

Effect of nitrogen dioxide and sulfur dioxide on viability and morphology of oak pollen

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Background: Nitrogen dioxide (NO₂) and sulfur dioxide (SO₂) generated by excessive coal combustion and motor vehicle emissions are major air pollutants in the large cities of China. The objective of our study was to determine the effects of the exposure of oak pollens (*Quercus mongolica*) to several concentrations of NO₂ or SO₂.

Methods: Pollen grains were exposed to 0.5 ppm to 5.0 ppm NO₂ or SO₂ for 4 hours and assessed for morphological damage by field emission scanning electron microscopy and for viability using the trypan blue stain. Morphological changes in pollen grains were also examined after contact with acid solutions at pH 4.0 to pH 7.0.

Results: Exposure to NO₂ or SO₂ significantly damaged pollen grains at all concentrations investigated, compared to exposure to air; with exposure to concentrations of 0.5 ppm to 2 ppm resulting in fissures or complete breaks in the exine and a concentration of 5 ppm resulting in complete breakdown and release of pollen cytoplasmic granules. Significantly greater amounts of pollen grain were damaged after exposure to SO₂ (15.5–20.4%) than after exposure to NO₂ (7.1–14.7%). Similarly, exposure to NO₂ or SO₂ significantly decreased the viability of pollen grains, compared

with exposure to air; with SO₂ being slightly more detrimental than NO₂. Exposure to acid solutions also induced pollen damage, which appeared to be pH-dependent (from 24.6% at pH 6.0 to 55.8% at pH 4.0; compared to 3.8% at pH 7.0).

Conclusion: Short-term exposure of oak pollen to high concentrations of SO₂ or NO₂ significantly increases their fragility and disruption, leading to subsequent release of pollen cytoplasmic granules into the atmosphere. These results suggest that heightened air pollution during the oak pollen season may possibly increase the incidence of allergic airway disease in sensitized individuals by facilitating the bioavailability of airborne pollen allergens. © 2015 ARS-AAOA, LLC.

Key Words:

air pollutants; sulfur dioxide; SO₂; nitrogen dioxide; NO₂; oak pollen; morphological damage

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The development of industry and urbanization in China has been coupled with the generation of large quantities of sulfur dioxide (SO₂) and especially nitrogen dioxide (NO₂), primarily as a consequence of excessive coal combustion and increased motor vehicle emissions, which can be detected as air pollutants at high concentrations in the large cities and urban areas. Although concentrations greater than 1 ppm NO₂ are not generally obtained in the environment, it is possible that such concentrations can develop in indoor environments, such as places where fossil fuels are burnt or during periods of extremely high traffic congestion. Indeed, a recent study showed that the

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monthly mean concentration of NO₂ in Beijing was 0.1 ppm, but reached 0.5 ppm or higher during heavy haze days, mainly due to motor vehicles and industrial emission pollution.¹ Considering that over one-half of China's population, which reached 1.4 billion in 2010, lives in cities and with megacities developing rapidly, it is possible that the concentration of NO₂ and SO₂ levels in some of these cities may reach even higher concentrations than those seen in Beijing on hazy days.

Substantial epidemiological evidence supports a relationship between air pollution and respiratory allergic diseases, with the pollutants exerting adjuvant or aggravating effects on the sensitization and elicitation phases of allergic immune responses.^{2,3} Several studies have indicated that air pollutants can also interact with and modify the allergen carrier itself; ie, the plant and its pollen grains. There is a large body of evidence that air pollutants influence gene expression and can provoke modifications in the membrane structures, interfering with the cellular mechanisms.⁴⁻⁶ In this regard, NO₂ present in the urban air has been shown to interact with pollen grains, inducing modifications in fertility and protein content that affect the reproductive cycle of spermatophytes.⁷⁻⁹ Furthermore, synergistic interaction between air pollutants and pollens may result in increased allergenicity and sensitization.^{8,10} Moreover, SO₂ and NO₂ present as pollutants in air can be converted to SO₄²⁻ and NO₃⁻, respectively, which are the main cause of production of acid substances, leading to the formation of "acid precipitation."¹¹ Although the effects of acidification can directly and indirectly impact the physiology and distribution of plants, there is relatively little information on the impact of pollution-related acidification on pollen allergenicity. One study has recently demonstrated that although exposure of Timothy grass pollen to NO₂, SO₂, or O₃ alone or in combination induced a decrease in allergen detected in pollen extracts, it was unclear whether the observed decrease was due to a mechanical loss of allergens from the altered pollen grains or due to posttranslational modifications which could affected recognition by immunoglobulin E (IgE).¹²

