



Characterization of allergenicity of *Platanus* pollen allergen a 3 (Pla a 3) after exposure to NO₂ and O₃[☆]

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ABSTRACT

Pollen allergens, widely present in the atmosphere, are the main cause of seasonal respiratory diseases that affect millions of people worldwide. Although previous studies have reported that nitrogen dioxide (NO₂) and ozone (O₃) promote pollen allergy, the specific biological processes and underlying mechanisms remain less understood. In this study, *Platanus* pollen grains were exposed to gaseous pollutants (NO₂ and O₃). We employed environmental electron microscopy, flow cytometry, western blot assay, enzyme-linked immunoassay, ultraviolet absorption spectrometry, circular dichroism, and protein mass spectrometry to characterise the subpollen particles (SPPs) released from pollen grains. Furthermore, we determined the immunogenicity and pathogenicity induced by *Platanus* pollen allergen a 3 (Pla a 3). Our results demonstrated that NO₂ and O₃ could damage the pollen cell membranes in SPPs and increase the amount of Pla a 3 allergen released into the atmosphere. Additionally, NO₂ and O₃ altered the structure of Pla a 3 protein through nitric oxidation and oxidation, which not only enhanced the immunogenicity of allergens but also increased the stability of the protein. *In vivo* analysis using an animal model indicated that NO₂ and O₃ greatly aggravated pollen-induced pneumonia. Thus, our study provides guidance for the prevention of pollen allergic diseases.

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1. Introduction

Pollinosis is an increasingly widespread allergic disease which is triggered by pollen grains, such as *Ambrosia* pollen, *Humulus scandens* pollen, *Platanus* pollen, and *Poaceae* pollen. Airborne pollen can cause severe allergies, including asthma, rhinitis, and lung inflammation (Hosoki et al., 2016; Wahn et al., 2019; Yan et al., 2019). More than a quarter of the world's population reportedly suffers from pollen allergies (D'Amato et al., 2007; Dahl, 2018). Therefore, pollen allergy is a common health problem that has attracted the attention of researchers worldwide (Asam et al., 2015; Dahl, 2018). A large number of pollen allergen proteins have been

isolated and identified from herbs (such as ragweed, *Humulus scandens*, and wormwood) and woody plants (such as birch, poplar, and plane tree) (Bouley et al., 2015; Wangorsch et al., 2016; Zhang et al., 2016; Hong et al., 2018; Wang et al., 2018; Guan et al., 2019; Wahn et al., 2019). Many advances have also been made in the exploration of the physiological mechanism of pollen allergy. Previous research has confirmed that pollen allergens can be released from pollen in the form of sub-pollen particles (SPPs) and inhaled into the lungs through the respiratory tract (Smiljanic et al., 2017; Yan et al., 2019; Grewling et al., 2020). The SPPs contained pollen allergens, NAD(P)H oxidases, and lipids, which significantly increased pollen allergy symptoms (Bacsi et al., 2006; Smiljanic et al., 2017; Yan et al., 2019). The SPPs are vesicle particles with of 0.1–1.0 μm diameter and could be released from pollen grains upon hydration and rupture of the grains. (Pazmandi et al., 2012; Smiljanic et al., 2017; Yan et al., 2019).

Epidemiological investigation and experimental research showed that air pollutants had a significant effect on pollen allergy

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(Peden and Reed, 2010; Grundström et al., 2017; Grewling et al., 2020). Exposure to both pollen and air pollutants would have more major health effects than exposure to only pollen or air pollutants (Peden and Reed, 2010). Air pollutants may function as stimuli to cause respiratory inflammation and damage to epithelial barrier homeostasis, thus facilitating allergen access to effector cells in an allergic immune response (Traidl-Hoffmann et al., 2009). Moreover, O₃, NO₂, and PM_{2.5} (particulate matter with an aerodynamic diameter of less than 2.5 µm) may induce the production of high levels of reactive oxygen species, thereby aggravating the allergic inflammatory response (Klein et al., 2012). In addition, O₃ and NO₂ could also affect the structure of allergen proteins (Franze et al., 2005; Lu et al., 2014) and change its stability and allergenicity, ultimately enhancing the allergic effect (Zhao et al., 2016; Hong et al., 2018).

Allergen proteins can interact with air pollutants after being released from SPPs. Some allergenic proteins are adsorbed onto the surface of ambient particles and spread with them in the air (Ribeiro et al., 2015; Süring et al., 2016). After the allergen is exposed to oxidising pollutants or nitrides, the structure of the protein changes (Frank and Ernst, 2016; Zhang et al., 2018). A change in the structure of an allergen may further influence its allergenicity, which enhances the ability of the protein to trigger immune responses (Grundström et al., 2017; Hong et al., 2018). Moreover, a structural change of allergen protein may increase its stability and prolong the existence of pollen allergens in the air, which increases the risk of exposure to an allergen in the allergic population and exacerbating the incidence of pollen allergy (Chassard et al., 2015; Zhao et al., 2016). Therefore, a thorough understanding of the effects of air pollutants on pollen allergies would help alleviate the threat of pollen allergy to the health of population.

With rapid urbanisation in China, the levels of air pollutants, such as O₃ and NO₂, in urban atmospheres are increasing (Xing et al., 2019; Hu et al., 2020). Therefore, understanding the mechanism of influence of O₃ and NO₂ on pollen allergen has practical significance in the development of treatment strategies for pollen allergy. *Platanus acerifolia* is a common tree species in cities across the world (Lu et al., 2014), and its airborne pollen has become a major allergy factor during its bloom in spring. *Platanus* pollen contains three main allergen proteins—Pla a1, Pla a2, and Pla a3 (Wang et al., 2018)—which induce allergic reactions in humans. In terms of biological function, Pla a3, with a molecular weight of between 9 and 10 kDa, is a non-specific lipid-transfer protein (ns-LTP) (Sankian et al., 2012; Sedghy et al., 2016). It is stabilised by four disulphide bonds and shows a characteristic hydrophobic cavity. It is the main lipid binding site that can transfer lipids between membranes. In our previous studies, we purified the Pla a3 protein and prepared a high-titre antibody (Zhou et al., 2019). To investigate the effects of air pollutants represented by NO₂ and O₃ on allergic pneumonia caused by the Pla a3 allergen of *Platanus* pollen, we used laboratory exposure devices to simulate the exposure of *Platanus* pollen to air pollutants. The effect of NO₂ and O₃ pollution on pollen allergy is described in terms of allergen production, allergen release, and allergenicity. This study provides reliable data and experimental support for understanding the mechanism underlying the effect of environmental pollution on pollen allergy.

2. Materials and methods

2.1. Fresh pollen collection

The sampling method has previously been described by Hong et al. (2018). Briefly, the mature *Platanus* globule inflorescences were sampled before they began to spread pollen grains, along

Shangda Road in Shanghai City. The globule inflorescences were placed in a cool, ventilated room. Two days later, the inflorescences were gently vibrated to disperse the pollen. The pollen was collected from foil paper on which it fell. Finally, the pollen was filtered through a 40 µm mesh and placed into a 15 mL sterile Eppendorf (EP) tube and stored at 4 °C.

2.2. Extraction of sub-pollen particles

Platanus pollen (2 g) was soaked in 5 mL deionised H₂O (dd H₂O) for 6 h and then centrifuged at 1600×g for 5 min. The supernatant was transferred into a new EP tube and centrifuged at 13,000×g for 15 min. A pellet was obtained and dissolved in phosphate-buffered saline (PBS) (pH 7.4). Then, a Pierce BCA protein quantification kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify the total protein amount in the SPPs.

2.3. Flow cytometry assay

Platanus pollen (0.2 g) was soaked in 1 mL dd H₂O, shaken gently for 2 h, followed by the addition of an equal volume of 1.0% glutaraldehyde to fix subpollen particles released from the pollen grains for 5 min. Flow cytometry (Moflo XDP, BECKMAN, USA) was used to analyse the change in the amount of SPPs released from the pollen after the hydration treatment. Pollen grains and SPPs were statistically analysed according to the size range of the detected particles, and the results are presented in a scatter plot.

2.4. Microscopic observation of pollen grains

Platanus pollen (0.2 g) was washed three times with dd H₂O, and then 1.0 mL dd H₂O was added to prepare the pollen suspension. Hydration of the pollen was carried out for 2 h in an MX-RL-E rotary mixer (SCIOGEX, USA). Next, an equal volume (1.0 mL) of 2.0% glutaraldehyde was added for fixation. Thereafter, 100 µL of the pollen suspension was The damaged pollen was covered with a cover glass and the grains were observed and counted under a DM2500 optical microscope (Leica, Germany).

2.5. Environmental scanning electron microscopy assay

Approximately 0.1 g of pollen grains were placed in a 1.5 mL EP tube to create a suspension with deionised water, and 200 µL of the pollen suspension was dripped onto the tin foil on the platform and observed under a Quanta200FEG environmental scanning electron microscopy (FEL, USA). The magnification, voltage, and working distance were 12,000 × 3.0 kV, and 4.9–5.1 mm respectively.

