

Changes in atmospheric CO₂ influence the allergenicity of *Aspergillus fumigatus*

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Abstract

Increased susceptibility to allergies has been documented in the Western world in recent decades. However, a comprehensive understanding of its causes is not yet available. It is therefore essential to understand trends and mechanisms of allergy-inducing agents, such as fungal conidia. In this study, we investigated the hypothesis that environmental conditions linked to global atmospheric changes can affect the allergenicity of *Aspergillus fumigatus*, a common allergenic fungal species in indoor and outdoor environments and in airborne particulate matter. We show that fungi grown under present-day CO₂ levels (392 ppm) exhibit 8.5 and 3.5 fold higher allergenicity compared to fungi grown at preindustrial (280 ppm) and double (560 ppm) CO₂ levels, respectively. A corresponding trend is observed in the expression of genes encoding for known allergenic proteins and in the major allergen Asp f1 concentrations, possibly due to physiological changes such as respiration rates and the nitrogen content of the fungus, influenced by the CO₂ concentrations. Increased carbon and nitrogen levels in the growth medium also lead to a significant increase in the allergenicity. We propose that climatic changes such as increasing atmospheric CO₂ levels and changes in the fungal growth medium may impact the ability of allergenic fungi such as *A. fumigatus* to induce allergies.

Keywords: allergenic potency, allergens, *Aspergillus fumigatus*, climate change, environmental allergens

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Introduction

Increased susceptibility to allergies in the western world in the last few decades has caused increased suffering, higher medical expenses, and loss of working days (Devereux, 2006). However, the reasons for this apparent increase remain yet unclear (Bartra *et al.*, 2007). One explanation offered is that the cleaner modern environment reduces the immune system's exposure to microbes, leading to a reduction in its efficacy (Romagnani, 2004). Alternatively, it was suggested that environmental changes may result in physiological changes affecting the potency of allergy-causing biological agents (Cecchi *et al.*, 2010; Wolf *et al.*, 2010; Low *et al.*, 2011; Blando *et al.*, 2012; Pawankar *et al.*, 2012). Qualitative and quantitative changes in nutrient sources, temperatures during growth, relative humidity, air pollution, and other environmental factors could potentially lead to changes in the physiology of allergenic plants and microorganisms (e.g., fungal spores, pollen, animal product etc.), thus impacting the production of

allergenic proteins. For example, changes in atmospheric CO₂ levels can directly change the physiology and morphological characteristics of fungi and spores, through changes in the environmental acidity (Caldeira & Wickett, 2003; Oh & Richter, 2004), as well as in the respiration rate (Fitter *et al.*, 2000). Therefore, it is possible that environmental changes may modify the allergenic properties of the fungi as well. This can be caused by either changes in fungal growth, accompanied by the dispersal of different amount of allergy-inducing spores, or by changes in the content of allergenic proteins per spore (Low *et al.*, 2011).

The rise in atmospheric CO₂ concentration is well documented (IPCC, 2007), and has been linked to changes in both vegetation biomass and chemistry in many plant species (Ainsworth & Long, 2005; De Graaff *et al.*, 2006; Taub *et al.*, 2008; Hikosaka *et al.*, 2010). The responses of individual species to increased atmospheric CO₂ concentrations are variable, but often include higher total biomass production (De Graaff *et al.*, 2006) and higher carbon-to-nitrogen (C : N) ratios of plant tissues (Taub & Wang, 2008). Consequently, the carbon and nitrogen content of soils composed of decaying organic matter may be modified as well. The elevated nitrogen concentrations

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due to intensive crop fertilization in agricultural areas also contribute to changes in biomass nutrient composition (i.e., C : N ratio) (Ring *et al.*, 2011). Previous studies showed that climate change may influence these parameters, impacting the future risk of chemicals and pathogens on human health (Boxall *et al.*, 2008). Therefore, primary consumers, including fungi that grow on living or dead plant material, are likely to encounter changes in substrate quality or quantity as global atmospheric CO₂ levels and the extensive use of nitrogen-based fertilizers rise (Reich *et al.*, 2006), possibly leading to changes in protein production rates and activity.

