

Research note:**Increasing Amb a 1 content in common ragweed (*Ambrosia artemisiifolia*) pollen as a function of rising atmospheric CO₂ concentration**

Ben D. Singer^{A,C}, Lewis H. Ziska^{B,D}, David A. Frenz^C, Dennis E. Gebhard^C and James G. Straka^A

^ADepartment of Biology, Macalester College, 1600 Grand Avenue, St Paul, MN 55105, USA.

^BCrop Systems and Global Change Laboratory, United States Department of Agriculture, Agricultural Research Service, 10 300 Baltimore Avenue, Beltsville, MD 20705, USA.

^CMultidata LLC, (a subsidiary of Surveillance Data Incorporated), 4838 Park Glen Road, St Louis Park, MN 55416, USA.

^DCorresponding author. Email: lziska@asrr.arsusda.gov

Abstract. Although the impact of increasing atmospheric carbon dioxide concentration ([CO₂]) on production of common ragweed (*Ambrosia artemisiifolia* L.) pollen has been examined in both indoor and outdoor experiments, the relationship between allergen expression and [CO₂] is not known. An enzyme-linked immunosorbent assay (ELISA) was used to quantify Amb a 1, ragweed's major allergen, in protein extracted from pollen of *A. artemisiifolia* grown at different [CO₂] values in a previous experiment. The concentrations used approximated atmospheric pre-industrial conditions (i.e. at the end of the 19th century), current conditions, and the CO₂ concentration projected for the middle of the 21st century (280, 370 and 600 µmol mol⁻¹ CO₂, respectively). Although total pollen protein remained unchanged, significant increases in Amb a 1 allergen were observed between pre-industrial and projected future [CO₂] and between current and projected future [CO₂] (1.8 and 1.6 times, respectively). These data suggest that recent and projected increases in [CO₂] could directly increase the allergenicity of ragweed pollen and consequently the prevalence and/or severity of seasonal allergic disease. However, genetic and abiotic factors governing allergen expression will need to be better established to fully understand these data and their implications for public health.

Keywords: allergenicity, climate change, public health.

Introduction

Common (short) ragweed (*Ambrosia artemisiifolia*) pollen is a major airborne allergen and significant cause of allergic disease. Ragweed growth is, like other plants with C₃-type photosynthesis, carbon limited, and consequently, recent and projected increases in atmospheric carbon dioxide concentration should result in greater growth and potential increases in pollen production. The impact of rising [CO₂] on ragweed biomass and reproductive effort has been demonstrated both in growth chamber (Ziska and Caulfield 2000) and in greenhouse experiments (Wayne *et al.* 2002). Urban–rural differences in [CO₂] and temperature have also confirmed outdoor, *in situ* stimulation of ragweed pollen production with global change scenarios (Ziska *et al.* 2003). Overall, changes in [CO₂] and temperature associated with global climate are anticipated to increase pollen exposure (Beggs 2004).

Allergic symptomology is related to atmospheric pollen counts in a dose–response manner (Frenz 2001). Although pollen counts are commonly used to represent pollen challenges, the ultimate triggers for allergic disease are protein allergens (antigens) associated with pollen grains. In the ragweeds, a family of allergens has been characterised and the major allergen, Amb a 1 (Antigen E), has been extensively investigated. The concentration of this allergen, localised within the cell wall of the pollen grain (intine) (Knox and Heslop-Harrison 1971) can vary geographically (Lee *et al.* 1979) and temporally (Maasch *et al.* 1987). However, the underlying environmental (Beggs 1998) or developmental (Kerim *et al.* 2003) influences for differences in pollen allergenicity have not been completely elucidated.

Initial work by Ziska and Caulfield (2000) offered a unique opportunity to investigate the effect of changing [CO₂] on both ragweed pollen production and Amb a 1 expression.

Abbreviations used: DAS, days after sowing; FACE, free-air CO₂ exchange.

Using an immuno-assay approach, total protein and Amb a 1 content were determined as a function of pre-industrial, current and projected [CO₂] and is reported here for the first time.

Materials and methods

Plant culture

Experiments were conducted in controlled environment chambers located at the Climate Stress Laboratory, USDA-ARS, Beltsville, MD, USA. Environmental chambers were used rather than field chambers or free-air CO₂ exchange (FACE) in order to simulate constant pre-industrial [CO₂] at a given temperature and consistent light and humidity for 24-h periods.