In view of these findings, the aim of the present study was to determine the effect of (1) exposure to several concentrations of SO₂ or NO₂; and (2) contact with acid solutions on the structure and viability of oak (*Quercus mongolica*) pollen.

Materials and methods

Pollen samples

Oak (*Quercus mongolica*) is a tree species that produces catkins each year during the spring months (usually around April) in Beijing, China. Anthers from oak trees were collected from the Botanic Garden of the Chinese Academy of Sciences and dried at 27°C. The pollens were released by gently crushing the dried anthers and sieved through 50-μm mesh, prior to storage at -20°C.

Exposure of pollen to NO₂ and SO₂

Exposure of pollen to air pollutants was carried out in a polypropylene cylinder (height 110 mm, diameter 31 mm), lined with a 15-μm polyamide microporous membrane above a porous plate situated at the bottom of the cylinder, as described.¹³ Pollen grains (1 g) were placed on the microporous membrane and mixtures of air and NO₂ or SO₂ at concentrations of 0, 0.5, 1, 2, and 5 ppm were introduced into the exposure chamber through the microporous plate of the chamber, at a constant rate of 1.0 L/minute for 4 hours. At the end of each exposure, the pollens were suspended in a rising current of air to dry the pollen until further analysis.

Assessment of pollen damage

Damage to the pollen was assessed using a Hitachi S-4800 field emission scanning electron microscope (Hitachi, Tokyo, Japan). Pollen grain samples exposed to air or the pollutants were sputter-coated with gold, and observed at a magnification of ×1200. The proportion of total damaged grains was determined in 6 randomly selected high-power fields by visual counting; a grain was considered to be damaged when the exine was broken or cracks were visible in the exine.

Assessment of pollen viability

Pollen viability was estimated using the standardized trypan blue staining technique, which stains cells with a damaged cell membrane. Briefly, 2 mg of treated pollen grains were mixed with 0.2 mL of 6% trypan blue for 5 minutes and then centrifuged at 1200 rpm (100g) for 3 minutes. Twenty-microliter (20-μL) samples of pollen were observed by light microscopy at ×400 magnification, using a DMLB microscope (Leica Microsystems, Wetzlar, Germany). The viability of pollen was determined in 5 randomly selected fields and expressed as the mean percentage of nonstained pollen relative to the total pollen grains.

Exposure of pollen to acid solution

Aliquots of pollens (10 mg) were suspended in 500-μL acid solutions (with pH 4, 5, and 6) and distilled water for 2 minutes, and 20 μL of each sample was placed observed under a light microscope at ×400 magnification, as in the previous section. The proportion of the damaged pollen grains was determined in 5 randomly selected fields by visual counting and expressed as the mean percentage of damaged pollen relative to the total number of grains in the sample.

Statistical analysis

All data were analyzed using Microsoft Office Excel 2007 spreadsheet (Microsoft Corp., Redmond, WA) and SPSS statistics version 21 (IBM Corp., Armonk, NY). Differences in the effects of air or pollutant exposure of pollen were considered statistically significant at a value of $p < 0.05$.