The fungus *Aspergillus fumigatus* is ubiquitous in many environments with well-documented allergic (and other) effects (Horner *et al.*, 1995). This fungus grows on decomposed biomass (Latgé, 1999), and its asexual conidia (spores) contain different proteins, of which 23 can induce allergic response in sensitized patients (International Union of Immunological Societies Allergen Nomenclature Sub-Committee, 2008). We use this fungi species as a proxy for outdoor allergenic fungal spores, as it is ubiquitous in the atmosphere (Lang-Yona *et al.*, 2012) and other environments. In this study, we investigated the changes in the allergenicity of *A. fumigatus* spores due to increasing atmospheric CO₂ concentrations. Both direct exposure to changing CO₂ levels during spore growth and development, and growth in media with a varying range of C : N ratios were considered. We determined the allergenicity of the spores through two types of immunoassays. The first assay is a general allergenicity test, in which allergens extracted from *A. fumigatus* spores grown under different environmental conditions were tested against a mixture of IgE from sensitized patients. In addition, we used a specific Asp f1-monoclonal IgE, for estimating the concentration of the major allergenic protein Asp f1. We accompanied these tests with expression analysis of genes that encode for several selected allergenic proteins, and quantification of the relative amount of these proteins.

On the basis of our results, we hypothesize that changing environmental conditions such as atmospheric CO₂ levels due to global climate change, and the associated C : N ratios in the growth medium, will impact protein production rate and content in the fungal spores, leading to a subsequent changes in their allergenicity.

Materials and methods

Fungi growth conditions

To simulate environments with different atmospheric CO₂ levels, a constant amount of *A. fumigatus* (ATCC, #34506) spores was seeded on a rich growth media [potato-dextrose Agar, Difco (Franklin Lakes, NJ, USA) with chloramphenicol,

100 mg l⁻¹; Sigma-Aldrich (Rehovot, Israel)]. The fungi were exposed to constant environmental conditions (i.e., 60% relative humidity, darkness, room temperature), with controlled CO₂ levels (Gordon Gas Chemicals Ltd, Tel-Aviv, Israel) as detailed in Table 1. The initial concentrated CO₂ was diluted to the required treatment concentration using a dilution system of N₂ gas controlled by mass flow controllers (MKS instruments, Andover, MA, USA). The stabilized gas flow was introduced into the condition-controlled chamber, where the seeded agar plates were placed. The CO₂ concentration was measured using a LI-6400 Photosynthesis System (Licor Inc., Lincoln, NE, USA).

A consistent amount of *A. fumigatus* (ATCC, #34506) was grown on a synthetic medium with a defined amount of nutrients (Czapek growth media with chloramphenicol, 100 mg l⁻¹; Sigma-Aldrich) allowing control on the carbon and nitrogen (and hence the C : N ratio) during growth. Two approaches were employed: (i) Changing the carbon (sucrose, C source) concentrations, while keeping the nitrogen (Sodium Nitrate, N source) concentration constant; and (ii) Changing the N source concentrations, while keeping the C concentration constant. The average C : N ratio which represents natural conditions in decomposed organic mineral soils ranges from 9 : 1 to 12 : 1 (Cooke, 1968). We therefore chose 10 : 1 as a representative ratio. The growth medium's C : N ratio was further modified to the following ratios: 5 : 1, 8 : 1, 12 : 1, 15 : 1, estimating past and future conditions with respect to the change in CO₂ concentration.

Protein extraction procedure

For both proteomic analysis and the immunoassays, proteins were extracted as follows: Conidia from cultures grown on PDA plates, exposed to different CO₂ concentrations, or on Czapek media, with different C and N content, were har-

Table 1 The different CO₂ concentrations for each treatment in the CO₂ chamber experiment

Treatment	CO ₂ concentration (ppm)	Remarks
1	280	Preindustrial equivalent concentration
2	325	Atmospheric CO ₂ levels during the year of 1968 (Tans, 2008)
3	360	Atmospheric CO ₂ levels during the year of 1993 (Keeling, 1999; Tans, 2008)
4	392	Atmospheric CO ₂ levels during the year of 2010 (Keeling, 1999; Tans, 2008)
5	450	Future prediction for the year of 2030 according to the A1F1 model (Johns <i>et al.</i> , 2003)
6	560	Future prediction for the year of 2050 according to the A1F1 model (Johns <i>et al.</i> , 2003)

vested after 7 and 14 days of incubation, respectively, due to lower growth rate in the Czapek medium. Harvesting was performed using flocked, nylon fiber-tipped swabs (Copan Diagnostics, Murrieta, CA, USA). The collected spores were resuspended in 2 ml phosphate buffered saline with 0.05% Tween 20, pH = 7.4 (PBS-T; Sigma-Aldrich), and the conidia suspensions' concentration was determined by direct counts using a 2×10^{-5} ml hemocytometer at $\times 400$ magnification.

