Original Article

Allergenic extracts from *Metarhizium anisopliae*: Obtainment and characterization

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Abstract. *Metarhizium anisopliae* is used as a biopesticide for insects that damage agricultural plantations like sugar cane and forage plants. In a previous study the sensitization to this fungus of asthmatic patients coming from sugar cane areas was showed. The aims of this work were: to compare crude extracts obtained with Tris-HCl and Coca liquid from several growth phases of *M. anisopliae* concerning the total content of proteins and their electrophoretic analysis profile; to evaluate *in vivo* allergic sensitization in Balb/c mice and allergic patients from a sugar cane area, and to characterize the allergenic fractions in the sera of patients positive for the prick test by means of Western-blotting. The extract obtained with Coca liquid on the 16th day was the one that presented the greatest number of proteic fractions, including all those present in the other extracts. Twelve fractions were verified in this extract with approximate molecular weights from 94 to 14 kDa. The allergenicity of the extract obtained on the 16th day was proven by the production of IgE antibodies in Balb/c mice, with titres of 200. Prick tests carried out with the extract of the 16th day in 79 atopic individuals (from sugar cane area), 35 atopic individuals (from urban area) and 11 non- atopic individuals showed respective positivity of 29%, 9% and 0%. The allergenic characterization *in vitro* was performed by means of Western blotting, and the fractions that reacted with the positive individuals' sera were those of approximate molecular weights of 67 kDa (95%); 20 kDa (55%); 94 kDa (36%); 34 and 36 kDa (23%); 43 and 48 kDa (14%); 16 kDa (9%) and 54kDa (5%). It was concluded that the crude allergenic extract, obtained with Coca liquid from the 16th day growth of *Metarhizium anisopliae*, contains allergenic fractions and can be used in diagnostic screening tests.

Key words: *Metarhizium anisopliae*, extracts, allergen, Balb/c mice.

Introduction

Metarhizium anisopliae, filamentous fungus of the Deuteromycotina subdivision, is found in the soil and commonly parasiting on insects. Amongst 700 species of entomopathogenic fungi [1], this fungus is considered one of the most important biological agents used in the control of insects that damage agricultural plantations of economic value as sugar cane and forage plants. Moreover, it has also been used in the control of cockroaches in closed places [2], and the possibility has recently been verified of its performance on eggs of *Bemisia* sp, a white fly, plague that causes losses of 40 to 70% of agricultural plantations at a world-wide level [3] and also in the control of *Psoroptes ovis* mites, a rabbit parasite [4].

The wide use of this fungus in the biological control of leaf and root hopper that attack sugar cane, besides its natural occurrence in regions that deal with this type of culture, added to the detection of asthmatic patients who live in those regions, have led to research on the possibility of this fungus to be an etiological agent of bronchial asthma. Allergenic extracts have been prepared with one strain of this fungus and 50 asthmatic patients from sugar cane regions of Sao Paulo State, Brazil, who were submitted to prick tests. Of these patients, 8

presented with strong positive reactions, and sensitisation was shown in 3 of them by means of the bronchoprovocation test [5].

Recently, two cases of sinusitis in 6 immunocompetent patients were reported [6], and one case of hyphamycotic rhinitis in a cat [7]. Ward *et al.*, in series of studies [2, 8, 9], have carried out crude allergenic extract inoculations, obtained from a *Metarhizium anisopliae* strain, into Balb/c mice and have shown that this extract contains components that induce immunological, inflammatory, histopathological response, the characteristics of allergy.

In the course of the pioneering work showing the sensitization of individuals to that fungus [5] and the necessity of allergenic extract obtainment with standardization adjusted for use in diagnostic selection tests, this research aims: to compare crude extracts obtained with Tris-HCl and Coca liquid from several growth phases of *M. anisopliae* concerning the total content of proteins and their electrophoretic analysis profile; to evaluate *in vivo* allergic sensitization in Balb/c mice and allergic patients from sugar cane areas, and to characterize the allergenic fractions in the sera of patients positive for the prick test by means of Western-blotting.