Seeds of common ragweed (*Ambrosia artemisiifolia* L.) were broadcast in vermiculite containers with a volume of 21.2 L for mature plants. All plants were watered and fertilised to maintain optimal growth. The set points for control of CO₂ concentration were 280, 370 and 600 $\mu\text{mol mol}^{-1}$ CO₂. Actual values determined at 10-min intervals over a 24-h period for each treatment were 281 ± 23 , 374 ± 14 and 603 ± 13 $\mu\text{mol mol}^{-1}$. These concentrations were intended to simulate the pre-industrial concentration, the current concentration, and the carbon dioxide concentration projected for the middle of the 21st century, respectively. Within a chamber plants received 14 h of 1.0 $\text{mmol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD) from a mixture of high-pressure sodium and metal halide lamps for the first 35 d after sowing (DAS). After 35 DAS, PPFD was altered to 12 h of 1.0 $\text{mmol m}^{-2} \text{s}^{-1}$ PPFD to induce flowering. For additional details regarding the methodology and experimental design see Ziska and Caulfield (2000).

After anthesis and before seed set, 10 terminal staminate floral spikes were selected on each of five plants from each [CO₂] treatment and labelled. A polyethylene bag (5 × 25 cm) was placed over each spike with a 2–2.5-cm slit cut ~2 cm from the bottom of the bag, into which the floral spike was placed. After placement of the bag, the slit was taped so the floral spike was inside the bag with at least 5 cm of space from the top of the open bag. Tops of bags were left open for air circulation and ventilation. Floral bags were tapped gently each day until all flowers had completely dehisced. The floral structure was then removed from the bag after tapping out any residual pollen. Total pollen for a given spike was calculated by subtracting the initial bag weight from the bag and pollen weight. At maturity for each plant from each [CO₂] treatment, the total number of floral spikes was recorded, then spikes were harvested and the dry weight (without pollen) recorded. The ratio of pollen collected to dry weight of the floral structure resulted in a consistent ratio that was used to estimate pollen production per plant. Following pollen collection, all pollen collection bags from each mature plant for a given [CO₂] treatment were kept frozen at –80°C until pollen analysis.

Protein extraction of pollen

Samples of *A. artemisiifolia* pollen (10 mg) were suspended in 200 μL of a 0.5% NaCl, 0.25% NaHCO₃, w/v in water (Coca 1922) with the following modifications. (1) Pollen suspensions were sonicated at 0°C with a Fisher Model 550 Sonic dismembrator (Fisher Scientific, Pittsburgh, PA) fitted with a micro-tip (3.2 mm diameter). The sonicated suspensions were held on ice with occasional mixing for various periods. (2) Following the extraction period, particulates were removed by centrifugation for 10 min at 13 000 g and 6°C. The supernatant fluid was carefully withdrawn from the pellet, and the volume of each extract measured. An equal volume of 50% (w/v) glycerol was added and the extracts were held at –20°C. We examined the following extraction conditions: (1) pollen was suspended in Coca's solution, sonicated as described, and incubated with occasional mixing on ice for 2 h or overnight; (2) pollen was suspended in Coca's solution supplemented with 1.0% Tween 20 non-ionic detergent, sonicated and held as above; (3) pollen was pre-wet with 0.1 volume 95% ethanol, 0.9 volume of Coca's solution containing 1.0% Tween 20, and the suspension was sonicated and held as above. The Bradford (1976) method was used for protein quantification. Overall, the use of detergent in the extracting medium led to an approximately 3-fold increase in protein extracted compared with Coca's salt solution alone (Table 1).