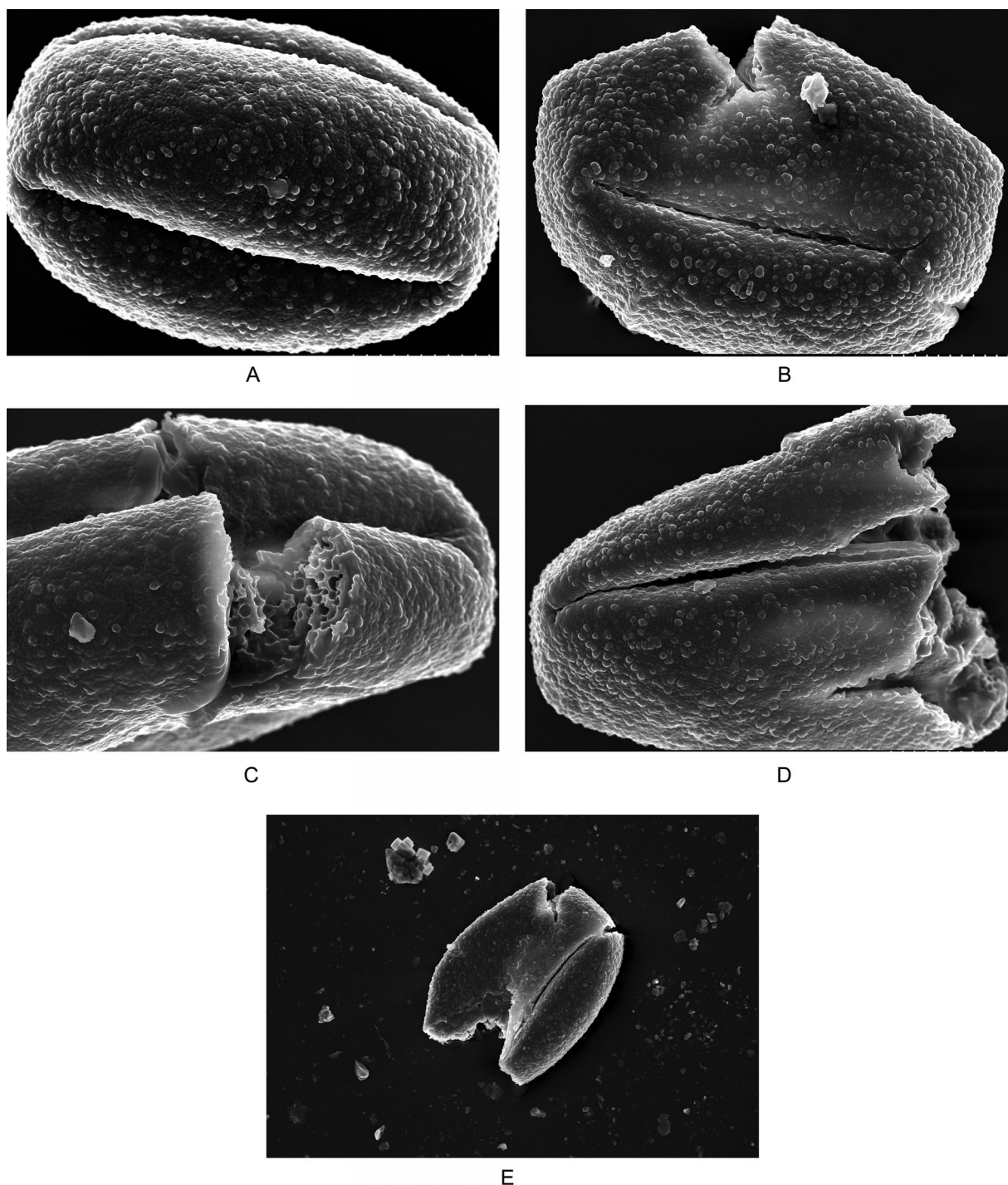


FIGURE 1. Scanning electron micrographs of oak pollen grains after exposure to air (A; $\times 3500$ magnification), 0.5 ppm NO₂ (B; $\times 3000$ magnification), 1 ppm NO₂ (C; $\times 4500$ magnification), 2 ppm NO₂ (D; $\times 3000$ magnification), and 5 ppm NO₂ (E; $\times 1200$ magnification).