2.6. Production and purification of Pla a3 protein

Considering that there are numerous types of proteins in pollen grains, and Pla a3 is not a dominant protein, it is difficult to isolate sufficient amounts of pure Pla a3 allergen directly by naturally extracting pollen grains. Therefore, a recombinant Pla a3 and tandem six-histidine fusion protein were expressed in a prokaryotic system and purified by affinity chromatography (Zhou et al., 2019). The coding sequence (CDS) of *P. acerifolia* allergen Pla a3 was obtained from the GenBank database: <http://www.ncbi.nlm.nih.gov/genbank/> (ID number AJ427413.2) and cloned by polymerase chain reaction (PCR) using the forward primer GCGGAATT-CATGGCCTTCTCTAGAGTTGC and reverse primer GGGGTCCG-CTCACTTCACCTTGGAGCAGTCAATGGTGGGGC. After PCR amplification and double digestion with *Sall* and *EcoRV*, the Pla a3 CDS was ligated into the pET28a vector and transformed into the *Escherichia coli* TOP10 strain and the Rosita strain for plasmid proliferation and

Pla a3 expression, respectively.

Pla a 3 protein production was induced in the pET28a–Pla a 3–His Rosetta strain after 1 mM IPTG was added to the medium. As the OD₆₀₀ value of the bacterial cultures reached 1.8–2.0, bacteria were collected and disrupted by sonication. The crude protein extract was centrifuged at 13,000×g at 4 °C for 15 min. The supernatant was recovered and incubated with Ni-NTA beads for 1 h. Then, the sample was centrifuged at 700×g at 4 °C for 20 min, and the Ni-NTA beads adsorbing the target protein were collected. After washing it with wash buffer (10 mM NaH₂PO₄, 60 mM NaCl, 50 mM imidazole), the target protein was obtained and eluted in elution buffer (10 mM NaH₂PO₄, 60 mM NaCl, 250 mM imidazole).

2.7. NO₂ and O₃ exposure treatment

The pollen grains and Pla a 3 protein were treated with NO₂/O₃ in an exposure chamber (Lu et al., 2014; Hong et al., 2018). The pollen grains and protein were transferred into a septic fresh-keeping film and exposed to NO₂/O₃, NO₂, and O₃. The mass concentrations of NO₂ and O₃ in the chamber were set at 400 ppb and 750 ppb, respectively. After 3 h of exposure, the working concentrations of NO₂ and O₃ decreased to 200 ppb and 350 ppb, respectively (Lang-Yona et al., 2016). After 6 h of exposure, the samples were collected and analysed.

2.8. Western blotting assay

After sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred onto nitrocellulose membranes through electrical transfer. Bovine serum albumin (1%) was added to seal the membrane for 1 h, followed by incubation of the blocked membranes with anti-rPla a 3 IgE antiserum from sensitised rats (HuaBio Co. Ltd, China) (1:2000 dilution) at 37 °C for 1 h. Next, the membrane was washed three times (5 min each) with Tris-buffered saline containing Tween-20 (TBST) (pH 7.4). Horseradish peroxidase (HRP)-labelled secondary antibodies (1:5000 dilution) (YESEN Biotechnology Co. Ltd, China) were added and incubated at 37 °C for 1 h. Next, the membranes were washed with TBST (pH 7.4) again. Finally, the substrate, tetramethyl benzidine (TMB), was added, followed by incubation at 37 °C for 10–30 min for colour development. The grey value of the protein bands was analysed using Image J software, and histograms were generated using GraphPad Prism5 software. Three independent experiments were performed.

2.9. Immunofluorescence localisation of Pla a 3 allergen

Pollen grains (0.01 g) were inserted in 1.5 mL EP tubes. After rinsing it three times with PBS, the pollen was fixed for 5 min using 500 µL of 2.5% glutaraldehyde paraformaldehyde. The pollen suspension was washed it three times with PBS, followed by incubation with anti-Pla a 3 antibody (1:500) at 4 °C overnight. After washing it with PBS again, the Alexa-488-labelled secondary antibody (1:5000) was added and incubated, avoiding light for 1 h at 25 °C. Finally, fluorescence intensity was observed under a Zeiss LSM 710 confocal microscope (Zeiss, Germany). Five visual fields were randomly selected from each sample, and three independent experiments were carried out.

2.10. Detection of membrane damage in the pollen grains

Pollen grains and deionised H₂O were mixed to prepare pollen grain suspension. Trypan blue (0.4%) was added to the solution (V:V = 1:9) and incubated for 3 min. The pollen grains were collected and washed three times with dd H₂O. Then, the pollen

suspension was dropped onto a slide. Finally, the slide was covered with glass, and the blue pollen was observed and counted under an optical microscope. The boiled pollen was stained blue as a positive control. Five fields of view were randomly selected under a microscope to observe the samples, and three independent experiments were performed.

2.11. Mass spectrometry of Pla a 3 protein after exposure to NO₂ and O₃

After exposure to NO₂/O₃, the purified Pla a 3 protein was dissolved in PBS (pH 7.2) at a concentration of 0.1 mg/mL, followed by SDS-PAGE to separate and recover the protein. After decolourisation, the protein was added to 40 µL trypsin buffer and enzymolysed for 16–18 h at 37 °C. The enzymolysis product was collected on a Thermo Scientific EASY column (75 µm × 100 mm × 3 µm; C18) for desalination and separation with capillary high-performance liquid chromatography (HPLC). Formic acid and formic acid acetonitrile (both 0.1%) were used as the A and B solutions, respectively. The HPLC products were analysed using a Q Exactive Mass Spectrometer (Thermo Fisher, USA). The data were searched using Mascot2.2 for the corresponding database (R554.fasta), and the identified protein results were extracted.

2.12. Ultraviolet absorption and circular dichroism analysis

The Pla a 3 protein samples (0.1 mg) exposed in the different treatment groups were analysed using an ultraviolet (UV)-vis spectrophotometer (UV759CRT, China) with a scanning wavelength of 250–420 nm and a scanning rate of 100 nm/min. The protein samples (0.1 mg) were dissolved in PBS for circular dichroism (CD) measurement using a Jasco J-815 spectropolarimeter (Jasco, MA, Japan) in the far-UV region from 190 to 250 nm. Ellipticity was recorded at a scanning speed of 500 nm/min, an accumulation of 3, and a bandwidth of 0.2 ± 0.1 nm. Three repeated samples were prepared for each test, and three experiments were carried out independently.

2.13. Enzyme-linked immunosorbent assay

Microtiter plates (96-well) were coated with 200 µL of the protein sample and incubated overnight at 4 °C avoiding light (AL). After rinsing with PBS three times, 100 µL of rat-derived anti-Pla a 3 antibodies (1:1000 dilution) was added into each well and incubated at 37 °C for 1 h AL. Next, the plates were washed three times with PBS, and 100 µL of HRP-labelled secondary antibodies (1:10,000 dilution) was added. The samples were again incubated at 37 °C (AL) for 0.5–1 h. After washing with PBS, 100 µL of freshly concocted TMB substrate solution was added to each well and incubated at 37 °C for 15 min AL. Finally, 50 µL of 2 M sulphate was added to stop the reaction. The positive cutoff was considered 2-fold the optical density (OD, at 450 nm) of the negative control. SARS software was used for statistical analysis.

2.14. Establishment of the mouse model of pollen allergic pneumonia

Pla a 3 protein (0.2 mg) and isopyknic Freund's complete adjuvant were intraperitoneally injected into pathogen-free male BALB/c mice (4 weeks of age). One week later, the mice were injected with 0.2 mg of Pla a 3 and isopyknic Freund's incomplete adjuvant. This procedure was repeated in the subsequent week. Mice in the control groups were administered PBS in Freund's incomplete adjuvant. Finally, the mice received a challenging dose of atomisation treatment with pollen extracts for 30 min every day,

continuing for a week. Each experimental group consisted of three mice.

2.15. Analysis of superoxide dismutase (SOD) and malondialdehyde (MDA) from mouse lung tissue

Here, 2 mL of extraction reagents were added to 0.2 g lung tissue homogenate and centrifuged at $12,000\times g$ for 5 min at 4 °C. The supernatant was collected, and the SOD was quantitatively detected using a total superoxide dismutase assay kit (Nanjing Jiancheng Bioengineering Institute, China). The experiment was carried out according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Inc.).

After 0.2 g lung tissue was lysed with 2 mL of lysate, the sample was centrifuged at $14,000\times g$ for 5 min to collect the supernatant. Next, three times the volume of TBA detection solution (working concentration of 0.37%) was added, and the mixture was boiled for 15 min. After cooling to 25 °C, the mixture was centrifuged at $1000\times g$ for 10 min. Finally, the absorbance of the supernatant was detected at 532 nm using an enzyme labelling apparatus (Varioskan Flash 3001, Thermo Fisher).

