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Effects of NO_2 on Inflorescence Length, Pollen/Seed Amount and Phenolic Metabolites of Common Ragweed (*Ambrosia artemisiifolia* L.)

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Abstract

Ambrosia artemisiifolia L. (common ragweed) is an annual ruderal plant that is native to Northern America but nowadays is also spreading across Europe, and its pollen is known to be highly allergenic. Air pollution, e.g. NOx and climate change may affect the plant growth, pollen production and duration of the entire pollen season. In this study, ragweed plants were grown over an entire vegetation period under 40 ppb NO_2/clean air (control) and 80 ppb NO_2 (treatment). The inflorescence length was not affected by this air pollutant. However, the pollen amount increased, while the seed production decreased in both populations upon elevated NO_2 concentrations. Regarding phenolic metabolites elevated NO_2 had no effect on the amount of total phenolic metabolites, while individual metabolites showed significant changes.

Keywords

Air Pollution, *Ambrosia artemisiifolia*, Flavonoids, Pollen, Ragweed, Seeds

1. Introduction

Ambrosia artemisiifolia (common ragweed) is native to North America; it is a monoecious and wind-pollinated herbaceous annual plant that belongs to the

Asteraceae family and has expanded its distribution out of its native range to Europe, Australia, Asia, South Africa and South America [1] [2] [3] [4]. Moreover, for Europe, the models of future expected climate change scenarios indicate a dramatic northward shift of *A. artemisiifolia*, also accompanied by an increase in pollen production [5] [6].

Environmental changes may increase the severity of pollen as stimulated atopic disease by influencing the large-scale distribution and local incidence of allergenic species, the flowering time, the pollen production and the allergenicity of individual pollen grains [7] [8] [9] [10]. It has been shown that elevated CO_2 concentrations result in an increase of *A. artemisiifolia* growth and pollen production [9] [10] [11] [12]. In addition to climate change parameters, air pollution might also influence the allergenicity of *A. artemisiifolia* pollen [13] [14] [15] [16]. However, the fumigation of *A. artemisiifolia* plants with 80 ppb O_3 did not alter the pollen amount [12]. Atmospheric NO_2 is either harmful or beneficial to plants, depending on the concentration and plant species [17] [18] [19] [20] [21]. For the vegetation the critical value is about 15 ppb per year (http://www.umweltbundesamt.de/sites/default/files/medien/1/dokumente/infoblatt_stickstoffdioxid.pdf). However, in urban traffic areas up to 90 ppb NO_2 can be measured, whereas in rural regions up to 20 ppb can be found (http://www.umweltbundesamt.de/daten/luftbelastung/aktuelle-luftdaten). A reduced pollen viability of *Pinus nigra* under ambient NO_2 levels was found in a field study [22]. Similarly *in vitro* fumigation of pollen from three tree species reduced the viability and germination [23]. The *in vitro* fumigation of pollen with NO_2 did not induce new allergens in birch or *A. artemisiifolia* and had no effect on the allergen release from grass pollen [24] [25]. Using high concentrations of NO_2 (ppm range), the content of several *Phleum pratense* grass allergens (Phl p) decreased [26]. However, treatment of pollen from four different tree species with moderate NO_2 concentrations (40 - 300 ppb) resulted in greater immunoglobulin E (IgE) recognition by immunodetection [23] [27]. Pollen isolated from *A. artemisiifolia* that was fumigated with realistic NO_2 concentrations (80 ppb) also showed a higher IgE recognition [16]. Similarly NO_2-fumigated pollen of *P. pratense* stimulated the production of chemokines by an increased Th2-cell response in human cells [28]. These studies suggest that changes in NO_2 concentrations will affect the allergenic potential of pollen and play a role in human health diseases that are related to allergic rhinitis and asthma.

Flavonoids are important secondary metabolites that protect pollen from UV-B irradiation, especially during the long-distance transport [29]. In addition, flavonoids are crucial for the germination process in many plant species [30] [31]. Moreover, these compounds may also be involved in the allergenic response of pollen [32] [33]. Flavonoids can interact with allergens [34] [35], and a direct link between the binding of a quercetin glycoside to Bet v 1 and the inflammation responses was recently reported [36].