Results

Pollen morphology following NO₂ or SO₂ exposure

Figure 1 shows representative scanning electron micrographs of pollen following exposure to air (Fig. 1A) or 0.5 to 5.0 ppm NO₂ (Fig. 1B–E). Exposure to 0.5 ppm to 2 ppm NO₂ resulted in fissures or complete breaks in the

exine (Fig. 1B–D), whereas exposure to 5 ppm NO₂ resulted in complete breakdown and release of pollen cytoplasmic granules (PCG) (Fig. 1E). Exposure of pollen grains to 0.5 to 5 ppm SO₂ also resulted in a similar damage pattern (data not shown).

Visual assessment of the proportion of pollen damaged following exposure to air or the pollutants showed that air induced damage to only 2.8% of the total grains (Table 1).

TABLE 1. The proportion of damaged oak pollens and pollen viability following NO₂ or SO₂ exposure

	Air ^a	NO ₂ ^a				SO ₂ ^a			
		0.5 ppm	1 ppm	2 ppm	5 ppm	0.5 ppm	1 ppm	2 ppm	5 ppm
Damaged pollens (%)	2.8 (0.4)	7.1 (0.5)*	10.2 (0.8)*	11.6 (1.3)*	14.7 (1.4)*	15.5 (2.1)*	15.6 (0.8)*	19.2 (2.0)*	20.4 (1.7)*
Viability (%)	95.8 (0.8)	90.1 (1.7)*	87.3 (1.6)*	84.9 (1.2)*	79.5 (2.1)*	84.9 (1.1)*	84.4 (1.3)*	79.8 (1.8)*	75.2 (1.0)*

^aStandard errors are in parentheses.

* $p < 0.05$, exposure to different concentrations of NO₂ and SO₂ vs exposure to air.

ppm = parts per million.

In contrast, exposure to NO₂ induced damage to between 7.1% of grains after exposure to 0.5 ppm NO₂ to 14.7% of grains after exposure to 5 ppm NO₂, whereas SO₂ induced an even greater level of damage, ranging between 15.5% of grains after exposure to 0.5 ppm SO₂ to 20.4% of grains after exposure to 5 ppm (Table 1).

Pollen viability following NO₂ or SO₂ exposure

Assessment of viability of the pollen grains following exposure to NO₂ or SO₂ indicated that the viability of the pollen grains significantly decreased compared to exposure to air (Table 1). Again, the effect of SO₂ appeared to be slightly more detrimental than the effect of NO₂ on viability of the pollen.

Pollen cytoplasmic granules release following contact with acid solution or water

Contact with acid solutions induced damage of the grains in a dose-dependent pattern, ranging from 24.6% at pH 6.0 to 55.8% at pH 4.0 (Table 2), and resulted in the release of PCG through pores and cracks developing in the grains (Fig. 2A, B). In contrast, the release of PCG was not observed after contact of the pollen grains with water.