Extraction of the conidial proteins was as previously described (Low *et al.*, 2011). The whole-conidium extract obtained was subsequently used as the source for proteins that are responsible for the allergenic reaction and that were studied here. Briefly, glass beads were added to the 2 ml suspensions and spores were lysed by bead beating (Mini Beadbeater-16; BioSpec, Bartlesville, OK, USA) for 30 s. Lysed cells were centrifuged and the protein-containing supernatant was collected and stored at -80 °C until further analysis. Protein content was determined using the bicinchoninic acid assay (BCA; Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's protocol and averaged for three replicates.

Immunoassay tests

The relative allergenicity immunoassay was performed as described in Low *et al.* (2011) with some modifications. Briefly, human serum samples from *A. fumigatus*-sensitized patients (PlasmaLab International, Everett, WA, USA) were pooled before use. A 50 μ l volume of both the fungal spore protein extract from the different treatments, and the diluted pooled serum were mixed in V96 MicroWell™ polypropylene Plates (NUNC, Rockford, IL, USA), and incubated at room temperature on a plate shaker for 2 h. The mixtures were then transferred to MaxiSorp flat-bottom 96-well plates (NUNC) coated with 46 μ g ml⁻¹ of *A. fumigatus* spore extract and incubated for an additional 2 h at room temperature, enabling binding of the free IgE to the coating allergens. The amount of bound specific IgE was determined by additional secondary antibody (horse-radish peroxidase-conjugated mouse antihuman IgE, diluted 1 : 2000; SouthernBiotech, Birmingham, AL, USA) and chromogenic substrate (tetramethylbenzidine/peroxide substrate color developer II), followed by colorimetric detection at a wavelength of 450 nm (Synergy HT Multi-Mode Microplate Reader; BioTek, Winooski, VT, USA). The relative allergenicity of the spore extracts were defined by the following equation:

$$\text{Relative Allergenicity} = \frac{\text{OD}_{\text{serum}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{serum}}} \times 1000 \quad (1)$$

where OD_{serum} is the optical density of the clean human serum (with no spore sample addition), and OD_{sample} is the optical density of the human serum mixed with fungal spore sample. The mean value of at least three replicates gives the allergenicity for each treatment along with the associated standard error (SE). Allergenicity per spore value was determined plotting the observed allergenicity vs. spore concentration for each treatment. In this study, we defined this parameter as the total allergenicity, as it accounts for all allergenic proteins produced in a spore.

The concentration of the major allergenic protein Asp f1 per spore was evaluated through immunoassay using a commer-

cial ELISA kit (Indoor Biotechnology Ltd, cardiff, wales, UK), according to the manufacturer's protocol. In this assay, allergenic proteins extracted from the environmental samples or standards are bound to a specific monoclonal IgE. The binding signal is measured by the addition of colorimetric, enzyme-labeled IgE antibodies. The optical density was measured at 405 nm, and the Asp f1 concentrations were calculated from a calibration curve and normalized to the spore number of *A. fumigatus* for each treatment.

Gene expression analysis

Mycelia grown on PDA plates (Difco) under different CO₂ concentrations, and on Czapek media, with different C : N ratios, were collected after 48 h and 5 days of incubation, respectively, due to lower growth rate in the Czapek medium. The total RNA was extracted from ground cultures (performed by a mortar and pestle, under liquid nitrogen) using the RNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA) according to manufacturer's protocol. RNA concentration was determined by measuring absorbance at 260 nm (Nanodrop ND-1000 Spectrophotometer; Thermo Scientific, Wilmington, DE, USA), and the purity and integrity of the RNA were checked using A260/280 and A260/230 ratios from the spectrophotometric readings.

