

Original article

Allergens of *Epicoccum nigrum* grown in different media for quality source material

Background: The *Epicoccum nigrum* (EN) extract used in allergy disorders exhibits batch-to-batch variations in protein composition and allergenic potency. In this study, the allergens of EN grown in different media were investigated.

Methods: EN was grown in five different nutrient media as stationary cultures at 25°C for 5–23 days. The growth pattern was characterized by measuring dry weight, protein and carbohydrate content. The antigenic and allergenic content of EN extract was evaluated with EN-positive patients' sera and antibodies raised in rabbit.

Results: The growth of EN in Czapeck Dox medium yielded insufficient material, while Sabouraud's broth with yeast extract (SBY) gave maximum spore-mycelial mass and protein content. Potato dextrose broth (PDB) and potato dextrose agar (PDA) showed higher dry weight and protein in 7–9-day cultures. SDS-PAGE resolved 26, 22, and 21 protein bands in EN extracts from cultures of day-13 SBY, day-7 PDB, and day-9 PDA, respectively. IgE/IgG immunoblots showed more allergenic (25)/antigenic (25) bands in EN cultured in SBY than in the others. Specific IgE ELISA and intradermal tests showed EN extract from day-13 culture in SBY to be the most potent.

Conclusions: The day-13 culture of EN in SBY was the most potent and may be selected for preparing EN extracts for diagnosis of allergy and future studies.

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Key words: allergens; antigens; *Epicoccum nigrum*; ELISA; immunoblot; intradermal test; media; standardization.

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Accepted for publication 8 November 1999

Airborne mold spores are widespread, outnumbering pollen grains in several places (1, 2). Many fungal spores have been recognized as important inhalant allergens inducing type I hypersensitive reactions in atopic individual (3). The prevalence of allergic diseases is reported to be 15–20%, of which more than 10% of those affected are sensitized to fungal allergen (4).

Epicoccum nigrum (EN) is a ubiquitous saprophytic mold with affinity for a damp environment and rotting vegetation (5). EN colonies on vegetation at the time of harvest have been found to cause respiratory allergic disorders in many predisposed individuals (5, 6). EN spores are considered an outdoor type, but in winter, in poorly ventilated damp houses, the indoor spore count may exceed that of outdoors (2, 5, 7). Various studies in India have reported 1–8% prevalence of this mold at different places in the months of December–May (8–13).

Fungal extracts are routinely used in clinical practice for the purpose of diagnosis and management of allergy disorders, but they are produced from insufficiently defined raw materials (14). A wide variation in the quality of EN extract has been reported by Portnoy et al. (15). The commercial EN extract detected sensitivity in 6% of cases, whereas the experimental

extract elicited positive response in 70% of cases. Various fungi such as *Cladosporium herbarum*, *Alternaria alternata*, *Aspergillus fumigatus*, *Fusarium solani*, and *Rhizopus nigricans* have been studied for the standardization of growth conditions to obtain reproducible allergenic potency (16–19). Dixit et al. identified allergens of EN extract from cultures in PDB by immunoblotting. They recommended the use of both spore and mycelium extract for skin testing/immunotherapy (5). In the present study, the growth characteristics of EN in different media have been investigated with parameters such as pH, dry weight, protein and carbohydrate content. Furthermore, the antigenicity and allergenicity of EN extracts prepared from cultures in different media were also studied.

Material and methods

Culturing of EN

EN (strain 4625, IARI, Delhi) was grown at 25°C in 500-ml flasks containing 100 ml of broth (medium) under stationary conditions. All flasks received 2 ml of inoculum containing 3×10^6 spores. Culture media such

as Sabouraud's broth (SB), Sabouraud's yeast extract broth (SBY), potato dextrose broth (PDB), potato dextrose agar (PDA), and Czapeck Dox medium (CDM) were used. At regular time intervals during cultivation, the spore-mycelial mass (surface growth) was harvested and utilized for measuring dry weight, protein and carbohydrate content, while the culture fluid was taken for pH measurement.