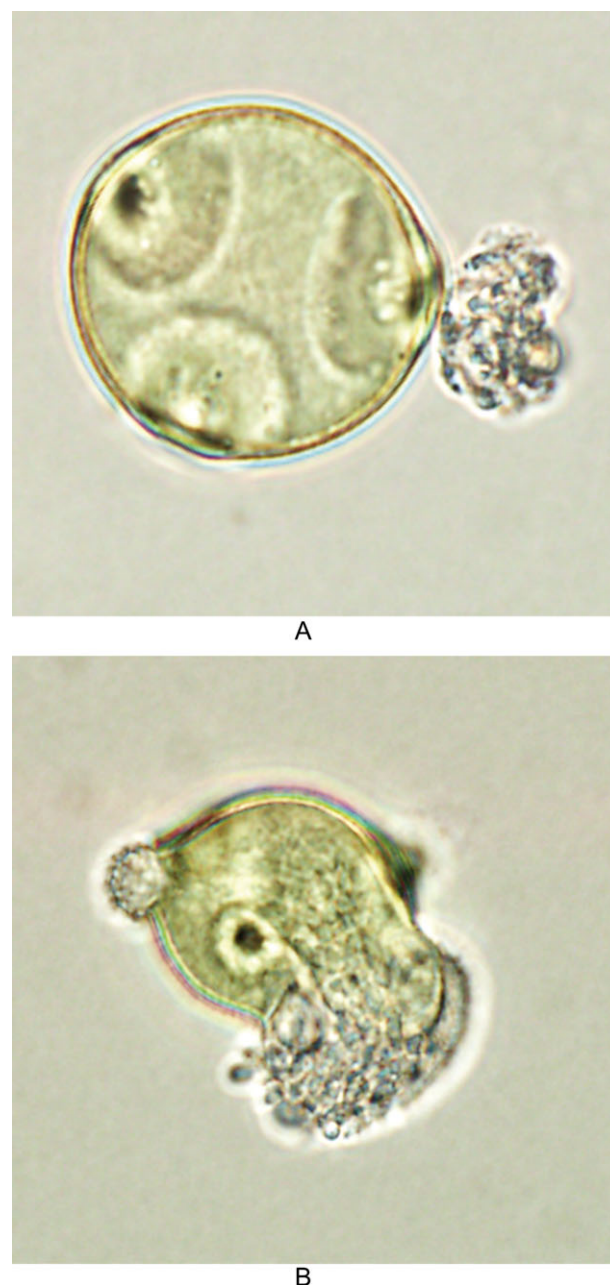
Discussion

Previous studies have shown that air pollutants induce airways inflammation, and play an adjuvant role in respiratory allergy.³ A recent study analyzed the effects of air pollutants on outpatient visits caused by allergic rhinitis (AR) in Beijing during the period 2009 to 2010, and demonstrated that particulate matter 2.5 to 10 μm in diameter (PM₁₀), SO₂, and NO₂ were associated with increased likelihood of hospital visits with every 10 $\mu\text{g}/\text{m}^3$ increase of pollutant concentration.¹³ Moreover, NO₂ was the most likely

TABLE 2. The proportion of damaged oak pollen grains after contact with solutions of various pH values

	pH values of the solutions			
	4.0	5.0	6.0	7.0
Damaged pollen (%)	55.8 $\pm$ 2.9*	53.2 $\pm$ 3.7*	24.6 $\pm$ 2.8*	3.8 $\pm$ 0.6

* $p < 0.05$ pH 4.0, pH 5.0, and pH 6.0 vs pH 7.0.

**FIGURE 2.** Release of PCG from oak pollen grain (A) from pores or (B) cracks in the damaged grains, after the contact with acid solution. PCG = pollen cytoplasmic granules.

of the 3 pollutants to increase the likelihood of hospital visits. Similarly, a more recent time series study investigating the association between ambient air pollution and hospital emergency admissions in Beijing has demonstrated that a $10\text{-}\mu\text{g}/\text{m}^3$ increase in concentration of PM_{10} , SO_2 , and NO_2 was associated with increases of 1.72%, 1.34%, and 2.57%, respectively, in respiratory disease emergency admissions at lag 2 day; and with increases of 1.39%, 1.56%, and 1.18%, respectively, in cardiovascular disease emergency admissions at lag 5 day.¹⁴ Also, preliminary data from our laboratory have indicated that nasal mucosa of mice exposed to inhaled pollutants have increased eosinophils and monocytes, compared to control mice not exposed to pollutants; and that exposure to inhaled pollutants lead to even greater increases in the numbers of eosinophils and monocytes in nasal mucosa of AR mice (data not shown).