Material and methods

Fungus strain

A sample of *Metarhizium anisopliae* strain, kept on agar-potato medium added with neutral mineral-oil at the mycotheque of the Laboratory of Mycology - Department of Microbiology (ICB -USP) under n° K-1-45, since 1987, was used in the experiments.

Obtainment of the fungal extracts:

For obtaining the extracts, 3 points were chosen corresponding to the logarithmic phase (16th day) and the stationary phases (24th and 32nd day) of fungus growth. These phases were detected through the previously sketched growth curve.

Inoculum was prepared in the following way: with the aid of a platinum wire loop, portions of fungus kept on modified Czapeck agar [10] were placed into 5 tubes containing 5 ml of Czapeck broth and incubated at 25^0 C for 5 days. After this period, the mycelial mass in suspension was ground and adjusted in the spectrophotometer with wave length of 550nm for absorbance between 0.150 and 0.168. Five ml of standardised inoculum were placed in Roux bottles containing 200 ml of Czapeek broth. The bottles were kept at 25^0 C, and after 16, 24 and 32 days of fungus growth, the total culture of 10 bottles for each extraction day was filtered through Whatman Nr/3.0 mm filter paper. The retained fungal mass was desiccated in a drying oven at 45^0 C until total desiccation; part of the mass was then submitted to the extraction with Coca liquid [11] and part of it with TRIS-HCl [10].

Biochemical analysis of the extracts

The total proteic content of the obtained crude extracts was evaluated according to Lowry *et al*. method 1951 [12], and fractioned by electrophoresis on polyacrylamide gel (SDS-PAGE) according to the technique described by Laemmli (1970) [13].

Allergenic evaluation of the extract

In laboratory animals

The induction capacity of IgE antibodies was verified in Balb/c mice ancestry, through inoculation of the extract obtained with Coca liquid on the 16th day of fungus culture, according to immunization protocol based on previous experience [14].

For antibody induction, 8 male mice 35 days of age were used. The inoculation was performed by intraperitoneal injection of 50 mg of the extract + 7.5 mg of aluminium hydroxide, and 5mg of the extract on the 28th day (booster).

After inoculation of the extract, bleedings were performed on the 28th and 35th day through punction of the retro-orbital plexus with a heparinized pipette. The blood extracted was diluted in PBS at ratio 1:1 and centrifuged for 5 minutes at 1,500 rpm. The plasmas were harvested and stored at -20° C.

The IgE content of the pool of the obtained plasmas was dosed by the reaction of Passive Cutaneous Anaphylaxis (PCA) in three Wistar rats [14, 15].

In allergic patients:

Prick tests were carried out [16] in three groups of individuals: group I: 79 atopic patients from the sugar cane area; group II: 35 atopic patients from an urban area, and group III as control consisting of 11 non-atopic individuals.

Blood was collected from patients with positive prick test, according to Lombardero *et al.* criterion, 1986 [17], for the allergenic evaluation performed by Western-blotting reaction [18]. The antiserum used in the immunoblotting was the anti-human IgE labeled with peroxidase.

Results

Biochemical Evaluation of the Crude Extracts

Table 1 shows the total protein dosages in the crude extracts. In the extractions with Tris-HCl, the maximum proteic content was obtained from the extraction of the 24th day, and in the extractions with Coca liquid, the

Table 1. Total proteins (mg/ml) of extracts of *Metarhizium anisopliae* obtained by extraction with Coca liquid and Tris-HCI from the fungus growth in Czapeck broth, modified by YUNGINGER et al. (1980).

Growth time	Tris HCI	Coca
16th day	0.257	0.405
24th day	0.321	0.380
32nd day	0.241	0.250

one of the 16th day was used. The proteic content was higher in the extractions carried through with Coca liquid.

Figure 1 shows the electrophoretic profile of the crude extracts, obtained in the different fungus growth phases with the two extracting liquids and the respective approximate molecular weights.