2.16. Analysis of inflammatory factors in mouse lung tissue

Measurement of allergic response from the mice was performed according to a previously described method (Hong et al., 2018). Briefly, after euthanasia, the mouse blood was collected into 1.5 mL EP tubes containing anticoagulant. The serum was collected by centrifuging the blood sample at $3000 g$ at 4 °C for 10 min. Next, the Pla a 3-specific IgE and IgG in the serum was analysed using an ELISA kit (Westang Biotechnology Co., Ltd, Shanghai, China), according to the manufacturer's instructions. Moreover, 0.2 g lung tissue was homogenised in 2 mL PBS and centrifuged at $12,000\times g$ for 5 min at 4 °C. The supernatant was collected for detection of histamine and cytokines using an Inflammatory Factor Detection Kit (Nanjing Jiancheng Bioengineering Institute, China), following the manufacturer's instructions.

2.17. Analysis of pathological sections from mouse lung tissue

The lung tip tissue (approximately 0.5 cm^3) was fixed overnight with 4% paraformaldehyde solution (0.1 M PBS, pH 7.4) at 25 °C. After rinsing three times with 0.1M PBS (pH 7.4), the samples were dehydrated, replaced, and waxed according to the method described by Zhou et al. (2018). Finally, the embedding blocks containing samples were sliced into 5 μm sections with Leica RM2235 slicers. Permanent slices were prepared by HE staining (Yan et al., 2019) and observed under a Leica DM2500 microscope. The lung tissue of three mice was analysed in each treatment group, and three experiments were performed independently.

2.18. Statistical analysis

SPSS version 11.5 was used to perform ANOVA for comparison among the experimental groups. Data from at least three independent experiments were analysed and expressed as mean $\pm$ standard error (SD). The values $*p < 0.05$ and $**p < 0.01$ indicated statistically significance.

3. Results

3.1. NO₂ and O₃ exposure changed the content and distribution of Pla a 3 allergen in *Platanus* pollen

The Pla a 3 allergen was mainly distributed in the cytoplasm

(Yan et al., 2019), and immunofluorescence staining with the polyclonal antibody of the anti-Pla a 3 showed a uniform dispersion state of the green fluorescence in the entire pollen cell (Fig. 1A-no treatment). This indicated that Pla a 3 was indeed distributed in the cytoplasm, and its distribution was homogeneous. However, after exposure to O₃ and NO₂, high brightness fluorescent spots were observed in pollen cells (Fig. 1A- O₃ treatment, NO₂ treatment, and O₃ + NO₂ treatment). Compared with the no-treatment group (Fig. 1A-no treatment, anti-pla a 3(-)), the exposed pollen groups showed a higher fluorescence intensity (Fig. S1A). The content of Pla a 3 in pollen was analysed by western blot (WB) assay. The results showed that the band of Pla a3 protein in the no-treatment group was shallow, whereas those in the three different treatment groups were deeper to varying degrees (Fig. 1B) The grey values of the Pla a 3 protein bands in the WB results were measured using ImageJ software and compared with that of Tublin protein. The grey values of Pla a 3 in the pollen from the O₃, NO₂, or O₃/NO₂ combination treatment groups were significantly higher than those in the no-treatment group (Fig. 1C). Additionally, the results of quantitative analysis of the total protein showed that there was no significant difference in the content of total protein in pollen of all experimental groups (Fig. S1B), but the content of the Pla a 3 allergen in pollen from the three different pollutant treatment groups detected by ELISA was significantly higher than that in the control groups (Fig. 1D). Further analysis of the proportion of the Pla a3 allergen in the total pollen protein showed that the Pla a3 content in the exposure groups was significantly higher than that in the control group (Fig. 1E). The amount and spatial distribution of Pla a 3 allergens in the pollen grains changed significantly after exposure to NO₂ and O₃. The Pla a 3 protein is a non-specific lipid transferase, therefore, Pla a 3 exposure to NO₂ and O₃ is likely to cause damage to the cell membrane of pollen grains.

3.2. Characterisation of pollen grain membrane after pollen exposure to O₃ and NO₂

The pollen from the different treatment groups was stained with Trypan blue. The experimental results indicate that some of the intact viable pollen grains were stained light blue without exposure to pollutants. Many pollen grains maintained a colourless and transparent state similar to those in the unstained group (Fig. 2A-No dyed pollen and dyed natural pollen); however, the boiled pollen grains (all pollen damaged) were dyed dark blue (Fig. 2A-dyed dead pollen). In the NO₂, O₃, and NO₂/O₃ combination treatment groups, the pollen grains were stained blue in varying degrees (Fig. 2A-dyed NO₂ treated pollen, dyed O₃ treated pollen and dyed NO₂+O₃ treated pollen), and the deepest blue could be found in the NO₂/O₃ combination treatment group (Fig. 2A-dyed NO₂+O₃ treated pollen). The ratio of blue pollen grains in the different treatment groups was $8.8\% \pm 1.9\%$ in the control group (unexposed), $55\% \pm 5.4\%$ in the NO₂ treatment group, $44\% \pm 6.1\%$ in the O₃ treatment group, and $82\% \pm 3.2\%$ in the NO₂/O₃ treatment group (Fig. 2B). This indicated that the integrity of the pollen cell membrane of *Platanus* pollen was damaged after exposure to pollutants. It was found that the exine of normal pollen was complete and the ornamentation was clear (Fig. 2C-control group). Surprisingly, in the NO₂ treatment group, the exine was significantly damaged. Part of the exine was badly torn, and the cell membrane was exposed directly (Fig. 2C- NO₂ treated; the ratio was approximately $12.5\% \pm 2.3\%$). In the O₃ treatment group, although there was no evident damage to the exine, some unknown substances filled the ornamentation gap of the exine (Fig. 2C- O₃ treated). In addition, in the O₃/NO₂ combination treatment group, the changes in exine had the characteristics of the above two groups (Fig. 2C- NO₂+O₃ treated; the ratio was approximately $26.7\% \pm 4.4\%$).