From these perspectives, a detailed analysis of the allergenic pollen and seed

production would allow to understand the anticipated changes in the pollen amount and seed dispersal in response to elevated NO_2 concentrations. In previous studies, we had shown that elevated levels of O_3 had no effect on the pollen production of *A. artemisiifolia*, whereas CO_2 increased and drought decreased the pollen amount [12]. In this study, we altered the gaseous air pollution by linking the pollen and seed production of *A. artemisiifolia* with elevated NO_2 levels. We emphasize that this environmental change affects the pollen amount, as well as phenolic metabolites, which is relevant to human health.

2. Material and Methods

2.1. Plant Growth Conditions

A. artemisiifolia seeds were collected from a single plant at an outdoor stand (Bad Waldsee, Baden-Württemberg, Germany) to prevent epigenetic-caused effects [37]. Seeds were sown in standard soil (Floradur®, Bayerische Gärtnerei Genossenschaft, München, Germany) in small multiflor palettes (6 × 6 cm) and transferred into four Plexiglas sub-chambers (1.1 m × 0.9 m × 0.8 m) that were placed in Phytotron walk-in chambers [38] (http://www.helmholtz-muenchen.de/en/eus/facilities/phytotron/index.html). All physical parameters, including the wind velocity were identical in the sub-chambers. After germination, the seedlings were planted in pots (Ø17 cm). One plant was grown per pot and 10 pots were placed into the sub-chambers. Plant growth and NO_2 fumigation were performed as described by [16]. Briefly, plants were treated with 40 ppb NO_2 (control) or 80 ppb NO_2 (treatment) for 61 d (10 h/d), and pollen was harvested during the last 28 d of fumigation (1st population), using a modified ARACON system (BETATECH, Ghent, Belgium) that covered the male inflorescences (**Figure 1**). Five inflorescences from each plant were randomly selected for the sampling of the pollen. The collected pollen

Figure 1. Sampling of ragweed pollen using a modified ARACON system.

samples were stored at −80˚C. In addition, seeds from the 40 ppb NO_2 treatment were collected and used in a second experiment the following year (2nd population). For technical reasons (an additional ozone experiment was carried out) the second population was treated with clean air (control) and 80 ppb NO_2 (treatment) [16]. For each population morphological data like inflorescence length, the pollen and seed amount was measured.

2.2. Analysis of Phenolic Metabolites

Frozen pollen (15 mg) was extracted with 1.5 ml of phosphate buffer saline (PBS) for 1 h at room temperature. After centrifugation, the PBS supernatant was kept, and the residue was re-extracted with 1.5 ml of methanol (high-performance liquid chromatography (HPLC) grade). The reverse-phase high-performance liquid chromatography (RP-HPLC) (Beckman HPLC System Gold, Beckman, Munich, Germany; column 240 × 4.5 mm ProntosilSpheribondODS2, NC, 5 µm, Bischoff, Leonberg, Germany) of both extracts was performed as described by [39], using 10 µl of PBS samples. In the case of the methanol extraction, 25 µl of H_2O was added to 75 µl of the methanolic samples, then centrifuged, and 10 µl were used for RP-HPLC separation. Solvents and gradient conditions for RP-HPLC separations were as described by [39]. Detection was at 280 nm with a UV/visible diode-array detector (Beckman Model 168).

2.3. Statistics

To calculate significant differences between samples, an unpaired t-test was carried out. The Shapiro-Wilk normality test or the Mann-Whitney rank sum test was used (SigmaPlot 12; Systat Software, Erkrath, Germany).

3. Results and Discussion

3.1. Morphological Plant Growth and Pollen Data

Regarding morphological parameters, increased NO_2 had no effect on the inflorescence length (**Table 1**), which is similar to the air pollution O_3, also showing no effect on this parameter, whereas elevated CO_2 resulted in an increased length of the main inflorescence [12]. Regarding allergenicity, the pollen amount clearly increased in both years of the study by approximately 70 to 80% (**Table 1**). This result parallels the increased number of flowers found in tomato plants upon NO_2 fumigation [40]. The increased pollen amount in *A. artemisiifolia*, similar to that under elevated CO_2 [12], may result in a higher pollen concentration in the air, with expected negative effects on the atopic population [41]. The total seed production and seed amount clearly decreased under elevated NO_2 (**Table 1**). This may be caused by a reduced pollen viability and germination rate upon NO_2 exposure [22] [23]. An important point for seed production in ragweed is the plant density [42]. As the plant density was the same in all sub-chambers the seed production could only be influenced by NO_2. This result is in contrast to

Table 1. Morphological data of ragweed plants that were grown in exposure chambers and fumigated with elevated NO_2 concentrations.