The presence of 8 fractions with approximate molecular weights of 94, 67, 48, 43, 36, 34, 30 and 14 kDa was verified in the extractions with Tris-HCI. These were also verified in the extractions with Coca liquid carried out on the 16th and 24th days.

In the extractions with Coca liquid, the largest number of fractions was achieved on the 16th day of the fungus growth, 12 of these with approximate molecular weights of 94, 67, 54, 48, 43, 36, 34, 30, 26, 20, 16 and 14 kDa. In addition, 11 fractions within this range of molecular weights were observed on the 24th day and 6, on the 32nd day of growth.

As a function of these results, the extract obtained with Coca liquid on the 16th day was chosen for allergenic evaluation.

Allergenic evaluation of the crude extract

In laboratory animals:

Figure 2 shows the IgE titration in Balb/c mice. The primary response at the first bleeding (28th day after the inoculation) presented low IgE titres (seven). However,

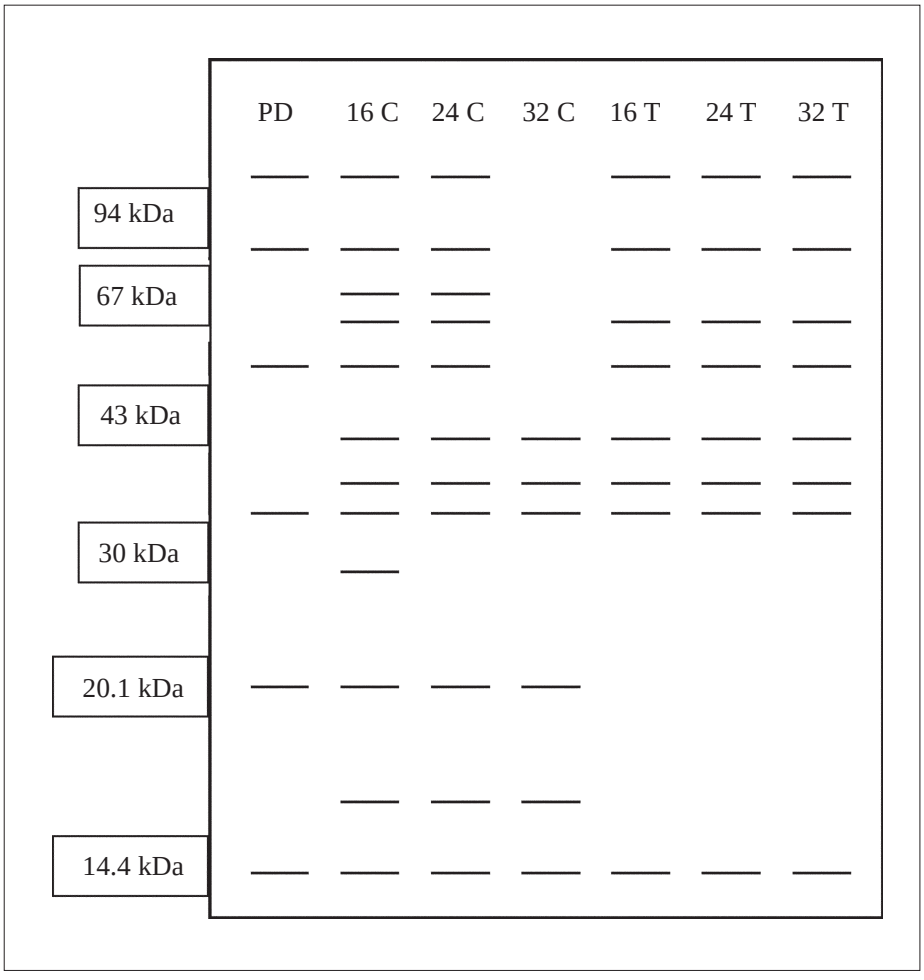

Figure 1. Electrophoretic profile of crude extracts of *Metarhizium anisopliae* obtained by extraction with Coca liquid (C) and Tris-HCI (T) on different days of growth (on the 16th, 24th and 32nd day) in Czapeck broth, modified by Yunginger et al. (1980).

