *F. solani* CF blotted strip was incubated with (1:10 v/v) diluted patient serum only. The strips were washed with PBS-Tween and then incubated with (1:1000 v/v) of peroxidase conjugated antihuman IgE (Sigma) for 4 h at 37°C. Other steps were the same as described above for immunoprinting.

The inhibition of individual bands of *F. solani* CF antigen by CF extracts of *F. solani*, *F. equiseti*, and *F. moniliforme* was assessed semiquantitatively (24), according to the following scale: no inhibition (0), partial inhibition with 500 µg inhibitor (1), total inhibition with 500 µg (2), total inhibition with 50 µg (3), and total inhibition with 5 µg (4).

Densitometry

Densitometric analysis was performed with a densitometric scanner (LKB, Pharmacia). The inhibition of the 14-kDa band, one of the most intensely immunoreactive components, was used to study the shared allergenicity among the three species of *Fusarium*. For the evaluation of results, the amount (µg) of each extract required for 50% inhibition was determined graphically by taking a peak area of the 14-kDa band in the control strip as 100%.

Results

F. solani CF was resolved into 37 antigenic components with rabbit antibodies raised against CF extracts of *F. solani* (Fig. 1a). However, *F. equiseti* CF and *F. moniliforme* CF exhibited 11 and 10 bands, respectively, with the same antibodies (Fig. 1b and c).

In ELISA inhibition, the concentration of *F. solani* CF antigen required to produce 50% inhibition was 0.40 µg, as compared with 1.1 and 7.0 µg for *F. equiseti* CF and *F. moniliforme* CF, respectively (Fig. 2).

The carbohydrate-to-protein ratios in CF extracts of *F. solani*, *F. equiseti*, and *F. moniliforme* were found to be 1.1:1, 0.8:1, and 1.3:1, respectively.

It was observed that CF extracts of *F. solani*, *F. equiseti*, and *F. moniliforme* contained 35–42 protein components on the IEF pI range 3–9 and the

