

aestivum L.), rice (*Oryza sativa* L.), and maize (*Zea mays* L.) production to lose 12.4%, 7.1%, 4.4%, and 6.1% per year, respectively, with a total loss of 27 million tons per year (Mills et al., 2018). Based on data from the Ministry of Ecology and Environment of the People's Republic of China (available at the website of the Ministry), the average O₃ concentration during the summer of 2023 (June to August) in 339 major cities in China was 70.27 nmol mol⁻¹, far exceeding the commonly recognized threshold of 40 nmol mol⁻¹, which is generally considered harmful to plants. With the development of economy and urbanization in East Asia, Southern Asia, and Sub-Saharan Africa, the future O₃ concentration in these regions will become even higher (Sicard et al., 2017; Gaudel et al., 2018; Sicard et al., 2023). Therefore, it is necessary to evaluate the ecological risk of O₃ pollution and try to take measures to mitigate the adverse effects of O₃ on plants.

There are several ways to mitigate the damage of O₃ to plants. For example, appropriate drought stress was able to close the stomata of plants and reduce the intake of O₃ by plants (Yin et al., 2022a), thereby weakening the reduction effect of O₃ enrichment on photosynthetic capacity (Yuan et al., 2016) and biomass (Gao et al., 2017). However, as a strong stress, drought has a huge negative impact on plant productivity (Xie et al., 2018; Arya et al., 2021; Dietz et al., 2021), so water stress for O₃ risk management has limited applications. In addition to drought, many chemicals have been tested for plant O₃ protection, such as flavonoids, polyamines, ascorbic acid and its derivatives, and Vapor Gard emulsion (di-1-p-menthene) (Didyk and Blum, 2011; Francini et al., 2011), among which ethylenediurea (N-[2-(2-oxo-1-imidazolidinyl) ethyl]-N'-phenylurea, EDU) is the most widely studied substance with reliable O₃ protection effect (Shang et al., 2022). A meta-analysis showed that EDU was able to reduce O₃ visible injury by 76%, increase photosynthetic rate by 8% and aboveground biomass by 7% (Feng et al., 2010).

Although the protective effect of EDU has been repeatedly verified (Zhang et al., 2021; Surabhi et al., 2022), the protective mechanism of EDU is still unclear. Several studies have attempted to unravel the O₃ phytotoxicity alleviation mechanism of EDU, covering various aspects: (1) EDU may be able to reduce stomatal conductance to minimize O₃ intake (Darrall, 1989). However, except for very few studies showing a reduction in stomatal conductance induced by EDU (Singh et al., 2009), most of research indicated that the effect of EDU on stomatal conductance was not significant (Lee et al., 1992; Singh et al., 2009). The meta-analysis results of Feng et al. (2010) also indicated that stomatal conductance might not be the underlying mechanism of EDU's protective effect. (2) The nitrogen (N) addition effect of EDU may serve as a protective mechanism. Since the N content in EDU

molecules accounts for 22.56% (Manning et al., 2011), plants receive a N supplement during EDU spraying. Previously, it was believed that the N addition would promote plant growth and enhance resistance to O₃ damage (Agathokleous et al., 2016). However, this perspective remains open for further consideration. Certain studies on poplar demonstrated that N addition did not enhance the O₃ resistance of plants (Shang et al., 2021; Agathokleous et al., 2023). Furthermore, a study combining the results of 273 experiments indicated that N addition did not influence the O₃ sensitivity of plants (Feng et al., 2019b), casting doubt on the notion that N addition reduces plant O₃ sensitivity. (3) EDU may help plants remove reactive oxygen species (ROS) more efficiently by improving the function of leaf antioxidant system. A meta-analysis integrating 50 articles also indicated that the protective mechanism of EDU was biochemical rather than biophysical. Particularly, antioxidant enzymes were more likely to be the primary reason for EDU's protective effect compared to other mechanisms (Feng et al., 2010). In addition, some experimental studies also showed that EDU was able to significantly increase the superoxide dismutase (SOD) activity of wheat and alter soybean peroxidase (POD) activity (Brunschonharti et al., 1995; Singh et al., 2009), which helps reduce O₃ damage to plants. Consequently, we believe that antioxidant system plays a crucial role in EDU alleviation of O₃ damage to plants.

Arbuscular mycorrhizal (AM) fungi can establish symbiotic associations with the majority of terrestrial plants (Wang and Qiu, 2006). In this relationship, plants supply lipids and sugars to AM fungi (Jiang et al., 2017), while the AM fungi aid plants in the absorption of mineral nutrients and water (Li et al., 2013; Li et al., 2014; Xie et al., 2019; Xie et al., 2022). A large number of studies have shown that AM fungi play an active role in helping plants cope with a wide range of abiotic stresses (Malhi et al., 2021), such as drought (Li et al., 2013; Li et al., 2014), heavy metals (Wu et al., 2015; Li et al., 2018), and salt-alkali (Santander et al., 2017). However, whether AM fungi can alleviate the damage of O₃ to plants and the underlying mechanisms are still unclear. Yin et al. (2022a) found that AM symbiosis reduced the O₃ sensitivity of poplar (*Populus euramericana* cv. '74/76'), while other studies performed O₃ fumigation on alfalfa (*Medicago sativa* L.), clover (*Trifolium subterraneum* L.) (Miller et al., 1997), and found that the biomass of AM plants compared with non-inoculated plants had a greater decline due to O₃ enrichment (Ho and Trappe, 1984). In addition, some studies showed that there was no significant interaction between AM inoculation and O₃, which suggested that AM inoculation could not affect plants O₃ sensitivity (Wang et al., 2017a). The inconsistency of these results remains confusing due to the lack of in-depth mechanistic research.