About 1 μ g of RNA from the different treatments was converted into first-strand cDNA using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Grand Island, NY, USA). The relative transcription levels of eight genes [including six encoding for allergenic proteins, found to induce allergy in more than 70% of *A. fumigatus*-sensitized patients (International Union of Immunological Societies Allergen Nomenclature Sub-Committee) (See Table S1)] were measured using real-time PCR (RT-PCR, StepOnePlus™ Real-Time PCR Systems; Applied Biosystems). The gene encoding for conidial hydrophobin RodB (a cell wall structural protein involved in the building of the conidial outer cell wall (Gautam *et al.*, 2008)) was chosen as a control for the sporulation stage of the fungi. Primer sets were designed and tested with the assistance of online primer design software (Primer 3, Table S2). The reagent Fast SYBR Green Master Mix (Applied Biosystems) was used, and the thermocycling conditions included an initial denaturation for 20 s at 95 °C, 40 cycles of 3 s denaturation at 95 °C, and 30 s for annealing and extension at 60 °C. Three replicates were carried out for each reaction, and negative no-RT-controls were performed on the source RNA to verify the absence of genomic DNA contamination. The endogenous reference gene 18S rRNA was used to normalize the quantity of RNA used, and the relative transcript levels (fold change) between the two sporulating cultures were determined using the 2^{- $\Delta\Delta$ CT} method (Livak & Schmittgen, 2001). The fold change was scaled for each gene by the sum of all treatments' change, termed as the relative fold change.

Proteomic analysis – label-free targeted analysis by selective reaction monitoring

Samples preparation included reduction of the extracted proteins by addition of dithiothreitol (Sigma-Aldrich) to a final

concentration of 5 mM and incubation for 30 min at 60 °C, and alkylated with 10 mM iodoacetamide (Sigma-Aldrich) in the dark for 30 min at 21 °C. The proteins were then digested using trypsin (Promega, Madison, WI, USA) at a ratio of 1 : 50 (w/w trypsin/protein) for 16 h at 37 °C. Digestions were stopped by addition of 1% trifluoroacetic acid. The samples were stored in –80 °C in aliquots.

For the liquid chromatography analyses ULC/MS grade solvents were used. Each sample was loaded using split-less nano-Ultra Performance Liquid Chromatography (10kpsi nanoAcquity; Waters Corp., Milford, MA, USA). Mobile phase was as follows: (i) H₂O + 0.1% formic acid; and (ii) acetonitrile + 0.1% formic acid. Desalting of samples was performed online using a reverse-phase C18 trapping column (180 µm i.d., 20 mm length, 5 µm particle size; Waters Corp.). The peptides in samples were separated using a C18 T3 HSS nanocolumn (75 µm i.d., 150 mm length, 1.8 µm particle size; Waters Corp.) at 0.4 µl min⁻¹. Peptides were eluted from the column and into the mass spectrometer using the following gradient: 3–30% B in 30 min, 30–95% B in 5 min, maintained at 95% for 7 min, and then back to initial conditions.

The mass spectrometry analyses were performed using nanoLC, coupled online through a nanoESI emitter (7 cm length, 10 mm tip; New Objective, Woburn, MA, USA) to a tandem quadrupole mass spectrometer (Xevo TQ-S; Waters Corp.). Data were acquired in selective reaction monitoring (SRM) using Masslynx 4.1 (Waters Corp., Milford, MA, USA). A list of all measured SRM transitions is detailed in the supporting information. Data were then imported into Skyline software (Maclean *et al.*, 2010; Stergachis *et al.*, 2011) for final processing and evaluation. Quantification was based on the area under the curve of extracted ion chromatogram from the most intense transition per peptide. At least three overlapping SRM transitions were acquired per peptide. For C-changed and N-changed media, we were able to identify 14 and 20 proteins, respectively, of the 23 known allergenic proteins of *A. fumigatus*.

Statistics

Data are presented as the mean ± SE of the mean (SEM). Statistical significance between groups was assessed using ANOVA with a Dunnett's post hoc test. $P \leq 0.05$ was set as the threshold for significance.

Results

Direct exposure to varying CO₂ levels

To examine the possible effect of changing environmental CO₂ concentrations, we chose CO₂ levels representative of the preindustrial era (280 ppm CO₂) up to double that level (560 ppm). Figure 1 shows the total allergenicity immunoassay results (Fig. 1a), Asp f1 concentrations (Fig. 1b), and PCR gene expression analyses (Fig. 1c) performed for fungi that grew under different CO₂ concentrations. The results indicate an increase in *A. fumigatus* allergenicity when cultured in the presence

of CO₂ concentrations from preindustrial CO₂ levels (280 ppm) to present CO₂ levels (392 ppm) followed by moderate decrease in allergenicity for higher CO₂ concentrations, as predicted for future climate (Keeling, 1999; Tans, 2008). The change was observed at all levels – from gene expression through protein abundance and actual allergenicity.