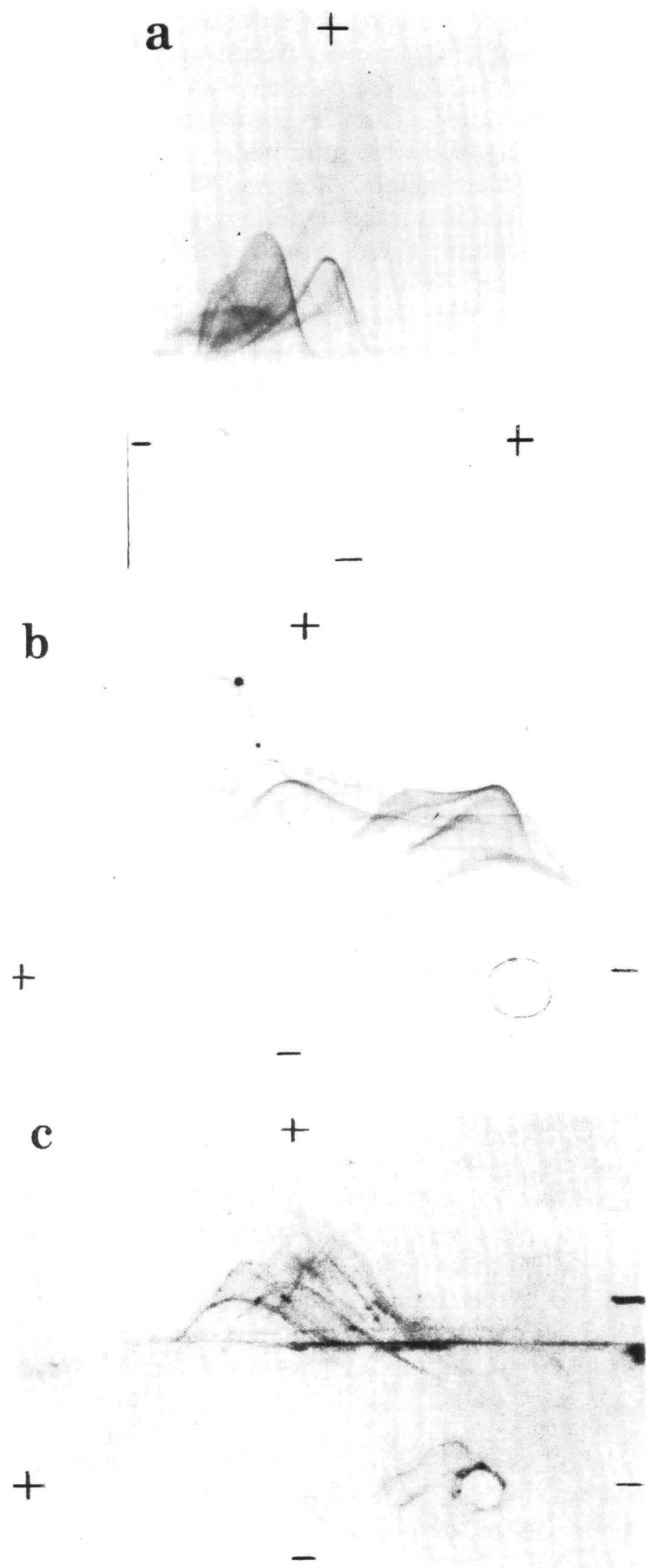


Fig. 1. CIE patterns of a) *F. solani* CF, b) *F. equiseti* CF, and c) *F. moniliforme* CF with rabbit anti-*F. solani* CF antibodies.

SDS-PAGE mol. mass range 14–100 kDa (Table 1).

Immunoprinting with pooled sera of *Fusarium*-sensitive patients revealed 18 IgE-binding proteins in *F. solani* CF with mol. mass ranging from 10 to

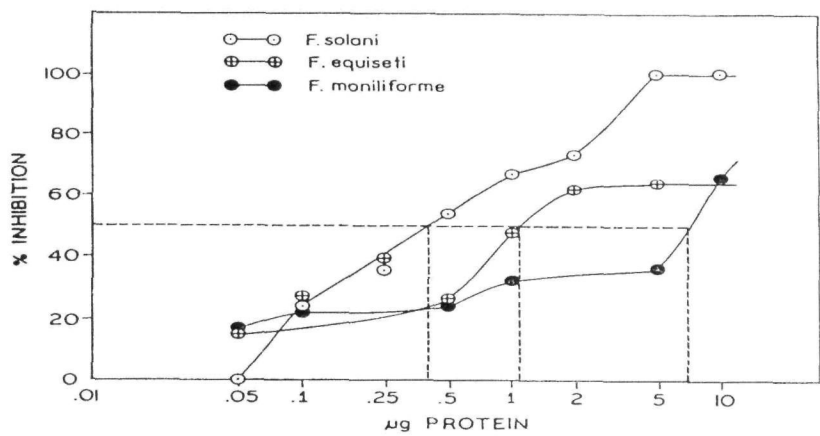


Fig. 2. IgG ELISA-inhibition, dose-response curves of CF extracts of (○----○) *F. solani*, (⊕----⊕) *F. equiseti*, and (●----●) *F. moniliforme* with rabbit anti-*F. solani* CF antibodies, with *F. solani* CF as solid-phase antigen, plotted on semi-logarithmic graph paper.

Table 1. Analysis of CF extracts of *Fusarium* species, giving different protein components separated on IEF, SDS-PAGE, and immunoblot

CF extracts	IEF pI (3-9)	SDS-PAGE (14-100 kDa)	Immunoblot with allergic serum pool
<i>F. solani</i>	42	37	18
<i>F. equiseti</i>	37	35	12
<i>F. moniliforme</i>	35	35	9

70 kDa. Similarly, 12 and 9 allergenic bands were identified in *F. equiseti* and *F. moniliforme* CF, respectively, in the same mol. mass range (Fig. 3; Table 1).

A number of allergenic components with mol. mass 14, 19, 35, 38, 45, 50, and 70 kDa were found