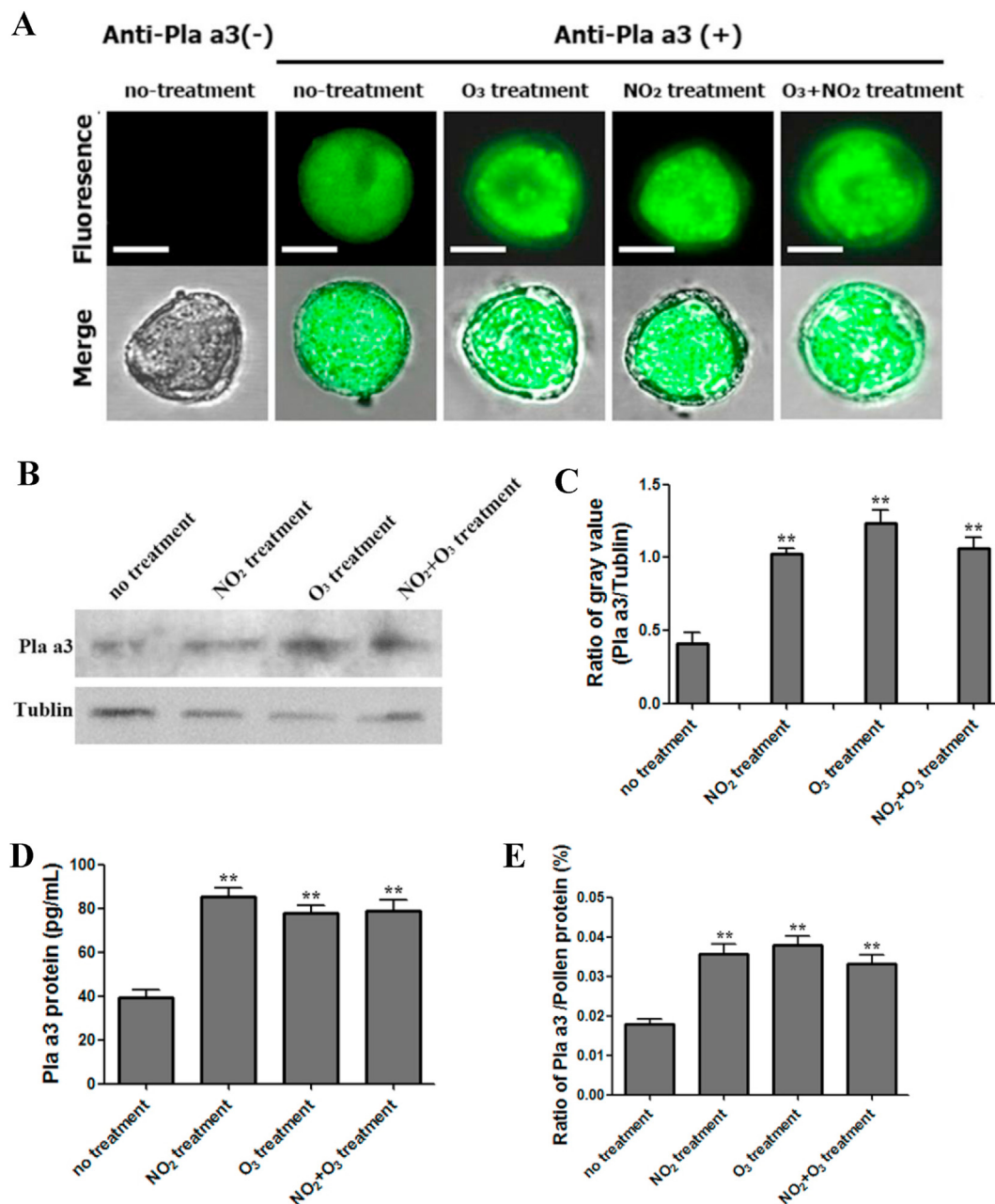


Fig. 1. Detection of Pla a3 allergen content and its distribution in pollen grains. (A) Immunofluorescence assay of Pla a3 allergen in pollen cells with different treatment. (B) Pla a3 protein content in pollen grain after exposure to different exposure groups by western blot. (C) The grey-scale comparing analysis of the WB protein band by using the internal reference Tubulin protein showed the content of Pla a3 protein. (D) ELISA assay showed that the content of Pla a3 protein in pollen of exposed group was significantly higher than that of non-exposed group. (E) Proportion of Pla a3 allergen in total pollen protein of different treatment groups. Each value represent mean $\pm$ standard errors ($n = 15$), ** $P < 0.01$, * $P < 0.05$, at least three independent replications were performed for all assays. Bar = 10 μ m.

After hydration, pollen could release sub-pollen particles, and the damaged pollen wall and cell membrane promoted the release of SPPs. Therefore, the release efficiency of SPPs could indirectly reflect the degree of damage of the pollen cells. Our flow cytometry analysis of SPP release from pollen from different treatment groups showed that the ratio of SPPs released in the unexposed group was approximately $7.33\% \pm 0.72\%$ (Fig. S2A-no treatment and B); however, in the NO₂ or O₃ exposure group, the proportion of SPPs changed significantly (Fig.S2A- NO₂ treatment to NO₂+O₃ treatment). It was approximately $14.09\% \pm 0.71\%$ in the NO₂ treated group, $5.42\% \pm 0.80\%$ in the O₃ treated group, and $8.08\% \pm 0.61\%$ in the co-treatment group (Fig. S2B). Meanwhile, the morphology of

the pollen was observed under a microscope, and the proportion of damaged pollen was counted. The proportion of ruptured pollen in the control group was approximately $10.14\% \pm 0.45\%$ (Fig. 3A-CK and Fig. 2S-C). The highest proportion of ruptured pollen ($19.95\% \pm 0.85\%$) was observed in the NO₂-treatment group (Fig. 3A- NO₂ treatment and Figs. S2-C). The proportion in the O₃ treatment and co-treatment groups were $8.68\% \pm 0.65\%$ and $11.99\% \pm 0.51\%$, respectively. Considering that the pollen allergen was mainly distributed in the form of SPPs, and the total allergens in pollen can be extracted by complete crushing with grinding, the ratio of Pla a 3 extracted by soaking was investigated. Compared with that in the unexposed group ($8.34\% \pm 0.42\%$), the proportion of

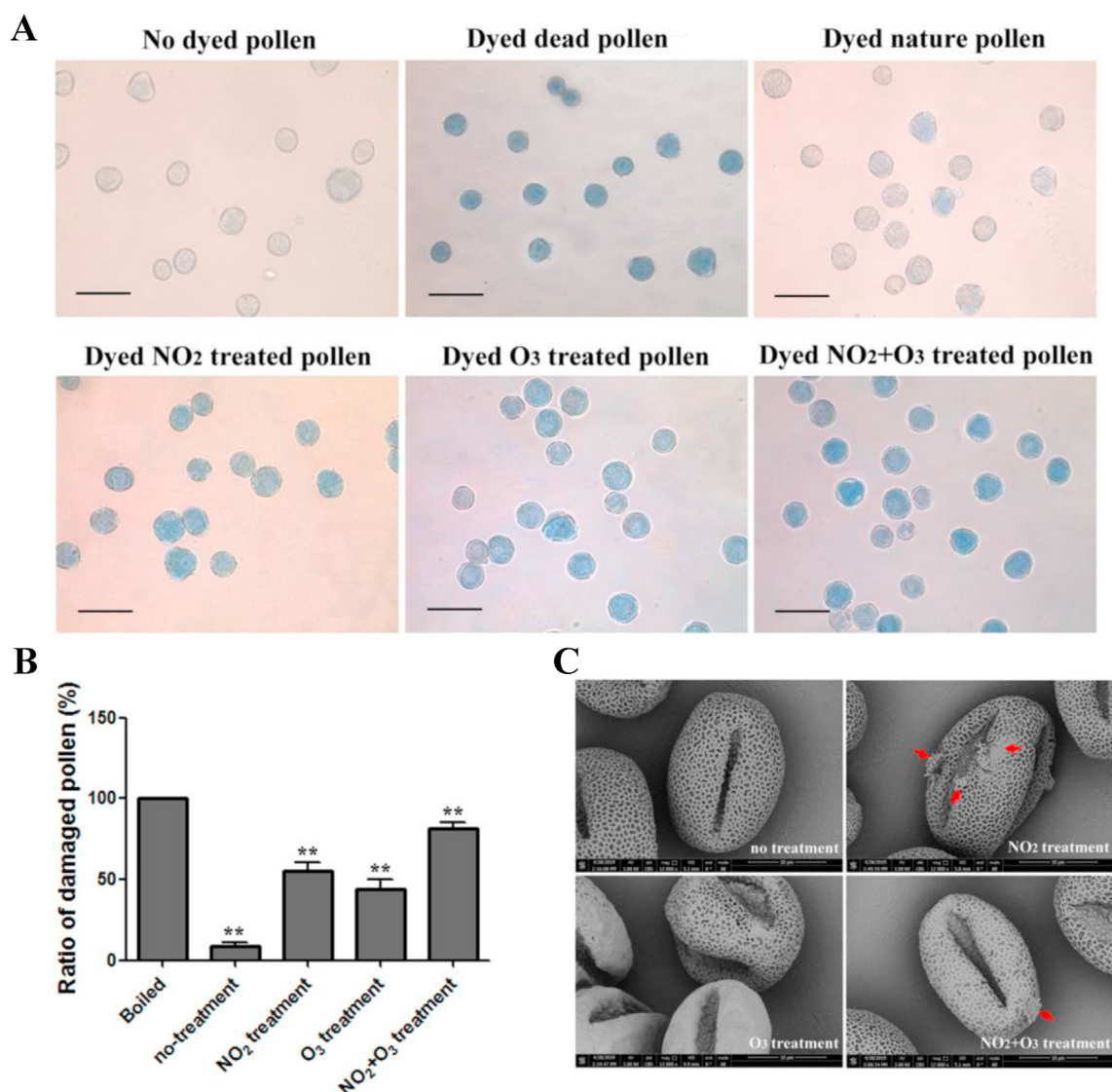


Fig. 2. Microscopic characterization of the *Platanus* pollen after treated with gaseous pollutants. (A) Analysis of permeability of pollen cell membrane by Trypan Blue staining. (B) Statistical analysis of damaged pollen ratio obtained by trypan blue staining analysis. (C) Scanning electron microscope (SEM) to observe the characteristics of extine in different treatment groups. Each value represent mean $\pm$ standard errors ($n = 15$), $**P < 0.01$, at least three independent replications were performed for all assays. Bar = 20 μm in (A) and 10 μm in (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

soaked Pla a 3 protein was significantly higher in the NO₂ and the NO₂/O₃ combination groups ($16.63 \pm 0.92\%$ and $9.57 \pm 0.79\%$) but lower in the O₃ exposure group ($3.92 \pm 0.43\%$) (Fig. 3B). These results suggest that when pollen is exposed to NO₂ and O₃, cell membranes and exine are damaged. Hence, pollen can easily break up after absorbing water from the air, resulting in more SPPs being released from damaged pollen and more Pla a 3 allergens being released into the air. Therefore, NO₂ and O₃ exposure greatly increased the risk of pollen allergy.