Both the general allergenicity (Fig. 1a) and the specific Asp f1 titer (Fig. 1b) peaked at approximately present-day CO₂ level (392 ppm). For total allergenicity, the value was not significantly different from that at 450 ppm value. However, the Asp f1 value at 392 ppm was significantly higher compared with all other conditions ($P = 0.0002$). In the gene expression analysis (Fig. 1c), the expression of six chosen allergen-encoding genes, along with that of a gene encoding for nonallergenic spore-specific protein (Rod B) is presented. The expression of the gene encoding for the Asp f1 protein (nomenclatures are detailed in Table S1) also peaked significantly at 392 ppm ($P = 0.0007$). All other allergenic encoding genes had maximum expression levels at 360 ppm, with statistical significance for Asp f11, 18, and 34 ($P = 0.0015, 0.0087, \text{ and } <0.0001$, respectively). Expression of the hydrophobin-encoding RodB gene was significantly higher at 260 and 392 ppm when compared with the other treatments ($P = 0.0003$). These results suggest that allergenicity (measured in this study per spore), as well as the sporulation efficiency is modified by atmospheric CO₂ concentrations. However, the reduced expression values above 392 ppm indicate an inhibitory effect that CO₂ can have on production and/or accumulation of allergenicity and sporulation factors by this fungus.

Exposure to a varying range of C : N ratios in the growth media

As carbon and nitrogen concentrations and their ratio can significantly affect fungal physiology and morphology, we further examined the possible influence of increased carbon source levels in the medium (due to increasing atmospheric CO₂ levels on the growth environment) on the allergenicity of *A. fumigatus* spores.

As can be seen in Fig. 2 (left panel), spores that grew on media with various C concentrations showed a consistent increase in the allergenicity values as the carbon (and the C : N ratio) increased, with a significant difference observed between the 5 : 1 treatment (0.61 g l⁻¹ carbon) to 8 : 1 and 10 : 1 (0.98 and 1.23 g l⁻¹ carbon, respectively), and between the last two treatments of 12 : 1 and 15 : 1 (1.47 and 1.84 g l⁻¹ carbon, respectively, $P = 0.0219$). The Asp f1 immunoassay also showed higher concentrations of this major allergen as the C : N ratio increased ($P = 0.0410$). For experiments