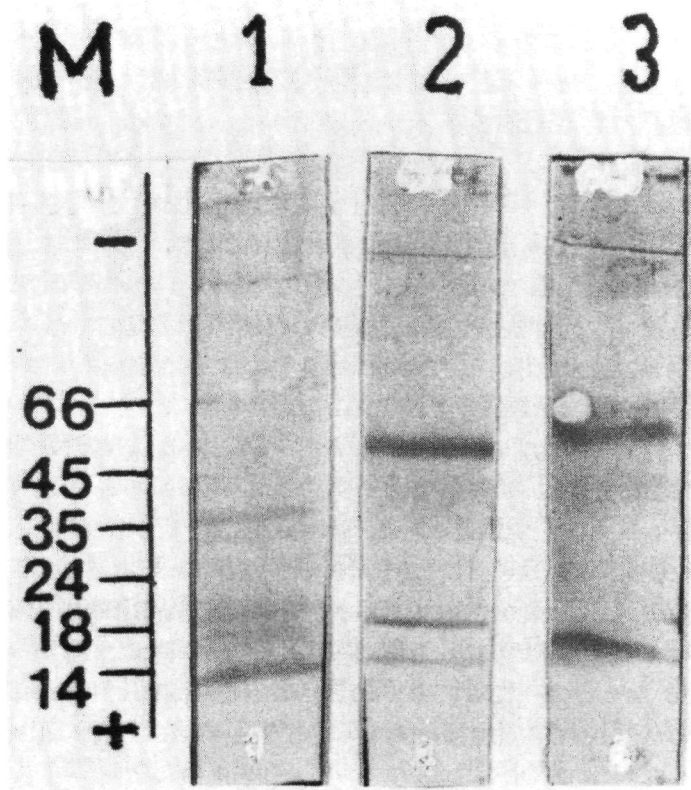


Fig. 3. Immunoprint analysis exhibiting IgE-binding components in CF extracts of 1) *F. solani*, 2) *F. equiseti*, and 3) *F. moniliforme* with pooled sera of patients allergic to *Fusarium*.

to be common among the three *Fusarium* species (Fig. 4). Of these, a 14-kDa allergenic band was seen as the most intensely immunoreactive band, indicating a high IgE-binding capacity of this protein in all three species.

A high degree of inhibition of immunoblots of *F. solani* CF with homologous antigen was observed, as compared with that with the heterologous antigens (Figs. 5 and 6). It was observed that different IgE-binding bands of *F. solani* CF on immunoprint could be inhibited with higher concentrations of CF of the other two species. *F. equiseti* CF required less protein to inhibit the binding of allergenic components of *F. solani* CF than did *F. moniliforme* CF. This indicated that *F. equiseti* CF antigen shared