Preparation of extracts

The harvested spore-mycelial mass was washed with distilled water to remove the media contents and treated with alcohol overnight (12). The spore-mycelial mass was then lyophilized and pulverized. The fungal powder was suspended in 1:20 w/v of 125 mM ammonium bicarbonate buffer containing 5 mM ethylenediamine-tetraacetic acid (EDTA) and 1 mM phenyl methyl sulfonyl fluoride (5, 20, 21). The suspension was sonicated for 5 min with a pause every 15 s, and extraction was carried out for 3 h at 4°C (18). After extraction, the suspension was centrifuged at 15000 rpm for 30 min at 4°C. Either the protein in the supernatant was precipitated with 90% ammonium sulfate followed by dialysis (mol. wt. cutoff 3500) against distilled water, or the supernatant was dialyzed against ammonium bicarbonate buffer. The dialyzed extract was then aliquoted, freeze-dried, and stored at -20°C.

Protein and carbohydrate content

The protein content of different day EN samples was estimated by a modification of Lowry's method (22). The EN samples were analyzed for carbohydrate content by the phenol-sulfuric acid method (23).

Rabbit antiserum

Antiserum was prepared by immunizing rabbits (New Zealander strain) with 1:1 v/v diluted antigen (day-13 extract in SBY) mixture in Freund's complete adjuvant. An amount of 150 µg/0.5 ml was used for rabbit immunization. A similar volume of antigen mixture in incomplete Freund's adjuvant was used for monthly booster injections until a good antibody titer was obtained.

Intradermal (ID) tests and collection of patients' sera

EN extracts from SBY, PDB, and PDA media were subjected to intradermal tests on nasobronchial allergy patients (aged 18–43 years) at the Clinical Research Centre, V.P. Chest Institute, Delhi (India), by the method of Shivpuri & Agarwal (24). The different day culture (EN) extracts were selected on the basis of higher protein content/OD values in ELISA. The extracts (1:500 w/v, 0.01 ml) were injected intradermally into the forearm of the patient, raising a bleb of

2–3 mm. Phosphate-buffered saline was used as a negative control. The wheal size was measured after 20 min in comparison to the negative control. The ID reactions more than three times that of the negative control were classified as 2+, those 3–4 times that of the control with 1–2 pseudopodia as 3+, and those with multiple pseudopodia as 4+. Histamine diphosphate, 100 µg/ml, was also injected as a positive control, producing skin reactions in the range of 11–16 mm (graded as positive). Blood samples were collected from patients showing 2+ to 4+ skin reactions.

Enzyme-linked immunosorbent assay (ELISA)

The IgG- and IgE-binding capacity of different day extracts of EN was determined by ELISA (25). Briefly, microtiter plates (Nunc) were coated (1 µg/100 µl in carbonate-bicarbonate buffer, pH 9.6, per well) with different day extracts of EN grown in SBY, PDB, and PDA by incubation overnight at 4°C. After washing with PBS-Tween 20 (0.1%), the plate was incubated overnight at 4°C with 1:5 v/v diluted pooled patients' sera or 1:1000 v/v diluted rabbit antiserum in PBS. The plate was washed with PBS-Tween and incubated with 1:1000 v/v diluted antihuman IgE or antirabbit IgG peroxidase for 3 h at 37°C. After washing, the substrate *o*-phenylenediamine, hydrogen peroxide in citrate buffer, was added, and the plate was incubated at 37°C for 40 min. The color development was stopped with 5 N H₂SO₄ and the absorbance was read at 490 nm on an ELISA reader (Spectramax).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The different day extracts of EN (days 7–17) were electrophoresed on 12% resolving gel by the method of Laemmli (26). An amount of 200 µg of EN extracts from cultures in SBY, PDB, and PDA was resolved. The separated proteins were stained with Coomassie brilliant blue (CBB) R-250 or used for immunoblot analysis.