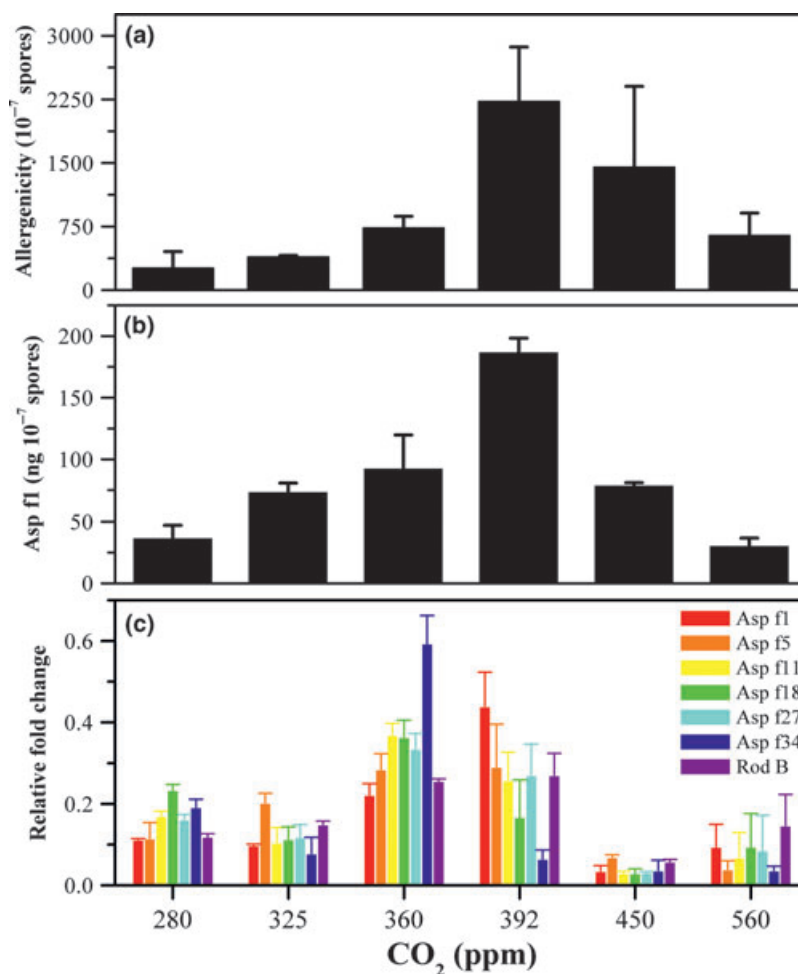


Fig. 1 The effect of changing CO₂ concentrations on the total allergenicity per 10⁷ spores of *Aspergillus fumigatus* (a), the major allergen Asp f1 concentration in ng per 10⁷ spores (b), and the gene expression by RT-PCR (c). The error bars represent the SE of the mean.

in which N was changed in the medium (Fig. 2, right panel), the allergenicity assay showed significantly higher values for the highest nitrogen concentrations (5 : 1 treatment, 10.16 g l⁻¹ Nitrogen) compared with the others ($P = 0.0273$), and in the Asp f1 assay, 5 : 1 and 8 : 1 (6.35 g l⁻¹ Nitrogen) treatments had significantly higher values compared with the others ($P = 0.0047$).

The observed changes in allergenicity are also reflected at the various stages of allergen gene expression. Similar to the experiments with varying CO₂ levels, we investigated the relative gene transcription using RT-PCR, presented in Fig. 3. Higher gene expression levels were observed for growth under increasing carbon concentrations. The expression of the Asp f1 gene positively correlated with increasing carbon concentrations in the growth medium, and a significantly higher value was detected for 15 : 1 treatment compared with the others ($P = 0.0006$). The gene expression of all other allergenic proteins, including hydrophobin Rod B but excluding Asp f34, peaked at

the 12 : 1 ratio. Asp f34 did not change significantly with respect to changes in carbon concentration. No significant trend could be identified for Asp f1, 5, 34, and Rod B genes ($P = 0.4726, 0.9518, 0.0745,$ and 0.8136 , respectively) for fungi grown under varying nitrogen concentrations. A significant decrease was observed in the 15 : 1 treatment (3.39 g l⁻¹ Nitrogen) for Asp f5, 11, 18, and 27.

In light of the N-altered medium results, suggesting that the observed increase in allergenicity for higher nitrogen concentrations may not be controlled at the transcription level, we performed proteomic analyses for the allergenic proteins in the experiments with varying C : N ratios. As can be seen in Fig. 4, for C-changed medium, all allergenic proteins reached the highest levels at the 15 : 1 treatment (0.84 g l⁻¹ carbon), consistent with the allergenicity test. Asp f1 protein showed a clear positive trend as the carbon levels increased, although not statistically significant at a 95% confidence threshold ($P = 0.0711$). For N-changed

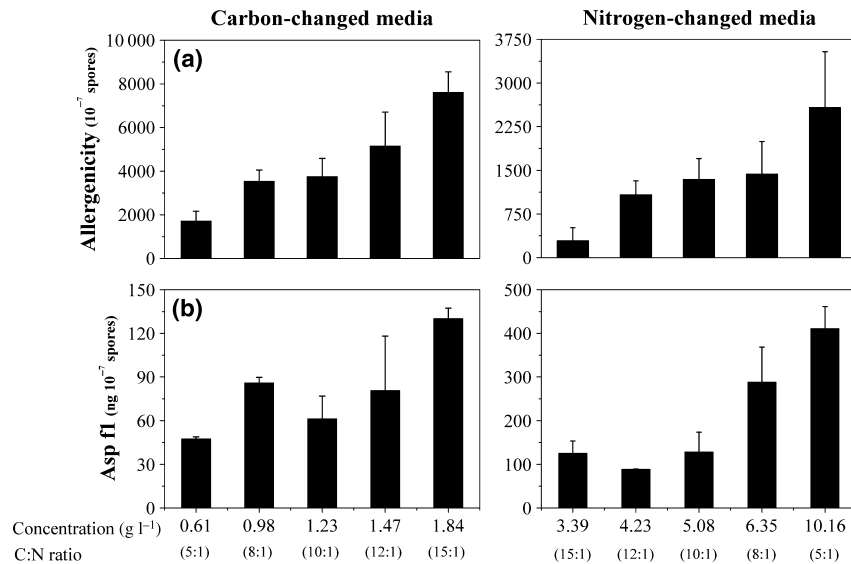


Fig. 2 The effect of changing carbon and nitrogen concentrations on the total allergenicity per 10^7 spores (a), and the major allergen Asp f1 concentration in $\text{ng } 10^{-7}$ spores (b) for: C-changed media (left panel), and N-changed media (right panel). The error bars represent the SE of the mean.