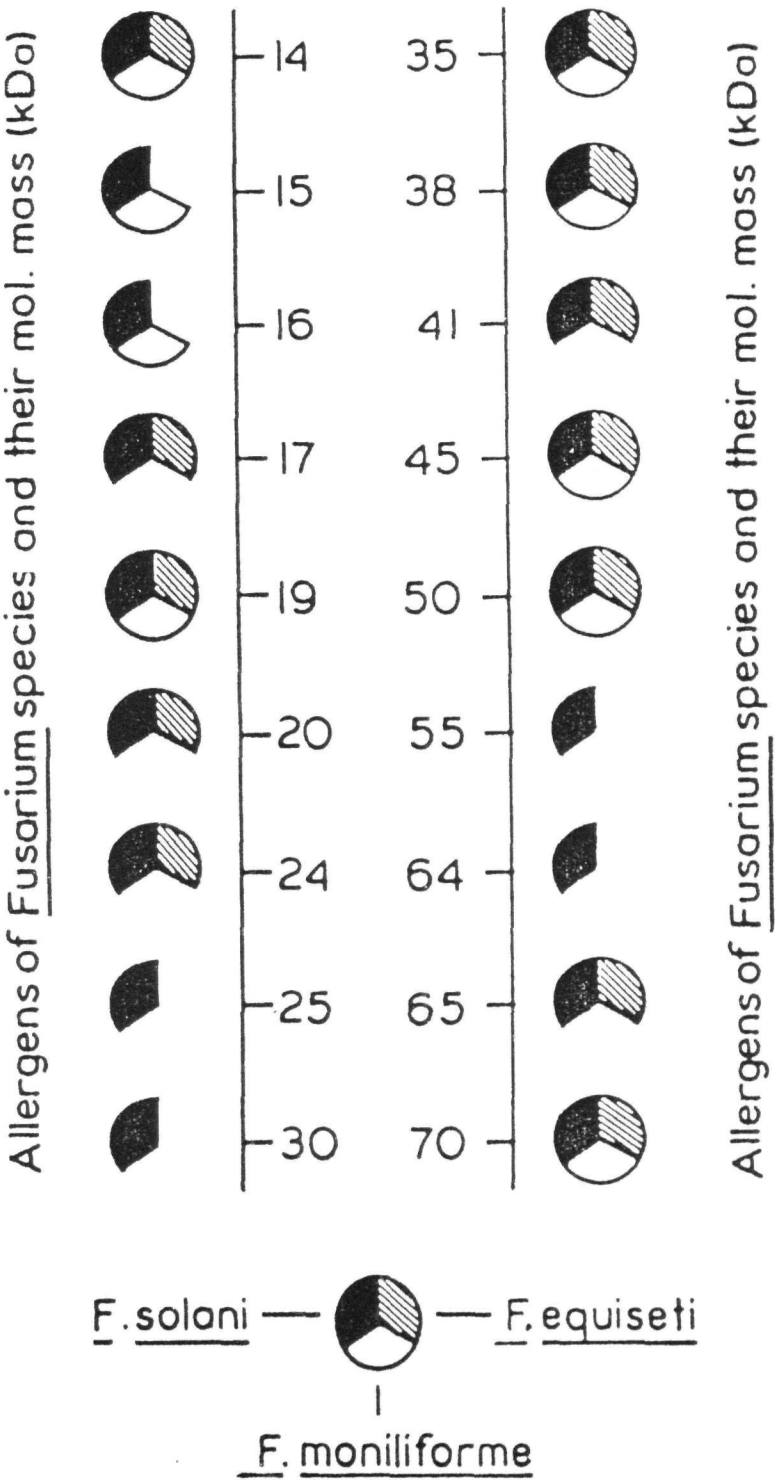


Fig. 4. Diagrammatic representation of shared and unique allergenicity in each CF protein of *F. solani*, *F. equiseti*, and *F. moniliforme*.

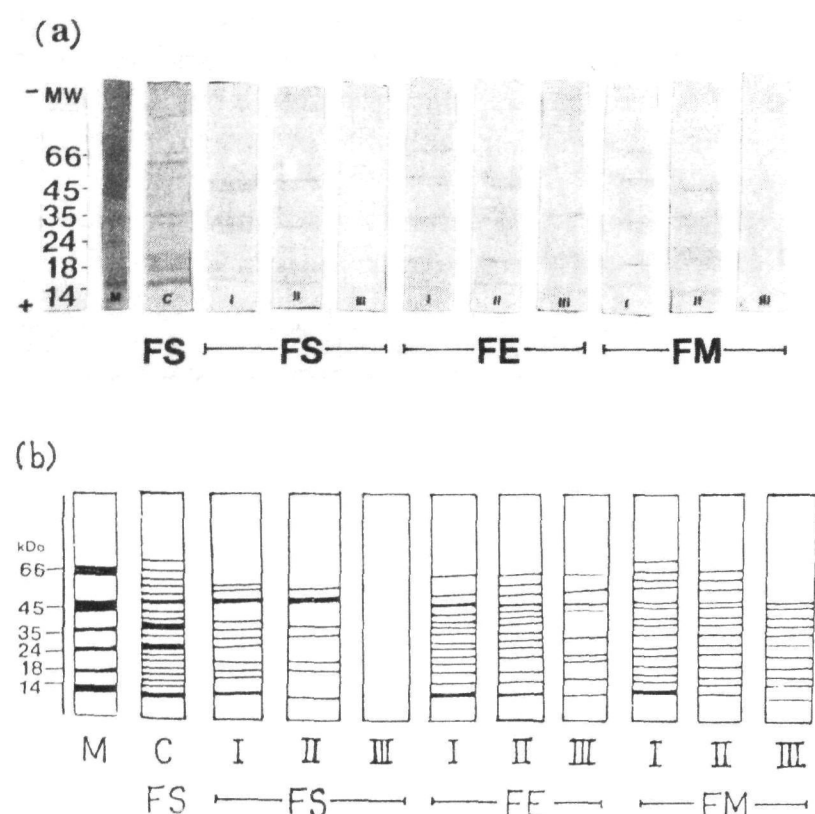


Fig. 5. a) Immunoprint inhibition analysis of *F. solani* CF by *F. solani* (FS), *F. equiseti* (FE), and *F. moniliforme* (FM) with 1:5 diluted allergic serum pool. In each set of inhibition, three different concentrations, 5 (I), 50 (II), and 500 µg (III), of antigen were used in the inhibition assay. b) Diagrammatic representation.