Immunochemical quantification of Amb a 1

Equivalent amounts of protein from the different extraction methods were evaluated for the concentration of Amb a 1 allergen by a kinetically analysed enzyme-linked immunosorbent assay (k-ELISA) (Engvall 1980; Tsang *et al.* 1983) as described by Straka *et al.* (1991). Pollen protein extracts were diluted to initial concentrations of 2.5×10^{-4} mg protein mL⁻¹ in 0.05 M sodium carbonate buffer, pH 9.6; linear dilutions over a 10-fold range were prepared in the same buffer as for ELISA analysis. Bovine serum albumin (BSA, Sigma, St Louis, MO) was used as a blocking agent to prevent non-specific protein binding. Sheep antibody-Amb a 1 was a gift from Dr JE Slater, CBER/FDA Laboratory, Rockville, MD; horseradish peroxidase (HRP)-labelled rabbit-anti-sheep IgG was from Kirkegaard and Perry (Gaithersburg, MD). Plates were developed with the addition of 1 mM H₂O₂ and 2 mM 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid, Sigma) in 0.20 M phosphate-0.10 M citrate (pH 5.0) buffer. The rate of colour development at 415 nm was measured with a 96-well plate reader (Bio-Rad Laboratories, Hercules, CA). We defined the amount of allergen present in a given sample using an arbitrary ELISA unit = $\Delta A_{415 \text{ nm}} \text{ min}^{-1}$. Plots of ELISA units against protein yielded straight lines; the slope of each line provided an estimate of allergen level as a function of protein, and is reported as ELISA units mg⁻¹ protein. A comparison of Amb a 1 as a function of the different extraction methods (averaged over CO₂ treatments) is given in Table 1.

Table 1. Comparisons of extraction methods for protein and levels of Amb a 1 antigen in ragweed pollen

Buffer used, and incubation time at 0°C is given in the first column. ELISA units are based on differences in absorption at 415 nm and were performed in triplicate for each sample; results are shown as mean ± standard deviation. See Materials and methods for additional details

Method	Protein ($\mu\text{g protein mg}^{-1}$ pollen)	Amb a 1 (ELISA units μg^{-1} protein)	Amb a 1 (ELISA units mg^{-1} pollen)
Coca alone, 2 h	6.6	3.68 ± 0.57	24 ± 4
Coca alone, 16 h	7.8	10.80 ± 1.20	84 ± 9
Ethanol pre-wet with Coca + 1%, Tween 20, 2 h	22.0	4.60 ± 0.58	100 ± 13
Ethanol pre-wet with Coca + 1%, Tween 20, 16 h	24.2	6.60 ± 1.07	139 ± 26

Design and statistics

Although several methods were evaluated, the extraction method (and corresponding ELISA) used for the analysis of the different CO₂ treatments was the ethanol pre-wet with Coca solution +1% Tween for 16 h. This was done in order to extract the most Amb a 1 per mg pollen (v. most Amb a 1 mg⁻¹ protein), since allergen exposure as a function of pollen was our ultimate variable of interest. A complete description of statistical design and analysis of the ragweed plants grown and pollen collected is given by Ziska and Caulfield (2000).

In the current paper, specific data analysis are included with the table or figure legend.

Results and discussion

Amb a 1 concentrations increased as a function of [CO₂] (Table 2). Relative to the pre-industrial [CO₂] condition, pollen contained 1.2 and 1.8 ($P<0.005$) times more Amb a 1 allergen at current and projected 21st century [CO₂], respectively. Relative to current [CO₂], pollen obtained at a projected 21st century [CO₂] contained 1.6 ($P<0.01$) times more Amb a 1 allergen. (Table 2). To our knowledge, this is the first investigation to demonstrate that [CO₂] *per se* can be associated with allergen expression and subsequent allergen content in ragweed (or any plant species, see Hjelmroos *et al.* 1995; Ahlholm *et al.* 1998).

The genus *Ambrosia*, which includes both *A. artemisiifolia* and *A. trifida* (giant ragweed) has long been recognised as one of the most significant cause of seasonal allergic disease (Wodehouse 1971). A large, random skin test survey demonstrated that 10% of the United States population was sensitive to this airborne allergen (Gergen *et al.* 1987). Among atopic individuals the rate of sensitisation exceeds 20% (Lewis and Imber 1975; Chapman 1986).

Although prior investigations (Ziska and Caulfield 2000; Wayne *et al.* 2002) demonstrated that rising [CO₂] increased ragweed pollen production per plant, any association between rising carbon dioxide and allergic disease could only be ascertained if Amb a 1 expression was known. By using previous estimates of ragweed pollen production (Ziska and Caulfield 2000) in combination with the current estimates of Amb a 1, we calculated that production of the allergen can increase in a logarithmic fashion as a function of both current

and projected [CO₂] relative to pre-industrial conditions (Fig. 1). Overall, the present investigation completes the causal chain by demonstrating that in addition to ragweed pollen production (Ziska and Caulfield 2000), Amb a 1 expression may also increase as a function of [CO₂].