3.3. NO₂ and O₃ exposure treatment enhanced the immunogenicity of Pla a 3 allergen

The results showed that the binding titre of Pla a 3 to antibody was the highest in the NO₂ treated group, and the NO₂-exposed Pla 3 protein had the lowest credible detection concentration under a certain level of antibody. The control group had the weakest binding ability to antibody, but the binding ability of the O₃ and

NO₂/O₃ combination treatment groups was intermediate between that of the other two treatment groups (Fig. 4A). Subsequently, BALB/c mice were immunised with different exposure-treated Pla a 3 proteins from the different treatment groups to establish a mice model of pollen-induced pneumonia. The levels of IgG and IgE antibodies in the mouse blood were analysed. The Western blot assay showed that the IgG and IgE bands were poor in mice immunised with natural Pla a 3 (without exposure) (Fig. 4B). However, the protein bands of IgG and IgE antibodies were enhanced in mice immunised with Pla a 3 protein exposed to NO₂ and O₃. The results showed that the IgG and IgE antibodies induced by unexposed Pla a 3 were the weakest (Fig. 4B). NO₂ and O₃ exposure enhanced the ability of Pla a 3 protein to induce an immune response in the mice and promoted more IgG and IgE antibody production, whereas NO₂/O₃ combined exposure made Pla a 3 stimulate the highest levels of IgG and IgE antibodies (Fig. 4C). The levels of anti-Pla a 3 IgG and IgE in the serum of BALB/c mice were quantitatively detected by ELISA. Compared with the non-treated

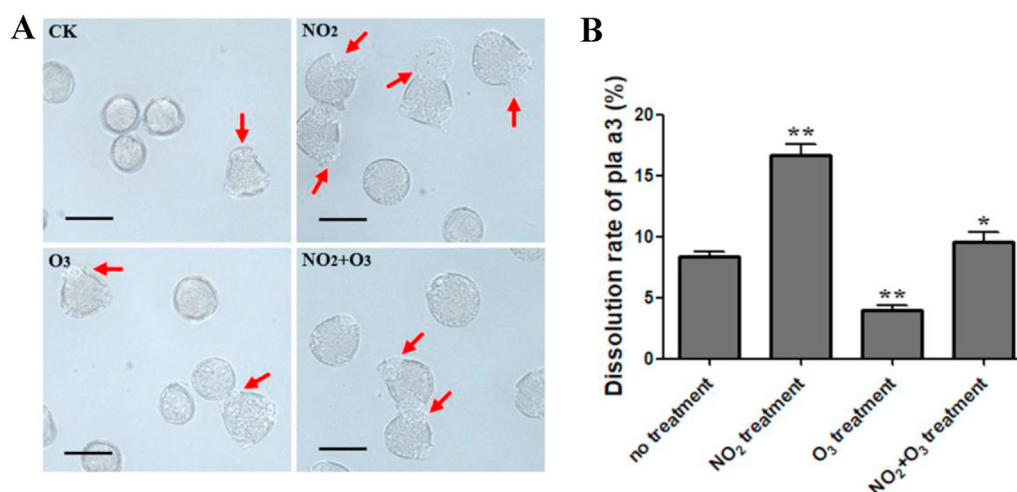


Fig. 3. Observation and statistical analysis of SPPs releasing from pollen. (A) Ruptured pollens in different treatment groups were observed under an optical microscope. The red arrows pointed to the SPPs releasing from broken site. (B) The proportion of Pla a3 protein released by SPPs from pollen. Each value represent mean $\pm$ standard errors ($n = 3$), ** $P < 0.01$, * $P < 0.05$. At least three independent replications were performed for all assays. Bar = 10 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

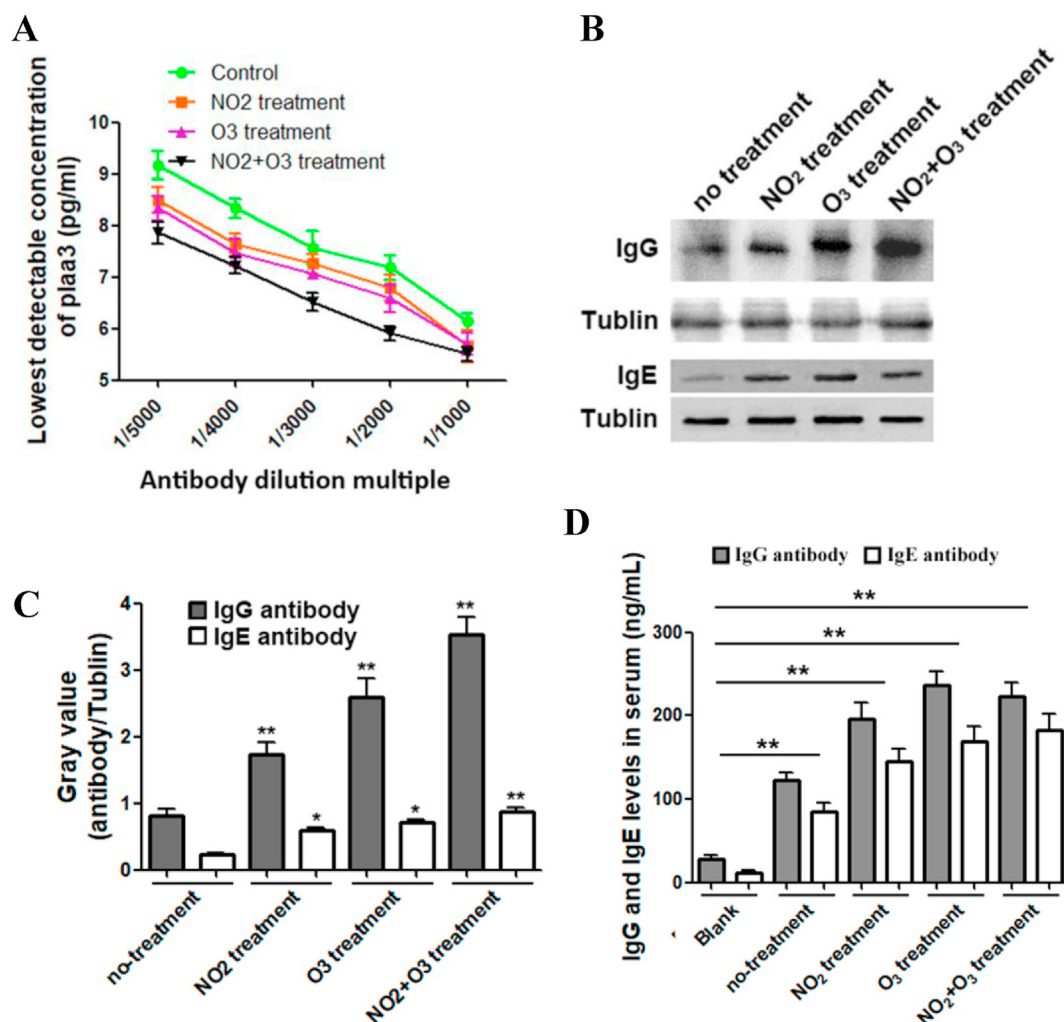


Fig. 4. Analysis of Sensitization Effect of Pla a3 allergen. (A) Analysis of immune titers of Pla a3 allergens with different exposure treatments. (B) Western blot analysis of IgG and IgE antibody proteins produced by immunizing mice with Pla a3 of different exposure treatments. (C) Grey Value Analysis of Western blot Detection. (D) ELISA analysis of IgG and IgE levels in serum of sensitised animals. Each value represent mean $\pm$ standard errors ($n = 9$), ** $P < 0.01$, * $P < 0.05$. Three independent replications were performed for all assays.