media, we could not identify a consistent trend of an increase in the relative amounts of proteins with increasing nitrogen concentration, excluding the case of Asp f1, which showed significantly higher relative amounts for the 5 : 1, 8 : 1, and 12 : 1 treatments ($P = 0.0284$), similar to the trend observed in the Asp f1 ELISA results.

Discussion

Aspergillus fumigatus conidia grown at varying CO_2 concentrations showed a consistent behavior of higher allergenicity values as well as Asp f1 concentrations for present-day CO_2 concentration (392 ppm) compared with preindustrial levels. These findings were further supported by the genomic analysis of the Asp f1 gene. The Asp f5, 11, and 27 genes showed insignificant difference between 360 and 392 ppm levels, compared to Asp f18 and 34, which showed significant peak at lower CO_2 level of 360 ppm, decreasing at the higher CO_2 concentrations. This difference between the immunoassays and some of the expressed genes may be attributed to the dominance of Asp f1 protein that reached the highest allergenic activity at the level of 392 ppm CO_2 .

It is apparent that there is an optimal CO_2 level for the allergenicity of *A. fumigatus* at ca. 400 ppm. The optimal condition may be attributed to different mechanisms, such as an increase in the growth medium acidity, or changes in the fungus respiration rates, nutrient production etc. Koizumi *et al.* (1991) reported decrease in the respiration rates of soil microbial populations

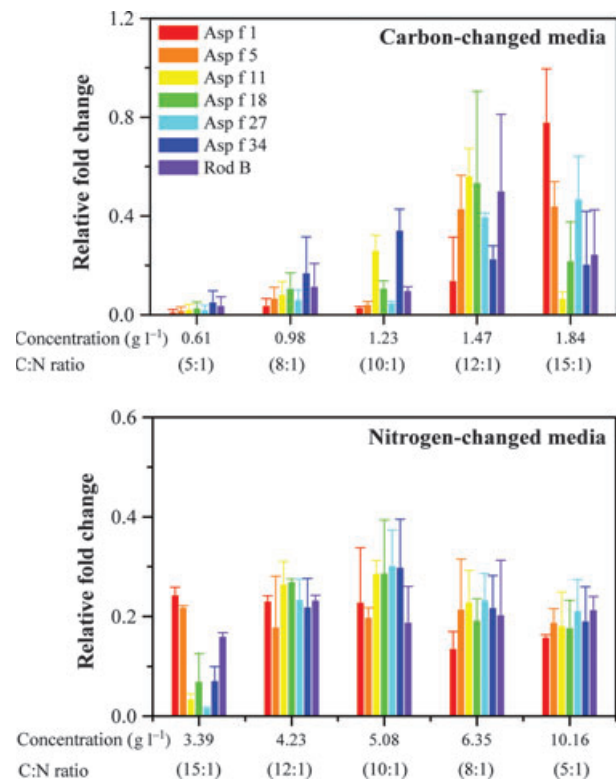


Fig. 3 The relative gene expression of selected *Aspergillus fumigatus* allergenic proteins due to changing carbon and nitrogen concentrations in the growth medium. The error bars represent the SE of the mean.