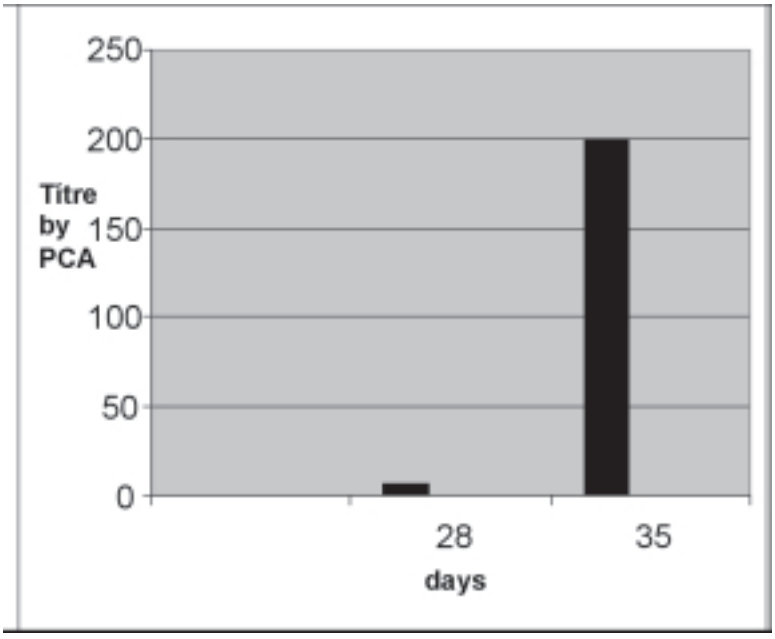

Figure 2. Titre of IgE antibodies dosed by passive cutaneous anaphylaxis (PCA) at primary (on the 28th day) and secondary (on the 35th day) responses in inoculated Balb/c mice with crude extracts obtained with Coca liquid, on the 16th day of growth of *Metarhizium anisopliae* in Czapeck broth, modified by Yunginger *et al*. (1980).

Table 2. Results of prick tests carried out with *Metarhizium anisopliae* extract obtained with Coca liquid, from the 16th day growth of the fungus in Czapeck broth, modified by Yunginger et al. (1980), in atopic and non-atopic patients grouped according to their clinical symptoms.

Symptoms	**Group I**			**Group II**			**Group III**		
	+	**-**	**T**	**+**	**-**	**T**	**+**	**-**	**T**
Asthma	1	11	12	0	1	1	0	0	0
Rhinitis	15	36	51	3	31	34	0	0	0
Asthma + rhinitis	7	9	16	0	0	0	0	0	0
Without symptoms	0	0	0	0	0	0	0	11	11
Total	23	56	79	3	32	35	0	11	11
%	29	71	100	9	91	100	0	100	100

+ = positive
- = negative
T = Total of patients
Group I: atopic individuals from the sugar cane area
Group II: atopic individuals originating from the urban area
Group III: control (non-atopic individuals).

the secondary response (after booster performed on the 28th day) presented a significant increase of IgE, with a titre of 200.

In allergic patients:

Table 2 shows the results of the prick tests performed with the extract collected on the 16th day of fungal growth. Of group I (79 atopic patients from sugar cane area), 23 (29%) were positive for the prick test, and of these 1 presented with asthma, 15 with rhinitis, and 7with asthma and rhinitis. Of group II (35 atopic individuals living in an urban area), only 3 (9%) were positive for the prick test, and all of them presented with rhinitis. Of group III (the control consisting of 11 asymptomatic individuals) all were negative at the prick test.