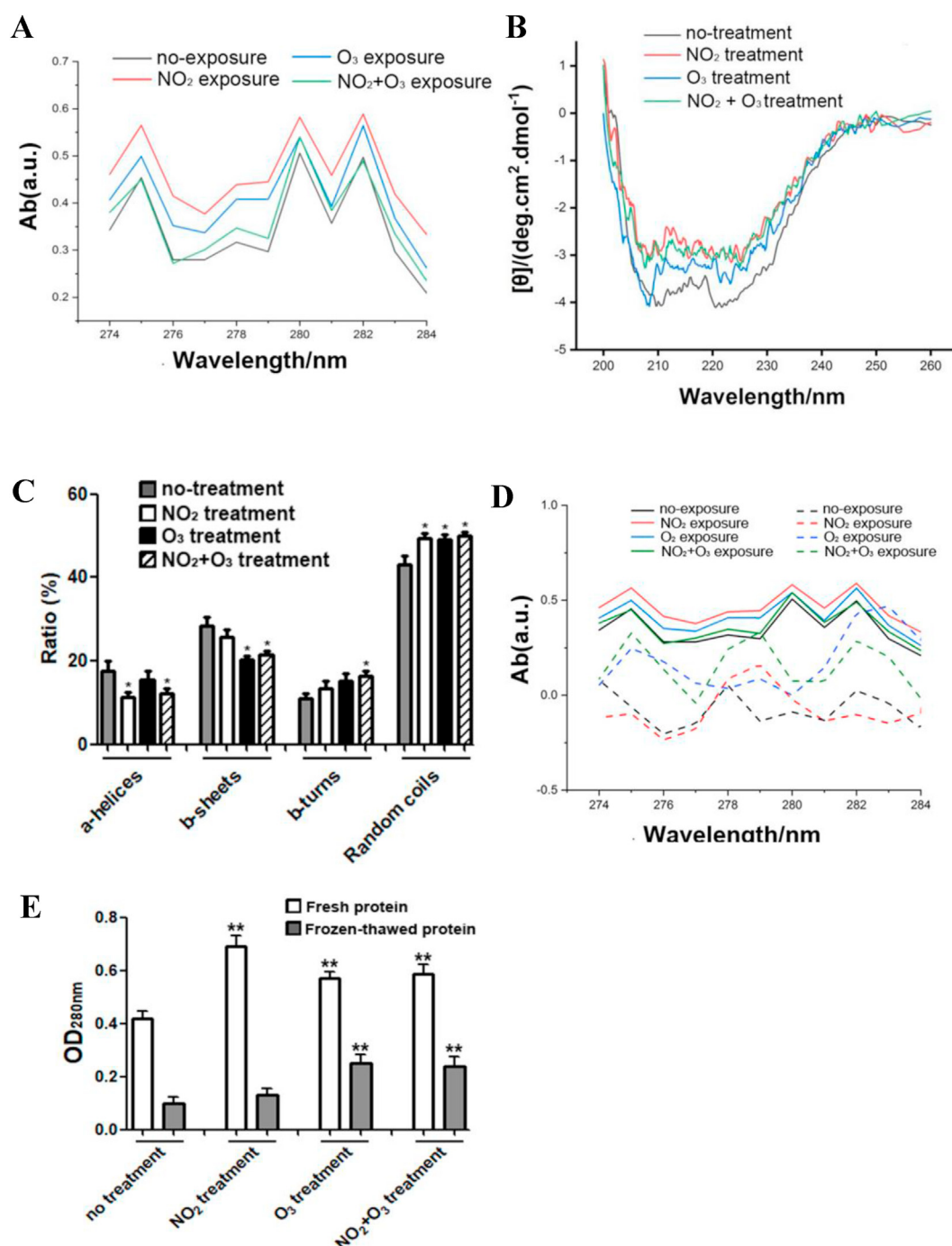


Fig. 5. Effect of NO_2/O_3 exposure on the structure and stability of Pla a3 protein. (A) UV Absorbance detection of Pla a3 with different treatment. (B) CD spectra of Pla a3 with different treatment. (C) The alteration of conformational units after exposure processing. (D) Comparison of UV Absorbance detection of Pla a3 Protein before (full line) and after (dotted line) repeated Freeze-thaw. (E) Effects of NO_2/O_3 exposure on the stability of Pla a3 protein, detected by Absorbance values at 280 nm of Protein with different treatment. Each value represent mean $\pm$ standard errors ($n = 9$), $**P < 0.01$, $*P < 0.05$, Three independent replications were performed for all assays.

group, NO_2 and O_3 exposure enhanced the allergenicity of Pla a3 protein, which increased the levels of IgG and IgE by $59.8 \pm 1.4\%$ and $72.6 \pm 2.1\%$ in the NO_2 treatment group, $93.4 \pm 3.5\%$ and $98.8 \pm 3.4\%$ in the O_3 treatment group, and $81.9 \pm 3.6\%$ and $115.2 \pm 2.7\%$ in the NO_2/O_3 combination treatment group (Fig. 4D). It suggested that the exposure of NO_2 and O_3 to Pla a3 allergen enhanced its immunogenicity and resulted in a stronger immune response triggered by Pla a3 in BALB/c mice.

3.4. Structural change in the Pla a3 allergen after exposure to NO_2 and O_3

Previous studies have shown that pollen allergy may be closely related to changes in the structure and stability of allergens. Therefore, ultraviolet (UV) and circular dichroism (CD) spectroscopy assays were employed to investigate the protein structure and molecular stability of the Pla a3 allergen. Our results demonstrated that the UV absorption peak of the exposed Pla a3 was significantly

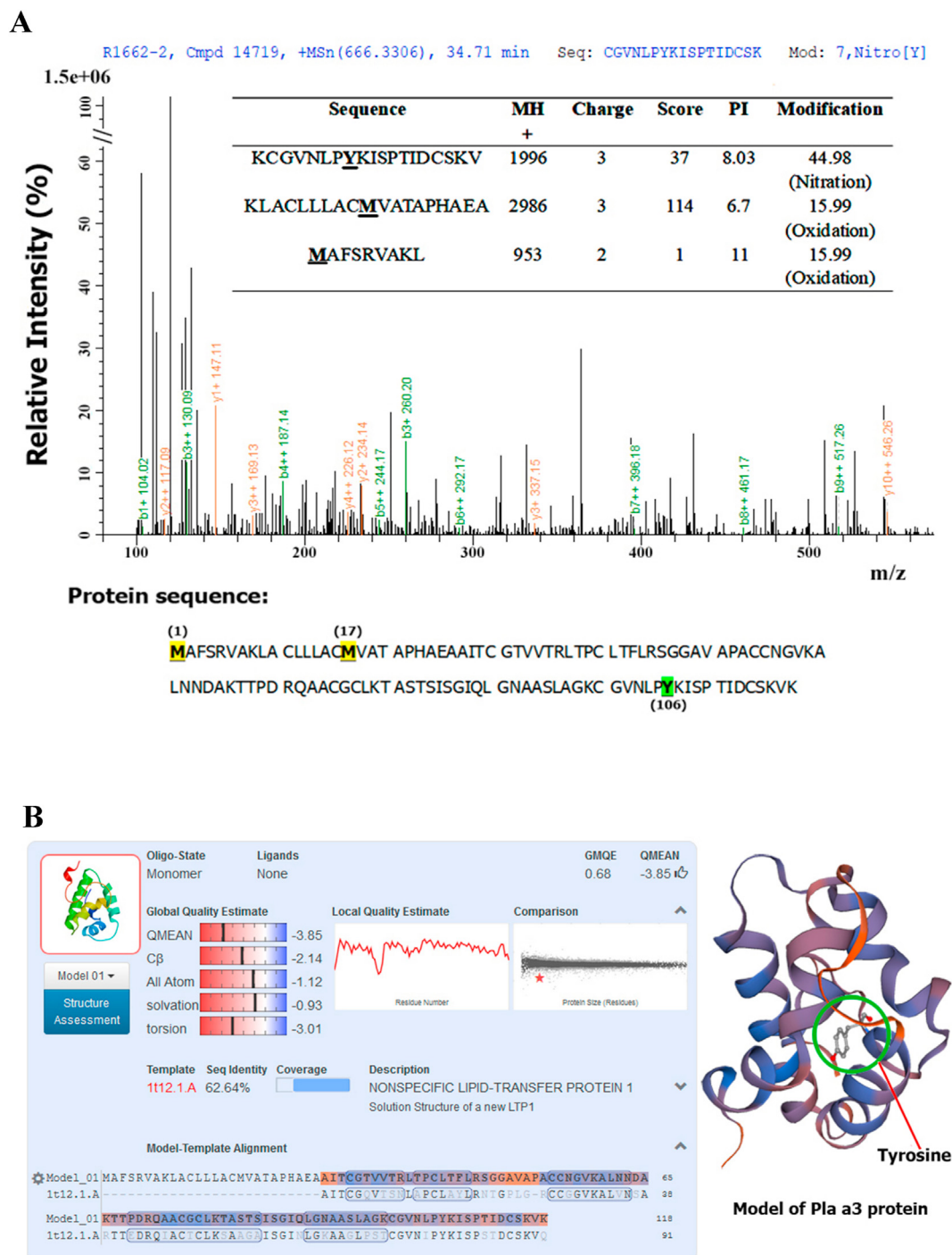


Fig. 6. Mass Spectrometry and Structure Analysis of Pla a3 Protein. (A) Mass spectrometry analysis showed that methionine at first and 17th sites was oxidized and tyrosine at 106 sites was nitrated. (B) A Prediction Structure Model of Pla a3 Protein, the site of nitrated tyrosine in Pla a3 protein structure model was pointed with green circle. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

higher than that of the unexposed group (Fig. 5A), suggesting that the protein structure of the exposed Pla a 3 was looser than that of the non-exposed group. Moreover, the CD assay on the secondary structures demonstrated that the number of alpha helices decreased, whereas parallel/anti-parallel beta strands and random coils increased by joint O₃/NO₂ exposure (Fig. 5B and C). Furthermore, many portions of the non-exposed Pla a 3 protein degraded after repeated freeze–thaw treatment, and its UV absorption value at 280 nm wavelength dropped much more dramatically than the

exposed Pla a 3, suggesting that protein homeostasis was also reinforced by O₃/NO₂ exposure (Fig. 5D and E).