NO_2 (ppb)	Inflorescence (cm) ± SD	Pollen/Inflorescence (mg) ± SD	Seeds/plant (mg) ± SD	Weight of 50 grains (mg) ± SD
1st 40	23.73 ± 1.12	79 ± 6	1329 ± 85	229 ± 18
1st 80	25.18 ± 1.57	132 ± 11	905 ± 69	199 ± 10
p-value	0.327	$1.8 \times 10E^{-5}$*	$2.3 \times 10E^{-3}$*	$1.9 \times 10E^{-3}$*
2nd 0	24.48 ± 1.35	85 ± 8	1637 ± 77	237 ± 16
2nd 80	24.61 ± 1.77	159 ± 12	1024 ± 53	209 ± 9
p-value	0.568	$2.7 \times 10E^{-5}$*	$3.5 \times 10E^{-3}$*	$2.9 \times 10E^{-3}$*

Number of plants N = 20, 5 inflorescences per plant, t-test, *p-value < 0.05. Five inflorescences from each plant were randomly selected.

black turtle bean (*Phaseolus vulgaris*), which showed an increased seed number and seed weight [19].

3.2. Secondary Metabolites

The total amount of analyzed phenolics is shown in **Figure 2(a)**. However, no significant changes between the controls and treatments in either year were evident. This result is similar to that of *A. artemisiifolia* plants that were fumigated with ozone or elevated CO_2 or were grown under drought stress [14] [43]. Typical RP-HPLC diagrams of water-soluble extracts revealed six prominent compounds (**Figure 3(a)**). Diode array spectra of the respective peaks indicated compounds DA 1, 2, 4 and 6 as quercetin derivatives and compound DA 5 as a kaempferol derivative, whereas compound DA3 could not be identified (**Figure 3(b)**, **Figure 3(c)**), and the highest amounts were found for DA 2, 5 and 6 (**Figure 2(b)**). Methanol-extractable phenolics additionally also showed six compounds that were characterized as hydroxycinnamic amides according to their diode-array spectra (**Figure 3(d)**, **Figure 3(e)**). Significant changes with an increased amount were only observed in the 2nd population for the water-soluble metabolite DA 6 and the methanol-extractable metabolites DA 5 and 6 (**Figure 2(b)**). In contrast, in the 1st population, the water-soluble metabolites of DA 1 and 4 clearly decreased under elevated NO_2 (**Figure 2(b)**). These differences might be explained by the different control treatments in both years: 40 ppb NO_2 in the first year and clean air in the second year. Changes in individual phenolic metabolites have also been reported in *A. artemisiifolia* pollen upon CO_2 and/or drought stress [43], whereas no changes were observed upon ozone fumigation [14]. The pollen of several other plants species that were sampled from polluted and less-polluted areas showed an increased amount of individual flavonoids [44] [45]. These data indicate that individual stress will differentially affect the flavonoid composition. As we did not find significant differences in the total flavonoid amount, it is unlikely that there is a direct effect of flavonoids on IgE