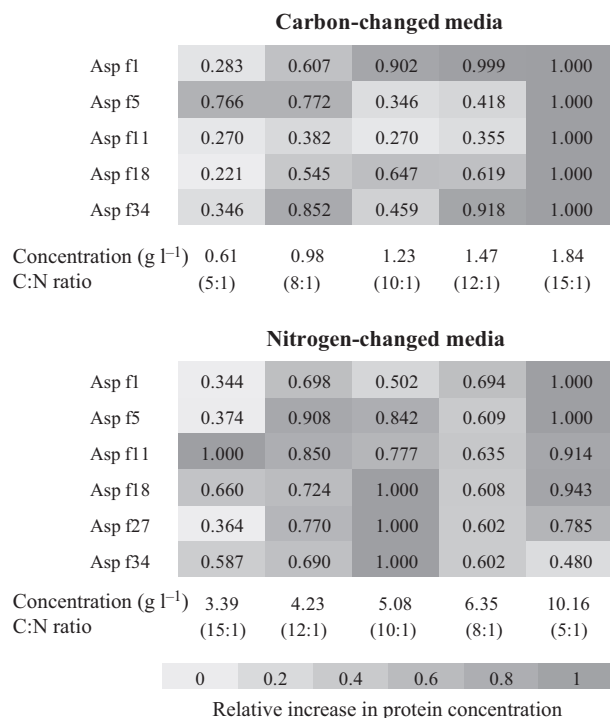


Fig. 4 The relative amount of main allergenic proteins per spore in both carbon and nitrogen changing concentration treatments.

(including filamentous fungi) exposed to elevated CO₂ levels, possibly leading to a general decrease in the growth and buildup of fungal biomass. An additional physiological process was observed by Cotrufo *et al.* (1998), who demonstrated a significant reduction in the nitrogen content of plant tissues with increasing ambient CO₂ concentrations. This probably affects the fungal protein production, including the allergenic ones. On the basis of these observations, we conclude that exposure to elevated CO₂ levels may lead to a reduction in allergenicity due to physiological processes in the fungus. However, it is noted that the allergenicity of fungi growing under these high CO₂ levels is still higher than for those that grew under preindustrial CO₂ levels.

Although it was hypothesized that global change may affect the allergenicity of *A. fumigatus* by modifying C : N ratios in the growth medium (soil, plants), our results suggest that it is rather the absolute concentration of carbon and nitrogen in the growth media that governs this mechanism rather than the C : N ratio itself.

Higher carbon concentrations in the growth medium linearly correlated with increasing allergenic activity of the conidia. This trend was supported by the gene expression and the proteomic results as well, suggesting that a future rise in CO₂ concentrations may lead to an increase in *A. fumigatus*'s allergenicity due to higher carbon consumption.

The different response of allergenic proteins when exposed to elevated atmospheric CO₂ levels compared with increasing carbon concentration in the growth medium can be qualitatively combined to estimate the influence of the total carbon increase during fungal growth on the allergenicity. The increase in the allergenicity of *A. fumigatus* with increasing carbon concentrations could partially explain the wider phenomenon of increase in allergy cases.

Elevated nitrogen concentrations in the growth medium (possibly due to agricultural land use and fertilization use changes) also lead to an increase in the total allergenicity and, specifically, the Asp f1 protein. As no significant change in Asp f1 gene transcription was observed, an increase in the abundance of the protein suggests that this process is not controlled at the transcription level. Regulation of allergenic genes at the transcription level may not always be a prerequisite for increases in allergenicity. Protein concentrations are commonly regulated during translation and allergenic proteins can also undergo posttranslational modification in the environment (Franze *et al.*, 2005). The study of translational regulation and posttranslational modification was not within the scope of these experiments, however, it is noted that such effects are potentially fruitful areas of further research for attempting to understand how the environment impacts allergenicity. There was an inconsistency between the appearance of allergenic proteins in the proteomic analysis and the immunoassays results, although the Asp f1 protein did increase with increased nitrogen concentrations in the medium. This could be explained by a quantitative increase in the main allergenic protein (e.g., Asp f1), which, in this case, is the dominant contributor for the allergenicity increase.

We observed a correlation between the expression of RodB and the allergenic proteins, both when atmospheric CO₂ levels were increased and when increasing carbon concentration in the growth medium. This may indicate that in addition to the allergenicity, spore production itself is also influenced by changes in CO₂ and higher carbon concentrations in the growth medium. Wolf *et al.* (2010) reported similar trends of increased sporulation of *A. alternata* at higher carbon concentrations in the growth medium. These results may indicate that changes in atmospheric CO₂ levels will have a combined effect of both changes in sporulation and in the allergenicity per spore.

The results of this study suggest that environmental changes such as elevated CO₂ concentrations and anthropogenic changes that modify the carbon and nitrogen content of the growth media can modify protein production in the fungus *A. fumigatus* quantitatively and structurally, influencing the allergenicity of

this species. We suggest with caution that the increased allergenicity identified here may partially explain the increase in allergies observed in the last few decades and that such changes and their consequent health effects may also be observed in other pathogens.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Genes used for RT-PCR assays.

Table S2. Primer sets used in the RT-PCR analysis.

Table S3. Peptides detected in the proteomic analysis.