The allergenic evaluation of the sera obtained from the individuals belonging to each group was performed

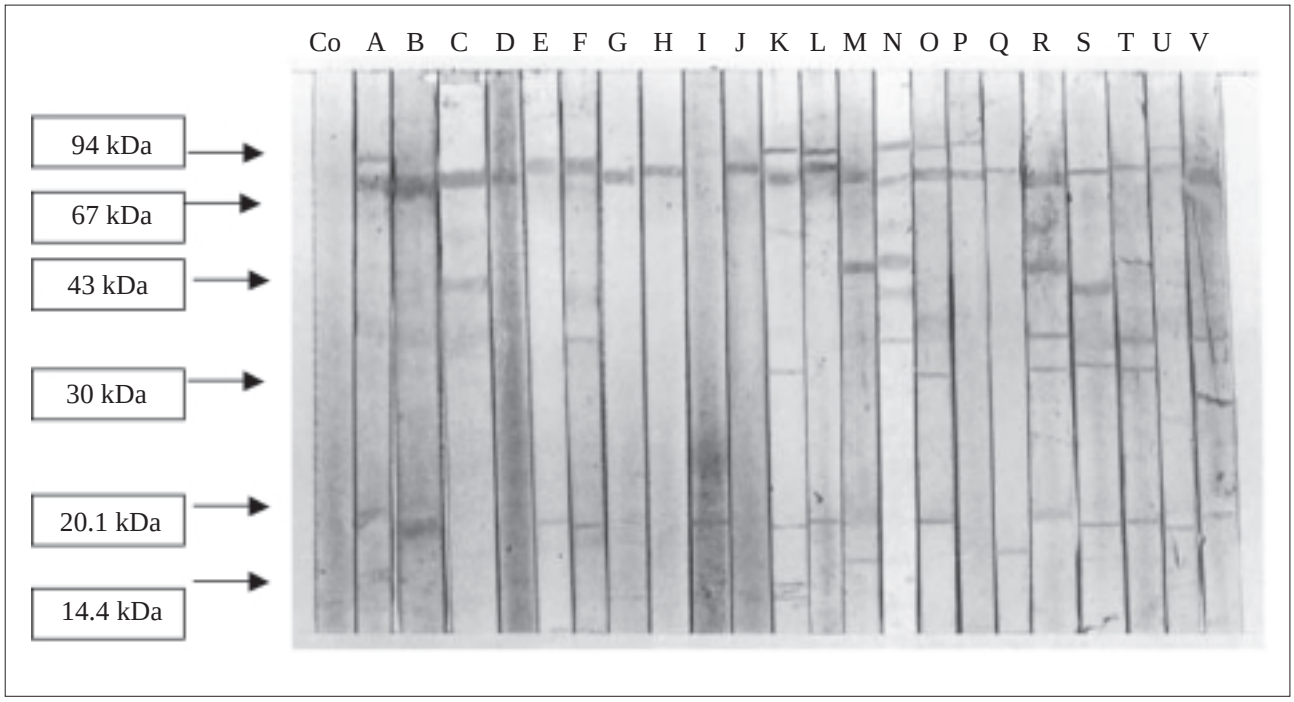

Figure 3. Western blotting of extract obtained with Coca liquid from the 16th day of *Metarhizium anisopliae* growth on nitrocellulose ribbons incubated with sera of 22 atopic patients and one control.
A-M, O and R-V - Group I: atopic individuals from sugar cane area, positive for the cutaneous test.
N, P and Q - Group II: atopic individuals from urban area, positive for the cutaneous test.
Co - Group III: Control (non-atopic individuals).

by the Western blotting technique (Figure 3). Table 3 shows the results of the observed reactive fractions. In group I, 18 (95%) patients sera reacted with the fraction of approximate molecular weight 67 kDa; 12 (63%) reacted with the 20 kDa fraction, from which only one (patient I) did not react concomitantly with the 67 KDa fraction. Of the 7 sera, negative for the 20 kDa fraction, one (patient A) reacted with the 94 kDa fraction, one (patient C) with the 43 kDa fraction, one (patient M) with the 48 and 16 kDa fractions, and the other four (patients D, G, H and J) did not react with any other fraction, besides the 67 kDa one.