To determine the reasons for the change in secondary structure and stability of Pla a 3 protein at molecular level, a tandem LC-MS/LC spectrometric assay was performed. The results showed that the joint NO₂/O₃ exposure resulted in oxidation of methionine at sites 1 and 17 and nitration of tyrosine at site 106 (Fig. 6A). The three-dimensional structure of Pla a 3 protein was simulated using the Swiss PDB Viewer website (<https://swissmodel.expasy.org/>)

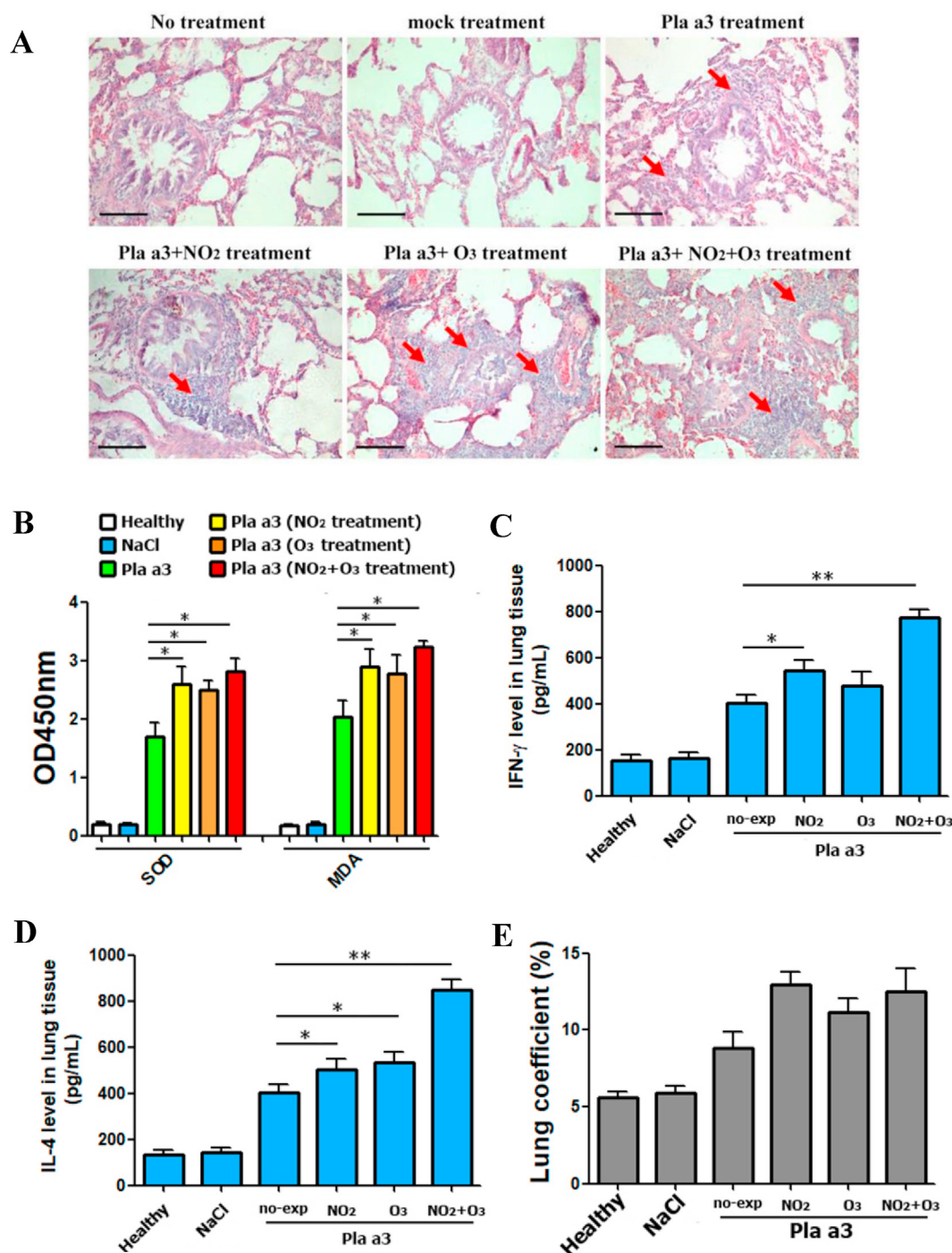


Fig. 7. Detection of physiological indexes in Balb/c mouse model of pollen allergic pneumonia. (A) Observation of pathological sections of healthy mice, mock treatment group, Pla a3 treatment group, Pla a3+NO₂ treatment group, Pla a3+ O₃ treatment group and Pla a3+ NO₂+O₃ treatment group. (B) The oxidative stress response of mouse lung tissue was analysis by SOD and MDA detection. (C) Detection of IL-4 levels in lung tissue. (D) Detection of IFN- γ levels in lung tissue. (E) Analysis of lung coefficient of mice in different treatment groups. Each value represent mean $\pm$ standard errors ($n = 9$), ** $P < 0.01$, * $P < 0.05$, Three independent replications were performed for all assays. Bar = 50 μ m.

interactive/). Due to limited existing protein structure information data being available, we only obtained a partial structural model of Pla a 3 protein, without the front 26 amino acids (Fig. 6B). In this model, the 106th tyrosine was in a position surrounded by five helical structures (Fig. 6B, indicated by a green circle); hence, the nitration of this amino acid would have an important effect on the protein structure. Our data suggested that NO₂ or O₃ exposure

could lead to the oxidation and nitration of certain amino acids, which could change the protein spatial structure and further change protein stability. Considering that the Pla a 3 allergen could be exposed in the urban atmosphere, high mass levels of NO₂ or O₃ could contribute to the increased stability of the protein, and allergenicity caused by the protein might be aggregated.

3.5. NO₂ and O₃ exposure aggravated pathogenic effects of Pla a 3 allergen

To further compare the allergenicity of Pla a3 in the exposed groups with that in the non-exposed group, BALB/c mice were treated with exposed (treated with NO₂, O₃ or NO₂/O₃) and non-exposed Pla a 3 were used to immunise the BALB/c mice. The results showed that the exposed Pla a 3 caused more severe allergic symptoms in mice. The pathological sections showed that the number of inflammatory cells in the lung tissue of the mice immunised with exposed Pla a3 was significantly higher than that in mice treated with non-exposed Pla a3 (Fig. 7A, as indicated by red arrows). Simultaneously, the level of SOD and MDA showed that the oxidative stress in the lung tissue of mice treated with exposed Pla a3 was stronger than that in mice treated with non-exposed Pla a3 (Fig. 7B). Furthermore, Pla a 3 could cause an increase in the levels of interferon γ (IFN- γ) and interleukin 4 (IL-4) in sensitised mice. NO₂/O₃ exposure could promote further elevation of these two cytokines. (Fig. 7C and D). Using the ratio of wet weight to the body weight of the mice, the results showed that pollen allergic pneumonia led to increased lung coefficients, and NO₂/O₃ exposure treatment exacerbated this change (Fig. 7E). The *in vivo* detected results indicated that the allergenicity of the exposed Pla a 3 was significantly higher than that of the non-exposed Pla a 3.

4. Discussion

Platanus acerifolia is a common tree species in urban areas of cities worldwide (Lu et al., 2014), and its airborne pollen has become a major allergy factor during bloom in spring (Zhou et al., 2019, 2021). The pollen allergens found in sub-pollen particles (SPPs) (0.1–1 μ m) can be released under conditions of relatively high humidity, and the SPPs are transported to the deep lung through the respiratory tract (Yan et al., 2019). Therefore, anaphylaxis risk was closely related to the amount of SPPs released and the allergen levels in the atmosphere.

NO₂ and O₃ are two major pollutants in the urban atmosphere. Effects of O₃ and NO₂ on pollen protein have been intensively studied recently (Franze et al., 2005; Zhao et al., 2017). These data showed that O₃ could up-regulate the allergen in perennial ryegrass and rye pollen, and enhance allergenicity of Bet v 1 allergen from birch pollen (Beck et al., 2013), while elevated NO₂ level could increase the Amb a 1 allergen amount in the ragweed pollen and up-regulate transcript levels of the Amb allergens (Zhao et al., 2016, 2017). Our results confirmed that air pollutants could destroy the outer wall of pollen and significantly affect the stability of the cell membrane (Fig. 4). Considering that the mass level of NO₂ and O₃ in the urban atmosphere is typically above the national air quality standard and is maintained at a high level, the molecular structure of allergenic pollen protein could be modified by air pollutants.