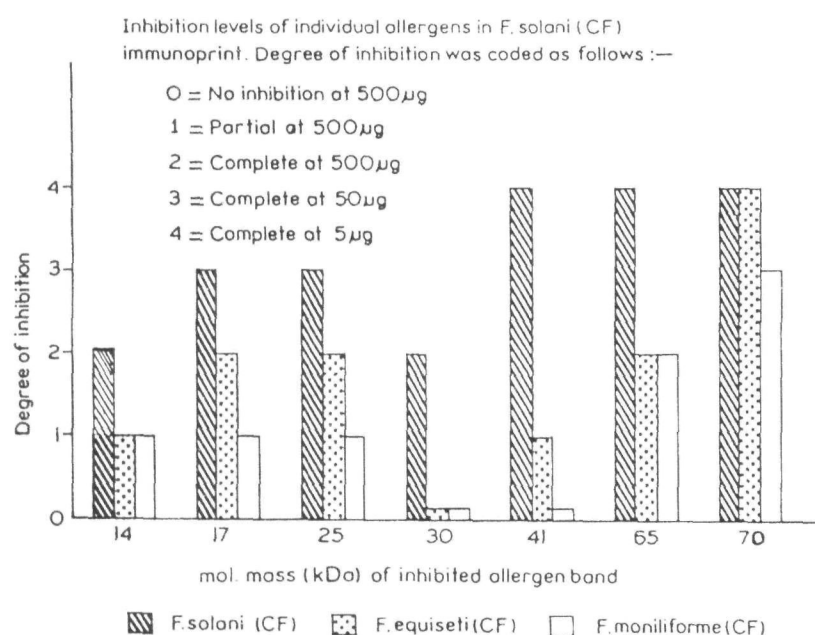


Fig. 6. Histogram showing inhibition levels of individual allergens in *F. solani* CF. Height of bar indicates degree of inhibition of *F. solani* allergens with *F. solani* CF, *F. equiseti* CF, and *F. moniliforme* CF.

more allergenic epitopes of *F. solani* CF antigen than those of *F. moniliforme*.

Dose-response curves for inhibition of the 14-kDa protein component of *F. solani* CF against CF antigens of *F. solani* and those of the other two species from densitometric scans were plotted (Fig. 7). It was observed that the concentration of *F. solani* CF antigen needed to produce 50% inhibition of band 14 kDa was 120 µg, as compared with 280 and

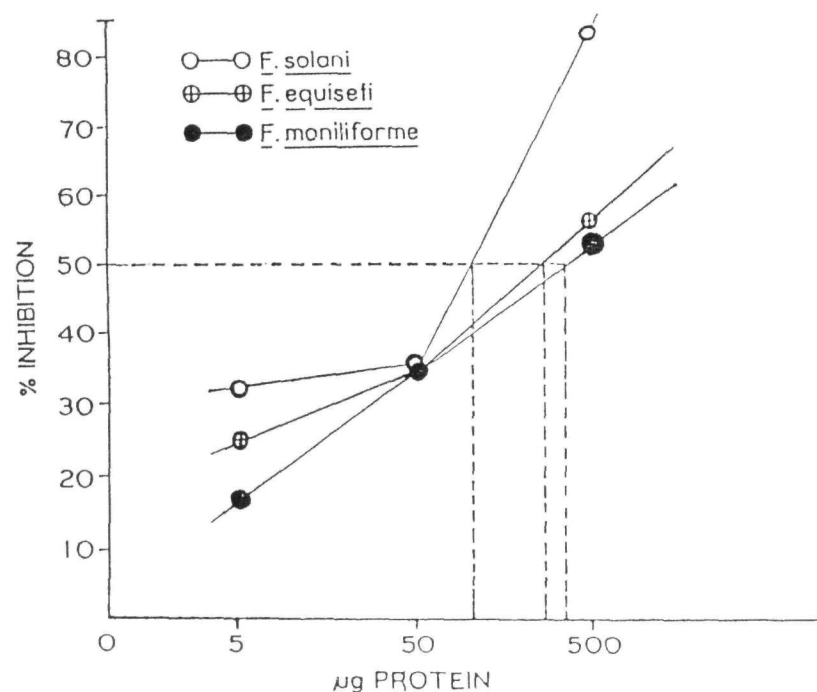


Fig. 7. Inhibition curves of band 14 kDa from *F. solani* CF by (○---○) *F. solani* CF, (⊕---⊕) *F. equiseti* CF, and (●---●) *F. moniliforme* CF. Percent inhibition was derived from analysis of peaks from densitometric scans of immunoprints.

360 µg of CF proteins of *F. equiseti* and *F. moniliforme*, respectively, confirming the shared allergenicity among the species of *Fusarium*.