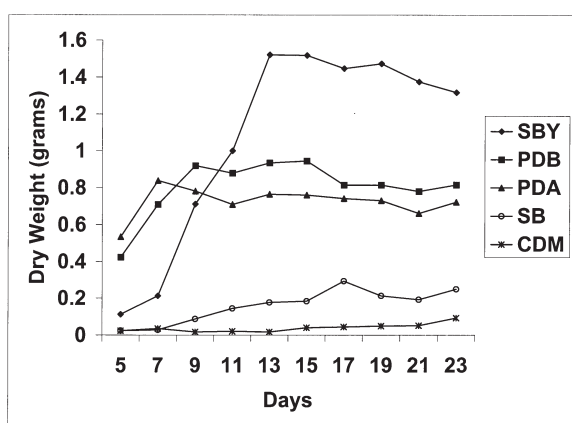
Immunoblot

SDS-PAGE separated proteins were transferred onto nitrocellulose paper using tris-glycine buffer with 25% methanol, as described by Towbin et al. (27). The nonspecific sites were blocked with 3% BSA in PBS and incubated overnight at 4°C with pooled patients' sera (1:5 v/v diluted in PBS) or rabbit antiserum (1:1000 v/v diluted in PBS). After washing with PBS-0.1% Tween 20, nitrocellulose strips were incubated with 1:1000 v/v diluted antihuman IgE/antihuman IgG/antirabbit IgG peroxidase. IgE/IgG-binding proteins were detected by substrate diaminobenzidine (28) with hydrogen peroxide in sodium acetate buffer (50 mM, pH 5).

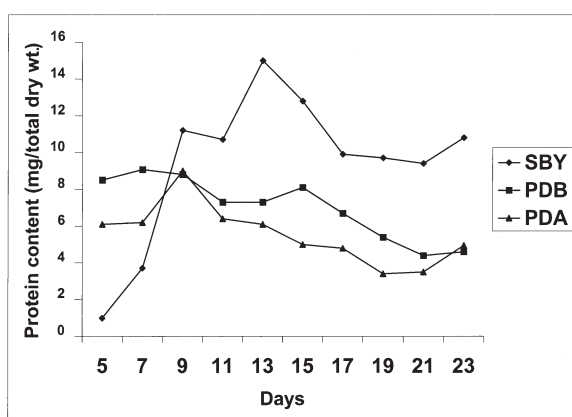
Results

The dry weight and protein content of spore-mycelial mass was higher in SBY, PDB, and PDA than in SB and CDM (Fig. 1A). Hence, SB and CDM were not included in further experiments. The protein content in spore-mycelial mass (Fig. 1B) continued to increase until day 13 of growth when EN was cultured in SBY, while it was maximum in cultures on days 7 and 9 in PDB and PDA, respectively. Quantitatively, the protein content was highest in day-13 culture of SBY. The carbohydrate content was highest in cultures of PDB as compared to SBY and PDA. The pH of the culture filtrate decreased during the early-growth phase of EN in different media, and this decrease was accompanied by an increase in dry weight and protein content (data not shown).

The allergenicity of different day EN cultures in various media was determined by specific IgE ELISA with an allergic serum pool. As shown in Fig. 2A, the day-11–13 extract from SBY, day-9 extract from PDB, and day-7 extract from PDA were most allergenic. The



A



B

Figure 1. Growth pattern of EN cultivated in different media. A) dry weight; B) protein content. SBY: Sabouraud's broth with yeast extract; PDB: Potato dextrose broth; PDA: potato dextrose agar; SB: Sabouraud's broth; CDM: Czapeck Dox medium.

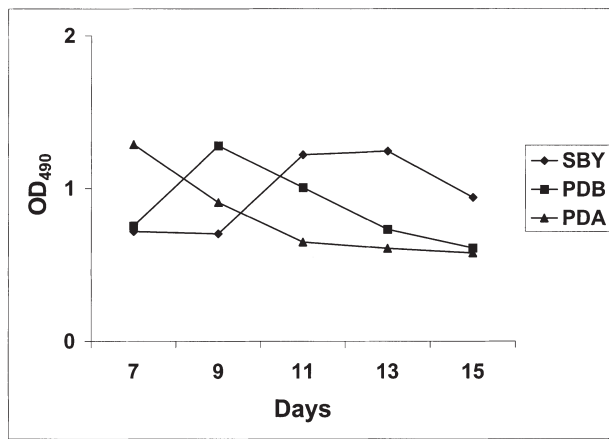
total yield of allergenic proteins was greatest in EN grown in SBY. Moreover, the day of harvesting the fungal material seemed to be critical in PDA and PDB. Intradermal tests were carried out with 7–15-day extracts of EN grown in SBY, PDB, and PDA. Out of 43 patients tested intradermally, three (6.9%) showed 2+ to 4+ skin response to EN extracts of day-7–15 cultures from different media. Skin reactivity (3+ to 4+) to extracts of day-13 SBY culture was highest, followed by day-7 SBY, day-9 PDB, and day 13 PDA. The antigenicity of EN extract using polyclonal rabbit antisera, as determined by IgG ELISA, was comparable in all the three media, although it was highest in day-13–15 extracts of EN grown in SBY (Fig. 2B).