In group II, 3 sera presented reactivity with the 67 kDa fraction, and one of them also reacted with the 36, 43, 48 and 94 kDa fractions; another one with the 94 kDa fraction and the other with the 16 kDa fraction.

Five sera of control group III (63%) presented reactivity with the 67 kDa fraction and the other three did not present any reactivity with any fraction.

Discussion

Beyond the natural occurrence of *M. anisopliae* in the environment, its use in the biological control of plagues implies the introduction of great amounts of particles of this fungus in one determined surrounding, thus suggesting the possibility that sensitization occurs in individuals living in such environments. Based on this fact, Zuppi *et al.* (1990), in a pioneering work, studied 50 patients with asthma, all of them from sugar cane areas and verified, through cutaneous tests using crude extracts obtained by extraction with Coca liquid, the possibility of sensitization by this fungus. Of the 50 patients, 8 presented a strong positive reaction, and in 3 of these patients their sensitization was proven through bronchoprovocation tests [5].

The methodology used in this article was based on previous works that had shown the need to establish an adequate and differentiated extraction and detection methodology, and subsequent standardisation, of the allergenic fractions of each species exhibiting specific characteristics, as the general use methods are not appropriate [5, 14, 19-29].

Electrophoresis on polyacrylamide gel showed that the extracts obtained with Coca liquid exhibited a larger number of proteic fractions, and that some of them also appeared in extracts obtained with Tris-HCl. This fact was already verified in other comparative works [28-30]. The extract obtained from the fungus growth on the 16th day was the one that presented the highest number of fractions.

The number of proteic fractions observed by other authors in studies with other fungi is also quite variable.

Table 3. Reactive fractions (+) by the Western blotting technique with sera of atopic individuals positive for the prick test, and of control individuals, negative for the same test using extract obtained with Coca liquid from the 16th day of growth of *Metarhizium anisopliae* in Czapeeck broth, modified by Yunginger et al. (1980).

Fractions	16	20	34	36	43	48	54	67	94
Patients									
A								+	+
B		+						+	
C					+			+	
D								+	
E		+						+	
F		+		+				+	
G								+	
H								+	
I		+							+
J								+	
K		+	+					+	+
L		+						+	+
M	+					+		+	
O		+	+					+	+
R		+	+	+		+		+	
S		+	+		+		+	+	
T		+	+	+				+	
U		+						+	+
V		+		+				+	
N				+	+	+		+	+
P								+	+
Q	+							+	
Co1								+	
Co2								+	
Co3								+	
Co4								+	
Co5								+	
Co6									
Co7									
Co8									

A-V-Group I: atopic individuals from the sugar cane area, positive for the cutaneous test
N,P and Q - Group II: atopic individuals from the urban area, positive for the cutaneous test.
Co - Group III - Control: non-atopic individuals, negative for the cutaneous test.

In genus *Alternaria*, from 12 [31,32]up to 40 fractions from some strains were assigned [33]. In the same way, in *Cladosporium* between 10 and 15 fractions were assigned [34-36]; in *Aspergillus* spp., 10 fractions [37]; in *Penicillium* spp, 15 fractions [38]; in *Dreschlera monoceras*, 7 fractions [14]; in *Saccharomyces cerevisae*, 15 fractions [29]; and in *Pleurotus ostreatus*, 13 fractions [28].

Some of these works established the kinetics of fraction production. Thus, in *Dreschlera monoceras*, the greatest production occurred on the 28th day of fungus growth [14], whereas in *Pleurotus ostreatus*, vegetative phase, the greatest production occurred on the 40th day [28], and *in Saccharomyces cerevisae*, on the 10th day [29]. The majority of the works do not establish the kinetics of production of such fractions, and this is one of the factors that justify the variability found in allergenic extracts and many times the lack of important allergenic fractions.

On the basis of the preliminary results, the extract of the 16th day, obtained with Coca liquid, was chosen for allergenic evaluation in laboratory animals.