The global implications regarding rising [CO₂] and increased allergic suffering should, however, be viewed conservatively. Other factors, both genetic and abiotic can also influence Amb a 1 expression. One study conducted in Illinois, USA, for example, demonstrated several-fold differences in allergenicity both among and within ragweed populations (Lee *et al.* 1979), although it was unclear whether these differences were genotypic or phenotypic. Year-to-year variation in ragweed pollen is also common (e.g. Goldfarb and Kaplan 1967; Maasch *et al.* 1987), presumably reflecting both genetic and abiotic differences (e.g. temperature, Wan *et al.* 2002). Therefore, while the current study suggests that changing [CO₂] is one possible environmental factor associated with allergenicity, it is not the only factor influencing levels of Amb a 1 expression. Any long-term assessment regarding the epidemiology of ragweed-induced allergic rhinitis will require a thorough understanding of how environmental parameters (either singly or in combination) alter pollen production and allergen expression. This will also require establishing the physiological function of Amb a 1, better appreciating the ecology of ragweed establishment and dynamics, and a more sophisticated delineation of the relationship between outdoor allergen exposure and resulting clinical symptomology.

Nevertheless, the demonstration that both pollen production and allergen levels can increase with rising [CO₂] suggests that climate change has portentous implications for seasonal allergic disease. The present data may offer a partial, but tantalising explanation for the changing epidemiology of allergic disease (Sly 1999) and the long-recognised variability in allergen extracts used for immunotherapy (Goldfarb and Kaplan 1967; Maasch *et al.* 1987). Further scientific investigation appears warranted to elucidate and clarify these potential associations between climate change and public health.

Table 2. Protein and Amb a 1 in extracts of ragweed pollen obtained from plants grown under controlled conditions of [CO₂]

The [CO₂] used correspond approximately to the pre-industrial concentration, the current concentration and that projected for 2050. Samples of pollen pooled from plants grown under the different [CO₂] were extracted as described in the methods. ELISA was performed in triplicate with each sample; results are mean ± standard deviation

[CO ₂] (μmol mol ⁻¹)	Protein concentration (μg mg ⁻¹ pollen)	Amb a 1 concentration (ELISA mg ⁻¹ protein)	Amb a 1 concentration (ELISA mg ⁻¹ pollen)
280	21 ± 2	4490 ± 960 ^A	93 ± 20 ^A
370	20 ± 2	5290 ± 560 ^B	103 ± 11 ^B
600	22 ± 2	8180 ± 900	178 ± 20

^A $P<0.005$ when compared with projected 21st century [CO₂], *t*-test using unequal variances.

^B $P<0.01$ when compared with projected 21st century [CO₂], *t*-test using unequal variances.

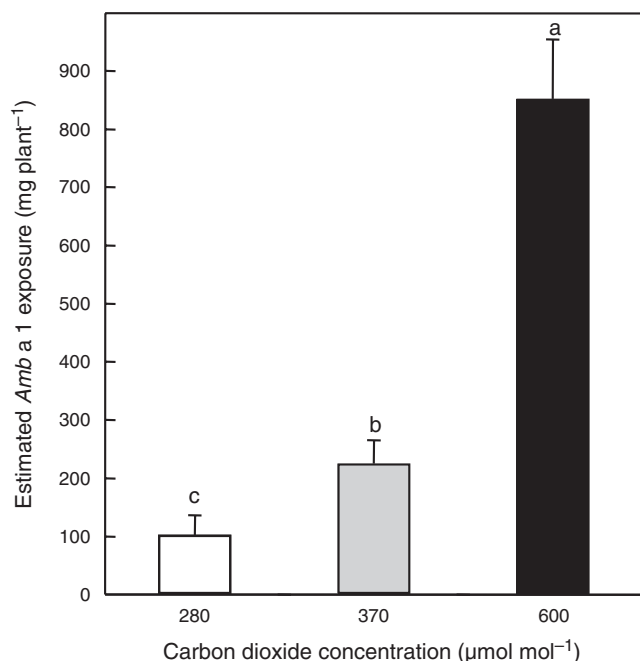


Fig. 1. Change in the relative exposure of Amb a 1 protein as a function of total pollen production estimated from Ziska and Caulfield (2000). Numbers of allergen Amb a 1 at the higher [CO₂] are derived using the 280 μmol mol⁻¹ baseline as a ratio. Bars represent ± SE. Different letters indicate significant differences between treatments ($P < 0.05$) as a function of pre-industrial, current and projected atmospheric [CO₂] as determined by Fisher's protected least significant difference test.

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