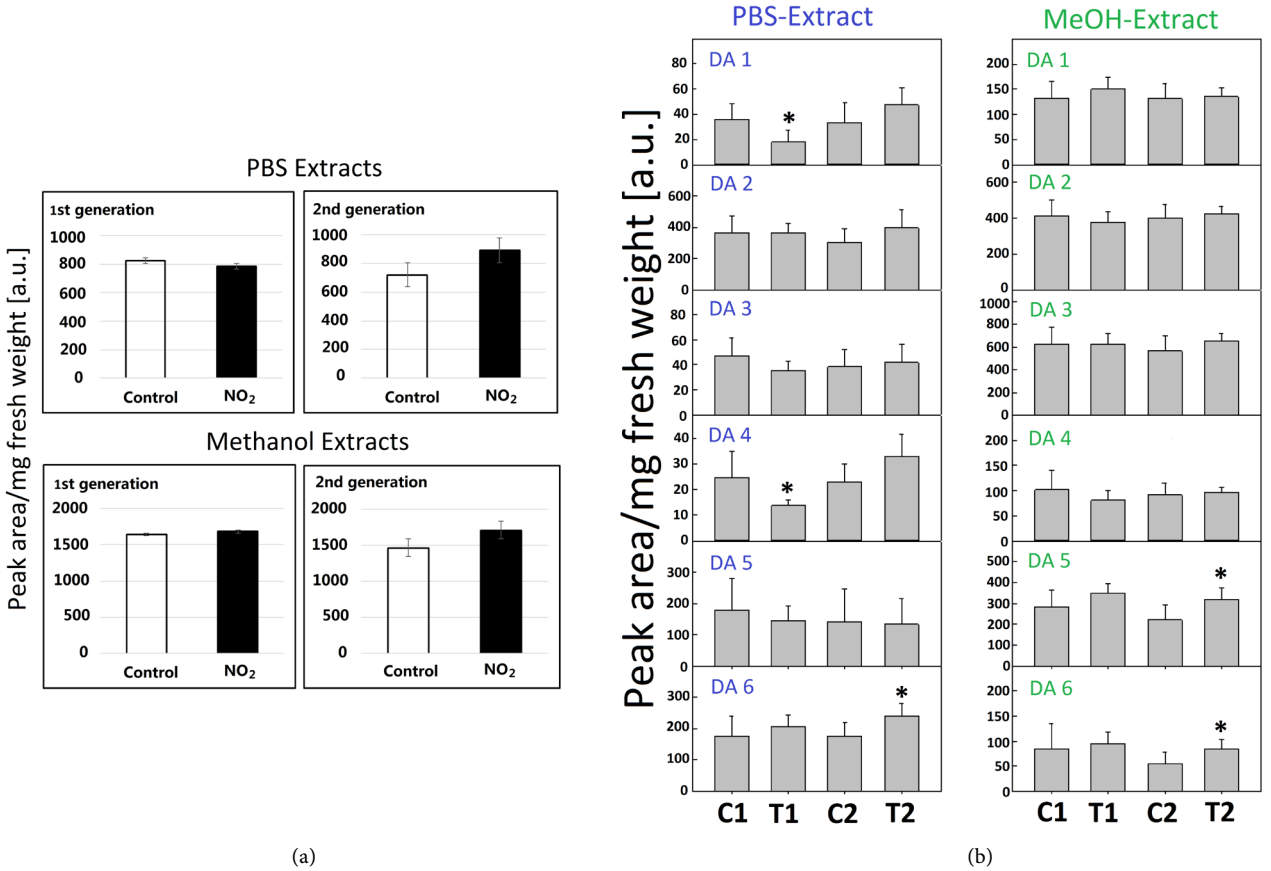

Figure 2. (a) Total amount of water-soluble and methanol-extractable phenolic metabolites in ragweed pollen. The separation was performed by RP-HPLC. Plants were fumigated with 40 ppb NO_2 (1st control) or clean air (2nd control) or with 80 ppb NO_2 (1st and 2nd treatment); bars indicate ± SD; N = 8 plants, 5 inflorescences per plant). (b) Amount of individual PBS-soluble and methanol-extractable phenolic metabolites in pollen of *A. artemisiifolia*. C1 = 1st population of control; T1 = 1st population of treatment; C2 = 2nd population of control; T2 = 2nd population of treatment. The bars indicate ± SD and significant differences are indicated by asterisks (N = 8 plants, 5 inflorescences per plant; t-test, *p-value < 0.05).

recognition, as elevated NO_2 resulted in a higher IgE recognition in immunoblots [16]. However, flavonoids can play a modulating role on immunity and inflammation [46] and may influence membrane translocation of allergens [47].

4. Conclusion

Our data on *A. artemisiifolia* fumigated with elevated NO_2 support the hypothesis that the overall allergenicity might be increased by an increased pollen amount. However, differences in the estimation of allergen exposure between pollen amount and ELISA data must be considered [48] [49]. As the overall seed production decreased under elevated NO_2, the dispersal of *A. artemisiifolia* should not be affected by this air pollutant.