The titre obtained at the primary response was low. This fact had already been verified in previous work with extracts of *Dreschlera monoceras*, which also showed low titre at the primary response, however, at the secondary response and after booster the titres increased significantly and reached 1,280 [14], verified

with the extract of *Metarhizium anisopliae*. The titres obtained at the secondary response with the extract of the 16 th day were 200. Other authors, using the same methodology with extracts of *Cladosporium cladosporioides*, have verified titres of 160 [39].

The only works in literature referring to the titration of IgE induced by *M. anisopliae* have been carried out by Ward *et al.*, [2, 8, 9] who had used another protocol of sensitization in Balb/c mice and had obtained titres of 14.7 of IgE in the serum. The authors have suggested that the crude extract of *M. anisopliae* contains one or more powerful allergens.

The prevalence of positive tests observed in group I of atopic individuals inhabiting sugar cane areas was 29%, a significantly higher result than the one observed in group II, atopic individuals of urban areas, that was 9%. This result indicates a greater sensitization in the areas where *M. anisopliae* naturally occurs in the environment. It must be pointed out that all the individuals of group III (control) were negative for the prick test, showing that the extract used displayed a discriminatory power in this test. The total protein concentration of this extract was 4 mg/ml. In spite of recommendations (for biological extract standardization of fungi through puncture tests/ prick tests) of the use of the total proteic concentration of 2 mg/ml [40], the concentration of 4 mg/ml was chosen based on results of previous studies with extracts of other fungi [27, 29].

The percentual variation of allergies attributed to fungi generally occurs depending on many variables, such as the studied population, the fungi species used in the extract preparation, and the methodology used in this procedure [41, 42]. The utilization of extracts that are produced adequately, the selection of atopic individuals from places where the fungus in question occurs at high concentrations, all of this increases the prevalence of positivity of the prick tests, suggesting a possible sensitization of those individuals. In this sense, prick tests, performed with extract of *Hemileia vastathrix* in atopic individuals from coffee culture areas where this fungus occurs, have shown a prevalence of 14% of positive tests [43]. In the same way, a positivity of 28% in prick tests with extract of *Pisolithus tinctorius* – basidiomycete associated to eucaliptus – was obtained in atopic workers from this culture areas [27] and 42.2% of positivity with extracts of *Saccharomyces cerevisae* in atopic individuals from the sugar cane area [29].

The characterization of the allergenic fractions achieved by Western blotting technique also showed that practically all the patients' sera (Groups I and II) reacted to the 67kDa fraction, including 5 controls (Group III). This suggests inespecificity of this fraction. On the other hand, this fact may also be due to sensitization in these five control individuals. As a function of this, this fraction must be further investigated.

In group I, the 20 kDa fraction reacted with 63% of the patients' sera, suggesting its importance as allergenic fraction in the produced extract. In group II, all the positive sera for the cutaneous test did not react with the 20 kDa fraction, one serum reacted with the 16 kDa fraction, and the other two with other fractions.

Studies carried out with other fungi have shown the existence of some allergenic fractions. The positivity of 4 fractions with approximate molecular weights of 14, 35, 40 and 55 kDa [44] and of 3 allergenic ones with approximate molecular weights of 25, 40 and 66 kDa [31] was detected in extracts of *Alternaria alternata*. Five allergenic fractions with approximate molecular weights of 75, 60, 43f, 26 and 20 kDa [45], 3 allergenic ones of 43, 36 and 20 kDa [46], and 2 fractions of 64 and 48 kDa [47] had already been detected in extracts of *Penicillium notatum*. Fifteen allergenic fractions were detected in extracts of *Cladosporium herbarum* [48], and 3 allergenic fractions of 57, 26 and 49 kDa were identified in extracts of *Saccharomyces cerevisae* [29].

In view of the results obtained, *Metarhizium anisopliae* extract, prepared as per the established conditions, presents with one important allergenic fraction of an approximate molecular weight of 20 kDa, capable of detecting most individuals potentially sensitised to this fungus by means of the screening cutaneous test. Further studies should be carried out in order to best clarify the significance of these fractions and the actual involvement of this fungus in the respiratory allergies in our environment.

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