The SDS-PAGE pattern of EN extracts, prepared from cultures grown in SBY, PDB, and PDA media, showed 26, 22, and 21 protein bands, respectively (Fig. 3A). The different day ammonium sulfate-precipitated extracts of EN cultivated in SBY showed an increasing number of protein bands until day 13 of growth. However, the lower molecular weight bands were faint or missing (Fig. 3B). The extracts of EN grown in PDB and PDA showed fainter and fewer bands after day 9 (data not shown).

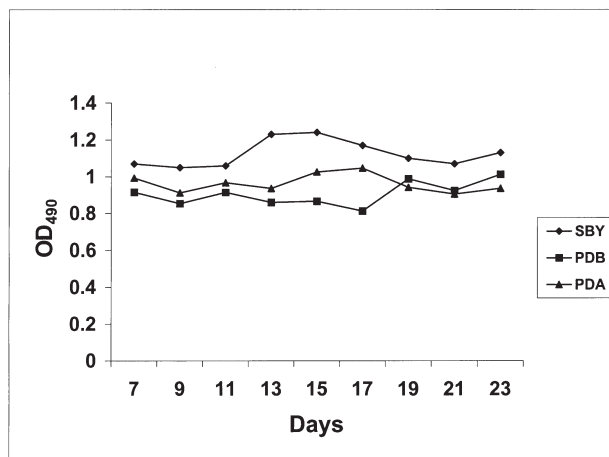
The allergenicity/antigenicity of EN extracts was further evaluated by IgE/IgG immunoblots. IgE immunoblotting exhibited 25, 18, and 10 allergenic bands, respectively, in extracts of EN grown in SBY, PDB, and PDA. Of the 25 allergenic bands in SBY, 18 reacted to human IgG (Fig. 4A, lane 1). IgG immunoblotting using anti-EN rabbit antibodies showed 25, 21, and 12 antigenic bands in EN extracts from day-13 SBY, day-7 PDB, and day-9 PDA cultures, respectively (Fig. 4B). Moreover, ammonium sulfate-precipitated extract of EN grown in SBY for day 13 showed a higher number of allergenic/antigenic bands on immunoblot than did extracts of other days (Fig. 5A and B).

Discussion

The knowledge of culture conditions is the first step toward the standardization of raw materials for fungal allergen extracts (3). Until now, the individual allergens of EN have not been identified, and the standardization requirements are unfulfilled. Hence, the present study was undertaken to develop standard culture conditions for source material (EN) with optimum antigenicity/allergenicity. Among the different media tested, Sabouraud's broth supplemented with yeast extract (semisynthetic medium) proved to be best for EN (Fig. 1A and B). The growth was quite favorable in PDB and PDA (natural media), as observed by Dixit et al. (5), but the profuse growth was accompanied by pigmentation of the spore-mycelial mass. Moreover, it was difficult to harvest EN cultivated in PDB and PDA because of the sticky nature of the media contents.