Pla a 3 is a ns-LTP (Wangorsch et al., 2016; Scala et al., 2017), and ns-LTPs have an important function that is responsible for repairing and maintaining the normal structure of the cell membrane (Salminen et al., 2016). If the cell membrane is damaged, ns-LTPs are produced and aggregated near the site of the damaged area (Salminen et al., 2016). Exposure to pollutants not only results in damage to pollen cell membranes but also stimulates the synthesis of membrane repair-related factors in cells, such as ns-LTP Pla a 3. Therefore, the direct effect of air pollutants on the pollen grains could increase the allergenic pollen allergen levels in the air and further contribute to allergic diseases.

We once reported that a novel disulfide bond between the sulfhydryl groups of two neighbouring cysteine molecules was formed after the rHum j1(*Humulus japonicus* pollen allergen) exposure to joint O₃/NO₂, and IgE-binding affinity was increased

and the allergenicity was reinforced. (Hong et al., 2018). Ackaert et al. (2014) reported that amino acid residue Y83, located in the structure of nitrated Bet v 1.0101 could favour a Th2 cell response and increase the allergenicity of birch pollen allergens. Our data demonstrated that the protein structure of Pla a 3 could be changed under the influence of NO₂ and O₃ (Fig. 5). The change in protein structure enhanced the immunogenicity (Figs. 6 and 7). Additionally, the stability of Pla a 3 protein could be increased after protein exposure to NO₂ and O₃. Pla a3 was not easily degraded owing to increased stability, which could lead to the suspension of more Pla a3 allergens in the atmosphere and an increase in the risk of pollen allergy.

5. Conclusions

To investigate the effects of air pollutants represented by NO₂ and O₃ on allergic pneumonia caused by Pla a 3 allergen of *Platanus* pollen, we designed an experiment to reveal the effect of typical gaseous pollutants on protein structure and enhance allergenicity of the pollen protein. Our results demonstrated that NO₂ and O₃ could damage pollen cell membranes and lead to an increase in Pla a 3 allergen released into the atmosphere in the form of SPPs. The amount of NO₂ and O₃ could change the molecular structure of Pla a 3 protein through nitration and oxidation. Moreover, proteins that have undergone structural changes can enhance the immunogenicity of allergens. Thus, our data provide new guidance for the prevention of pollen allergy diseases.

Credit author statement is following

Zhou Shumin: Methodology, Software, Writing – original draft. Wang Xingzi: Writing – original draft. Lu Senlin: Conceptualization, Data curation, Supervision. Yao Chuanhe: Data curation. Zhang Luying: Software, Validation. Rao Lanfang: Software, Validation. Liu Xinchun: Visualization, Investigation. Zhang Wei: Methodology, Software. Li Shuijun: Methodology, Software. Wang Weiqian: Reviewing. Wang Qingyue, Editing. All authors read and contributed to the manuscript. S.Z. and S.L. designed the study, and supervised all experiments. S.Z. drafted the manuscript. X.W. carried out the animal experiment. C.Y. conducted exposure experiment. Z.L. and R.L. analysed the data of Pla a 3 protein. W. Z and S.L. provided instruction on allergenic reaction. X. L, W.W. and W.Q. revised the manuscript.

Declaration of competing interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Acknowledgments

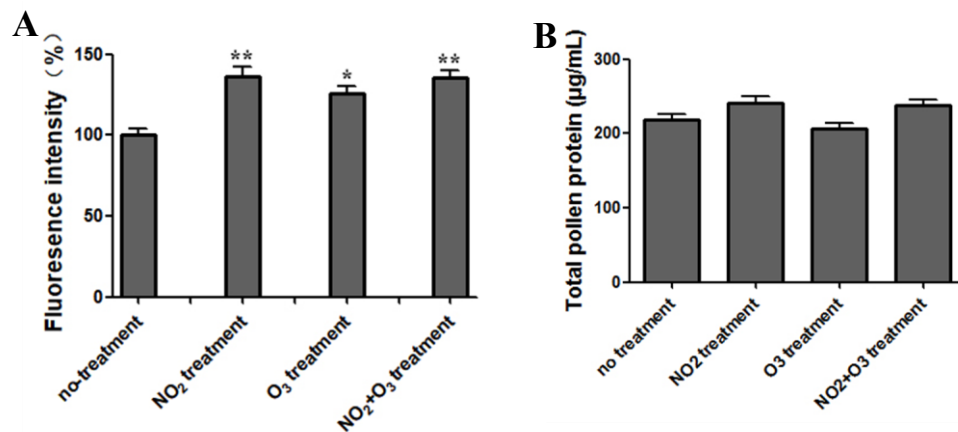
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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2021.116913>.

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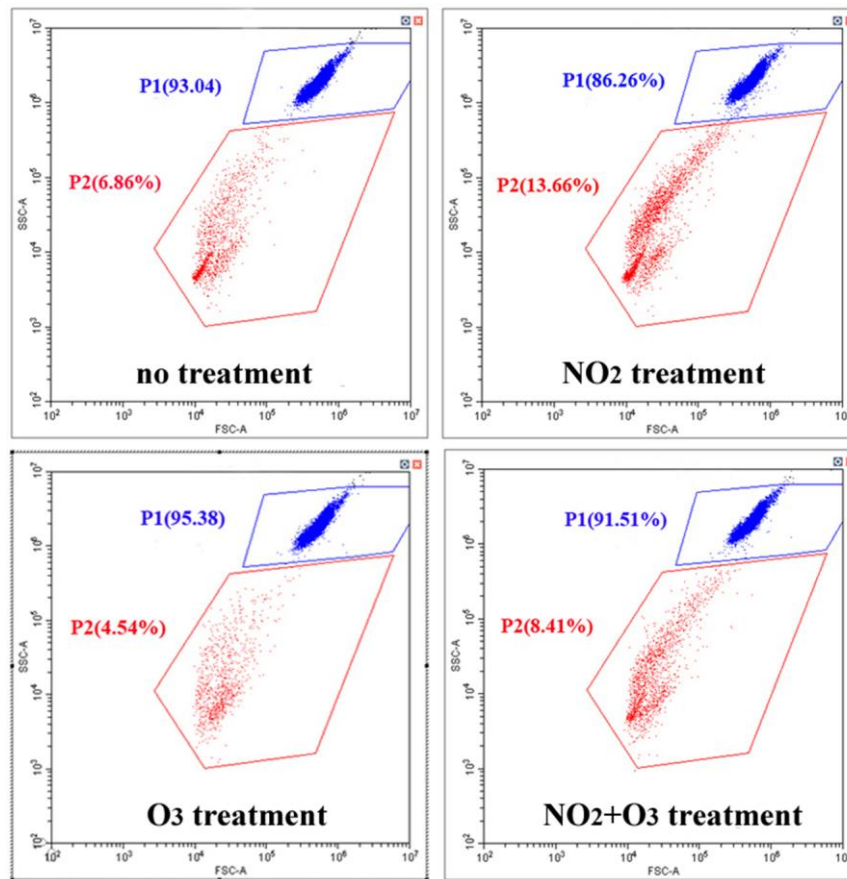
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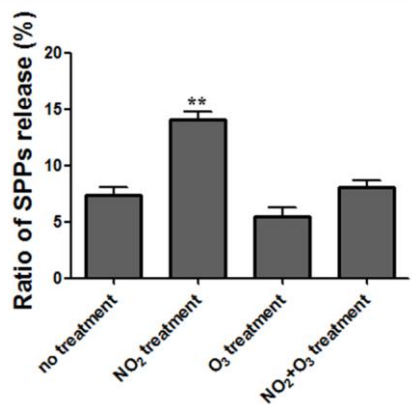
Supplementary figure 1. Statistical analysis of fluorescence intensity and protein content.

(A) Analysis of fluorescence intensity in pollen grains of different exposure groups after immunofluorescence staining, the fluorescence intensity of no-treatment group as 100%. (B) Quantitative detection of protein showed that there was no significant difference in total pollen protein content between exposed group and non-exposed group. Each value represent mean $\pm$ standard errors (n=15), **P<0.01, *P<0.05, at least three independent replications were performed for all assays.

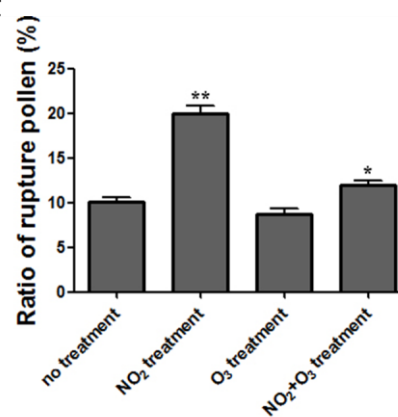
A



B



C



Supplementary figure 2. Analysis of SPPs release and pollen wall damage

(A) Flow cytometry analysis of SPPs release from pollen of different treatment groups. (B) A statistical analysis of three independent FLC assay showed the proportion of SPPs in the detected particles. (C) Statistical analysis of the proportion of ruptured pollen under optical microscope. Each value represent mean $\pm$ standard errors (n=3 in B and n=15 in C), **P<0.01, *P<0.05, At least three independent replications were performed for all assays.