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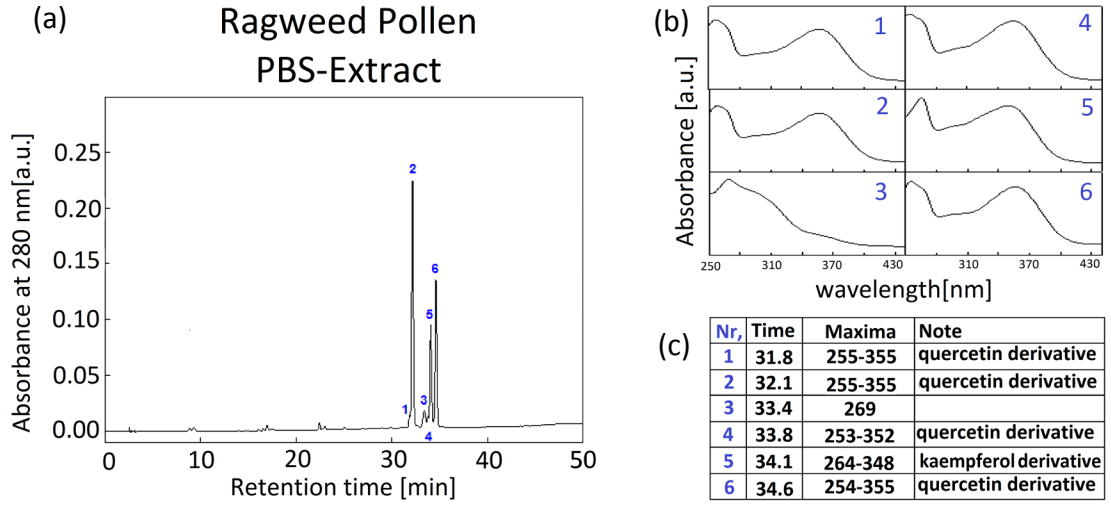
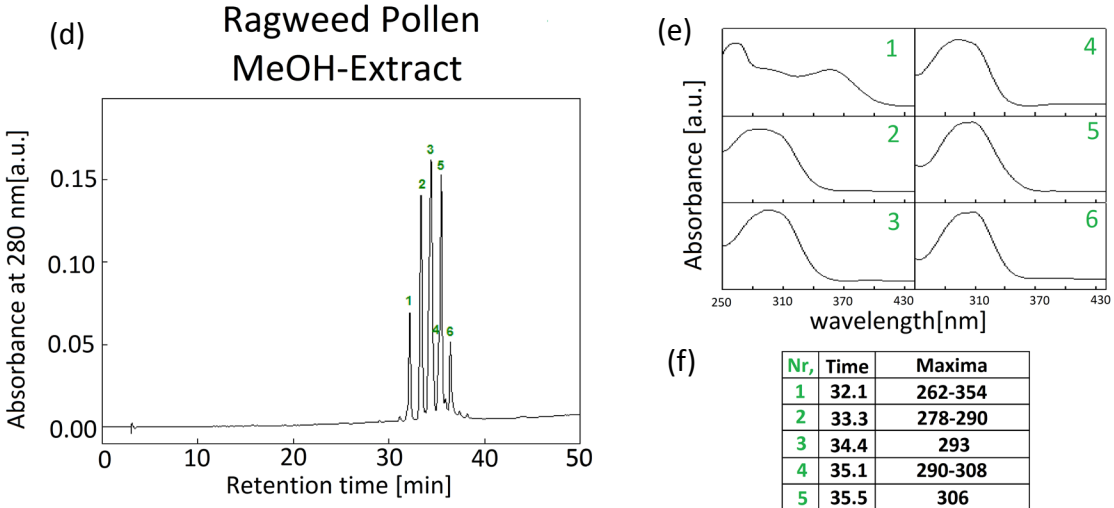


(c)

Nr,	Time	Maxima	Note
1	31.8	255-355	quercetin derivative
2	32.1	255-355	quercetin derivative
3	33.4	269	
4	33.8	253-352	quercetin derivative
5	34.1	264-348	kaempferol derivative
6	34.6	254-355	quercetin derivative



(f)

Nr,	Time	Maxima
1	32.1	262-354
2	33.3	278-290
3	34.4	293
4	35.1	290-308
5	35.5	306
6	36.5	297-307

Figure 3. RP-HPLC diagram of water-soluble and methanol-extractable phenolic metabolites. (a) shows a typical HPLC run of a PBS extract from *A. artemisiifolia* pollen at an absorbance of 280 nm, resulting in 6 distinct peaks; (b) represents the diode array spectra of the respective peaks between 250 and 430 nm; (c) indicates the elution times and maxima of the corresponding peaks, and preliminary structural assignments are given; (d) gives a typical HPLC run of a methanol extract, following the PBS extraction, from *A. artemisiifolia* pollen at an absorbance of 280 nm. The HPLC run resulted in six distinct peaks; (e) diode array spectra of the respective peaks between 250 and 430 nm; (f) indicates the corresponding retention time and the peak maxima for each single peak.

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Conflict of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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