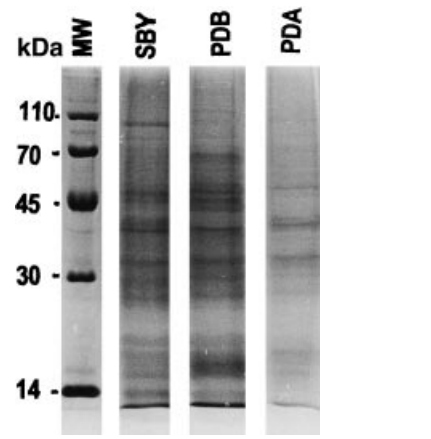
A



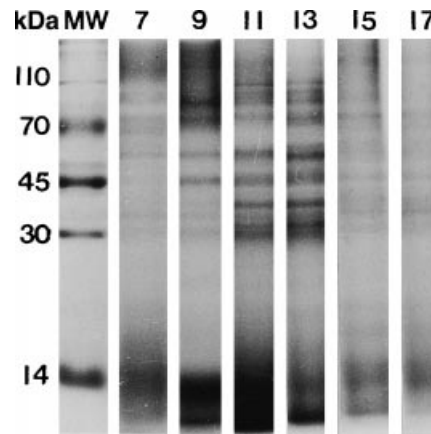
B

Figure 2. Allergenicity and antigenicity of EN extracts from following cultures: SBY: Sabouraud's broth with yeast extract; PDB: potato dextrose broth; PDA: potato dextrose agar. A) IgE ELISA using EN-positive pooled patients' sera; B) IgG ELISA using anti-EN rabbit antibodies. OD₄₉₀ represents absorbance value of color reaction obtained with antihuman IgE and antirabbit IgG peroxidase, respectively.

Similar results showing rapid growth without pigmentation were observed when *Alternaria* was cultured in Sabouraud's medium, as compared to growth in 2% malt extract (29). Pigmentation was associated with enhanced sporulation and precipitation of the allergens in growth medium (20). Probably for this reason, very faint and reduced number of protein bands were observed when EN samples grown beyond day 9 in PDB and PDA were resolved on SDS-PAGE (data not shown). High carbohydrate content in the sample(s) could also be the reason for faint bands, as it has been shown to interfere with CBB staining (30). However, there was a decrease in the number of protein bands after day 13 in SBY (Fig. 3B). This could be due to release of metabolites/allergenic components/intracellular protein into the culture filtrate during the later phase of growth (17). Similarly, the differences in the



A



B

Figure 3. SDS-PAGE showing Coomassie brilliant blue-stained bands. A) Crude extracts of EN from cultures in different media: lane 1) mol. wt. markers; lane 2) SBY – Sabouraud's broth with day-13 yeast extract; lane 3) PDB – potato dextrose broth day 7; lane 4) PDA – potato dextrose agar day-9 culture. B) Ammonium sulfate-precipitated extracts of EN for day-7–17 culture in SBY. MW: mol. wt. markers.

number of IgG/IgE bands observed on the immunoblot could be attributed to the same cause. Dixit et al. (5) observed 3–25 IgE-binding bands recognized by different patients' sera positive to EN, while, in this study, 10–25 IgE-binding bands were detected with pooled patients' sera. Kurup et al. (31) observed 15–20 precipitin bands with mycelial extract of *Aspergillus fumigatus* (AF), using anti-AF antibodies raised in rabbit, while Gupta et al. (32) observed 10–16 IgG-binding proteins with *Curvularia lunata* (CL) extract, using anti-CL antibodies raised in rabbits. However, in the present study, 12–25 antigenic bands with EN extracts were detected when anti-EN antibodies raised in rabbit were used for the immunoblot. It was also observed that lower molecular weight proteins appeared as faint bands on SDS-PAGE when the protein was treated with ammonium sulfate than with native extract (Fig. 3A and B).

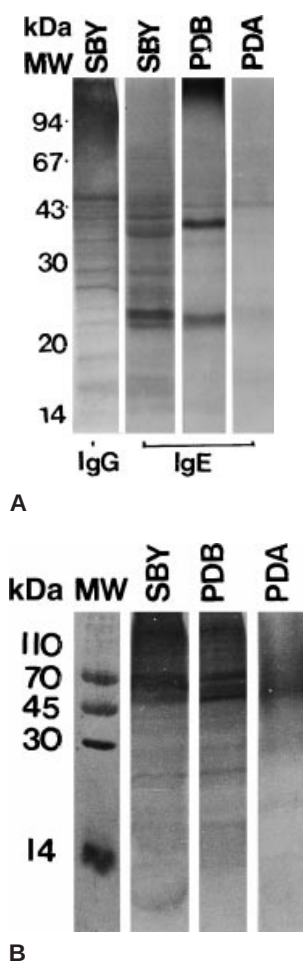


Figure 4. Immunoblots: A) IgG/IgE-binding components using anti-EN pooled patients' sera; B) IgG-binding components using anti-EN rabbit antibodies detected with EN extracts; SBY – Sabouraud's broth with day-13 yeast extract; PDB – potato dextrose broth day 7; PDA – potato dextrose agar day 9. MW: mol. wt. markers.