Discussion

The genus *Fusarium* belongs to the class Deuteromycetes and the order of moniliales. It has been discovered that *F. solani*, *P. notatum*, and *Asp. glaucus* have several common antigenic/allergenic determinants (17), as shown by RAST inhibition, CIE analysis, and skin prick testing. Within the *Aspergillus* genus, *Asp. fumigatus* and *Asp. niger* appear to be allergenically distinct from *Asp. versicolor*, *Asp. nidulans*, and *Asp. glaucus* (12). Karr et al. (11) determined that although species of *Aspergillus* (*Asp. fumigatus*, *Asp. glaucus*, and *Asp. flavus*) cross-reacted extensively in RAST inhibition, each species contained unique allergenic determinants. Similarly, in the genus *Cladosporium*, Honbo et al. (9) have demonstrated shared antigenic determinants between *C. bantianum* and *C. carrionii*, but these species were found to be antigenically distinct from *C. herbarum* and *C. cladosporioides*. Both the species cross-reacted extensively in gel-diffusion methods. In these studies RAST inhibition, skin prick test, immunodiffusion, and CIE methods showed the total biologic/immunologic cross-reactivity without giving an idea of individual allergenic components.

The present study, by the immunoprint inhibition method, shows the degree of cross-reactivity among three species of *Fusarium*. Zubiria et al. (24) have also studied allergenic cross-reactivity among six Basidiomycetes species (*Calvatia cyathiformis*, *Coprinus quadrifidus*, *Psilocybe cubensis*, *Pleurotus ostreatus*,

Ganoderma meredithae, and *Pisolithus tinctorius*) by an immunoprint inhibition method using proteins which were resolved on IEF pI (3.5–9.5), but in this study proteins of CF antigens of *F. solani*, *F. equiseti*, and *F. moniliforme* resolved on SDS–PAGE were used for the inhibition assay. Immunoprint analysis of three CF extracts with sera of *Fusarium*-positive patients showed that the mol. mass distribution of allergenic proteins was similar between 10 and 70 kDa with a number of common bands: 14, 19, 35, 38, 45, 50, and 70 kDa (Fig. 4).

Immunoprint inhibition of *F. solani* CF antigen by the antigens of the other two species showed that *F. equiseti* was more closely related to *F. solani* than *F. moniliforme* (Fig. 6). The degree of inhibition of IgE binding of 14-, 17-, 25-, 41-, 65-, and 70-kDa protein components of *F. solani* CF was stronger by *F. equiseti* than *F. moniliforme* under similar conditions.

There is evidence of shared allergenicity of the 14-kDa protein component among the species studied. This protein band was selected for semiquantitative densitometric analysis because it contains an IgE-binding epitope in all three species of *Fusarium*. Furthermore, there was enough separation between the 14-kDa band and other bands to distinguish the densitometric peak corresponding to the 14-kDa band from other bands. It was observed that at an antigen concentration of 50 µg, there was an almost equivalent inhibition of binding of the 14-kDa allergen (35%), indicating that the 14-kDa component could be common to all three CF extracts (Fig. 7). However, at a lower concentration (5 µg), the inhibition was 10–30%, and at a higher concentration (500 µg), *F. solani* CF inhibited the binding of the 14-kDa band completely while the other two species could inhibit only 50–60% of binding. This may happen because the concentration of 14-kDa-like component is different in other CF extracts, or inhibition of binding may occur because of the interplay of other components in the extracts with shared allergenicity.

ELISA inhibition and CIE were done with all three extracts of *Fusarium* species with rabbit antibodies raised against the CF antigen of *F. solani*. According to O'Neil et al. (17), *P. notatum* and *Asp. glaucus* showed three and two common antigenic determinants with *F. solani*, respectively, in CIE. But in our study *F. solani* shared 11 and 10 antigenic components with *F. equiseti* and *F. moniliforme*, respectively, on CIE analysis. ELISA-inhibition results also revealed that both *F. equiseti* and *F. moniliforme* cross-react with *F. solani*. The carbohydrate and protein ratios in all three species of *Fusarium* were virtually the same.

Thus, the present study provides strong evidence for a significant amount of allergenic cross-reactivity

among the three species of *Fusarium*. However, the unique allergenic components of both species do not show cross-reactivity with each other. This fact must be taken into account when one uses extracts of *Fusarium* in diagnosis or immunotherapy of allergic patients.

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