Furthermore, air pollutants also exert an effect on airborne pollen allergens. In China, the oak pollen is a major source of airborne allergens; releasing allergenic proteins present on the surface of pollen starch granules and within the PCG. Motta et al.¹⁵ have shown that following PCG released from grass pollen can form a respirable allergenic aerosol, which has been shown to be capable of reaching the deep airways and inducing local inflammatory and allergenic reactions in animal models of asthma and allergy.^{16–18}

In this study, we provide evidence that exposure of oak pollen to air pollutants NO_2 or SO_2 significantly damages the pollen grains, decreases their viability, and induces the release of PCG, compared to air exposure. Our study demonstrated that whereas a relatively low concentration of 0.5 ppm NO_2 or 0.5 ppm SO_2 both caused variable damage of the pollen exine, a higher concentration of 5.0 ppm of both pollutants resulted in severe damage and disintegration of the exine, leading to the likely release of PCG. In a study investigating the effect of exposure to NO_2 on Timothy grass (*Phleum pratense*) pollen, Motta et al.¹⁵ found that the proportion of the damaged pollen retained a plateau at 6.4% until the NO_2 concentration reached 10 ppm, when 9% of the pollen were damaged, and increasing to 15% damage at the maximum concentration of 50 ppm NO_2 . Unlike our studies, which were carried out using freshly collected oak pollen grains, these authors used commercially distributable pollens that had been stored at 4°C for more than 1 year. Thus, it is possible that the difference in our findings and those of Motta et al.¹⁵ are likely to be a consequence of not only the morphological differences in the pollen species used for investigation, but also caused by the changes in the characteristics of the pollen induced

by storage over large periods. Indeed, the pollen showed a marked decrease in viability with increased concentration of the pollutant. In another investigation, a decline in the pollen viability of *Betula pendula* and *Ostrya carpinifolia* was reported after exposure for 2 days to a lower concentration of NO_2 (0.034 ppm),⁸ suggesting that the viability and susceptibility of pollen to NO_2 -induced damage is likely to be dependent on a combination of the concentration of NO_2 , the period of exposure, and the pollen species. Compared to NO_2 exposure, there is comparatively little information of the effects of SO_2 exposure on pollen. However, an environmental concentration of 1 ppm SO_2 is frequently reached in many Asian countries or industrial zones, predominantly as a result of heavy coal combustion during the winter or on smog days.^{1,19} SO_2 , either alone or in combination with NO_2 , may lead to the production of acid rain with detrimental ecological consequences; including a negative impact on growth and productivity of forest trees and crop plants.²⁰

Some pollen grains release PCG via the pores following contact with water.¹⁵ However, in the present study PCG release from oak pollen was not observed on contact with neutral water, but was found to be released in a pH-dependent manner in contact with acid solutions. In acid solutions, PCG was expelled from the grain through the pores and cracks in the exine when the pollen grain was damaged. Although these findings might partly explain the cause for the higher frequency of allergic reactions in patients allergic to pollens following rainfall, the mechanisms involved in this phenomenon remain to be elucidated.

Conclusion

In summary, short-term exposure of oak pollen to high concentrations of SO_2 or NO_2 leads to a significant decrease in the viability of pollens and an increase in their fragility, leading to the subsequent PCG release. Similarly, SO_2 -mediated or NO_2 -mediated acidification of water leads to an increase in the level of PCG released from oak pollen. These results suggest that heightened air pollution during the oak pollen season may possibly increase the incidence of allergic airway disease in sensitized individuals by facilitating the bioavailability of airborne oak pollen allergens. It is possible that exposure to SO_2 or NO_2 may alter the allergenicity of the allergens released from the pollen. Thus, future investigations should assess the effect of PCG released from pollutant exposed-pollen on the immunomodulatory mechanisms underlying allergic disease. 

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