The influence of yeast extract was observed when EN was grown in SB. Absence of yeast extract slowed down the growth of the mold remarkably (Fig. 1). van der Heide et al. (17) also observed an incomplete growth cycle in the case of *A. fumigatus* and *P. notatum* cultured in the absence of yeast extract. Furthermore, higher IgG/IgE-binding capacity of mycelial antigens in yeast extract-enriched cultures of *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium* has been reported (33). In our study, EN showed scanty growth in CDM (synthetic medium). Other workers (32, 33) have also recorded the similar findings with cultivation of many fungi in synthetic medium. EN extracts grown in different media showed varying allergenic potency due to the nutrients available (media). Specific IgE ELISA showed EN extract from day-13 SBY culture to be more potent than other day extracts. This was further supported by ID testing. Agarwal & Shivpuri (12) reported 2+ to 4+ skin reactions to EN in 5.1% of the

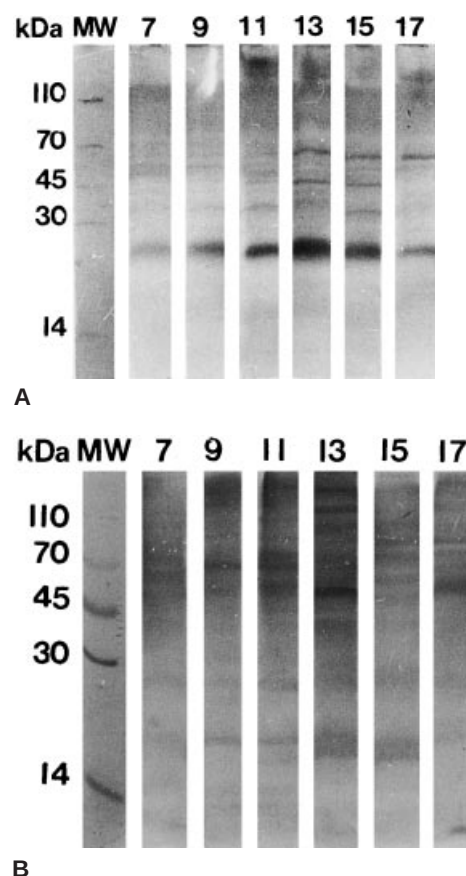


Figure 5. Immunoblots: A) IgE-binding components using anti-EN pooled patients' sera; B) IgG-binding components using anti-EN rabbit antibodies, detected on different days (7–17); ammonium sulfate-precipitated extracts of EN from SBY – Sabouraud's broth with yeast extract cultures. MW: mol. wt. markers.

patients, while 6.9% of subjects exhibited marked positive skin reactions in the present study.

This study describes the effect of culture media on the antigenicity and allergenicity of EN extract. The extraction of antigens was carried out in suitable buffer containing proteolytic inhibitors, so as to obtain the maximum number of proteins for IgE binding. Paris et al. (20) have also emphasized the importance of culture conditions and extraction procedure in obtaining good-quality allergen extracts. Recently, the number of biologically active recombinant allergens available for experimental, diagnostic, and therapeutic purposes has been increasing. However, until all the relevant allergens are cloned, native allergen extracts will continue to be used to determine patient sensitization and management of allergy disorders (34, 35). Valenta et al. (36) have suggested the use of standardized crude allergen extract for preliminary diagnosis that can be extended by the use of recombinant allergens to measure IgE levels directed against individual components if available.

Previous studies (5, 18, 32, 37, 38) with fungi suggest the use of 7–16-day cultures for the best representation of antigenic/allergenic proteins. Our study reports day-13 culture of EN in SBY to be the best in terms of allergenic content; hence, it may be used for diagnosis of fungal allergy, as well as for further studies.

Acknowledgments

One of us, V. Bisht, is the recipient of a CSIR (New Delhi) fellowship. We thank the Department of Biotechnology, Delhi, India, for financial support of this project. We also thank Mr S. K. Sharma, Centre for Biochemical Technology, Delhi, for his skillful technical assistance in culturing fungus and in animal experiments.

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