To reveal how AM symbiosis affects plant O₃ sensitivity, we summarized several possible mechanisms: (1) AM symbiosis affects stomatal conductance and thus alters O₃ uptake by plants (Auge et al., 2015; Yin et al., 2023). AM symbiosis tends to increase stomatal conductance (Auge et al., 2015; Xie et al., 2019; Yin et al., 2022a), which undoubtedly increases the risk of plants suffering from O₃ damage. (2) AM symbiosis reduces the O₃ sensitivity by increasing the plant's antioxidant capacity (Chandrasekaran et al., 2014), which has been confirmed in a study on poplar (Yin et al., 2023). (3) AM symbiosis improves plant nutritional status (Yin et al., 2022b), thereby enhancing plant resistance to O₃ stress. (4) AM symbiosis alter the leaf morphology of plants; for instance, AM inoculation may lead to a decrease in specific leaf weight (Yin et al., 2022a). A lower specific leaf weight indicates that leaves have a higher O₃ load per unit weight, which can increase O₃ damage (Li et al., 2016; Feng et al., 2018a).

Through the above review, it was found that EDU and AM symbiosis can both protect plants from O₃ damage by enhancing antioxidant enzyme activities. However, since EDU directly acts on the plant leaves while AM fungi directly affect the roots, their respective impacts on antioxidant enzymes might follow different pathways. Hence, there might be an interaction between EDU and AM symbiosis in their protective role against plant O₃ damage. However, there has been no previous research on the combined application of EDU and AM inoculation to alleviate plant O₃ phytotoxicity.

Alfalfa is a perennial forage leguminous plant widely planted in semi-arid and arid regions of China, with high economic value (Xu et al., 2022). In this study, alfalfa was used as experimental material to study the interactive effects and potential mechanisms of EDU and AM inoculation on plant O₃ sensitivity, and to explore the feasibility of combined application of EDU with AM inoculation in alleviating O₃ damage to plants. We hypothesized that: (1) EDU mediates antioxidant enzymes to reduce O₃ sensitivity of plants. (2) AM fungi improve plant performance by enhancing plant nutrition status without affecting plant O₃ sensitivity. (3) The combination of EDU and AM inoculation alleviates the adverse effects of O₃ on plants. This study is expected to deepen our understanding of the mechanism of how EDU and AM alleviate O₃ phytotoxicity, while exploring effective strategies to reduce the environmental risks associated with O₃.

2 Materials and methods

2.1 Experimental site and plant material

The experiment was conducted in the Tangjiapu County of

Beijing municipality, China (115°59' E, 40°29' N). The locale has a warm temperate and semi-humid continental monsoon climate, where the mean annual temperature and precipitation stand at 8°C and 467 mm, respectively. The growth substrate comprised local farmland soil, which was passed through a 2 mm mesh and sterilized using γ -radiation (20 kGy, 10 MeV electron beam). Plants were cultivated in 2.5 L round plastic pots with top and bottom diameters of 20 cm and 13 cm, respectively, and a depth of 14 cm. Each pot contained 2 kg of soil. The soil exhibited the following characteristics: pH of 7.58, available phosphorus (P) of 15.01 mg kg⁻¹, NH₄⁺ content of 5.54 mg kg⁻¹, NO₃⁻ content of 8.28 mg kg⁻¹, and an organic matter content of 4.42%. Prior to sowing, the soil was meticulously mixed with basal nutrients, including 60 mg kg⁻¹ of N (CON₂H₄) and 90 mg kg⁻¹ of K (K₂SO₄).

The plant material was alfalfa (*M. sativa* L. cv. Zhongmu No.2). The plant seeds were sterilized by soaking them in 10% H₂O₂ for 10 min and then germinated in a dark environment at 25°C. Sowing took place on July 1, 2021, with 10 seeds sown per pot. After one week, the seedlings were thinned to 6 plants per pot. After planting, the pots were watered by weighing every day to maintain a consistent 15% moisture content (75% field capacity) for each pot.

2.2 Experimental design and procedure

There were two O₃ levels allocated randomly across 8 open-top chambers (OTCs): ambient air O₃ (AO₃), elevated O₃ with 40 nmol mol⁻¹ O₃ enrichment (EO₃). Each O₃ level had 4 replicates (OTCs). OTC was designed with an octagonal base, providing a growth space of 12.5 m² and a height of 3.0 m. The O₃ was generated using a pure oxygen ozone generator (HY003, Chuangcheng Co., Jinan, China), mixed with air, and then circulated into the OTCs using a fan (1.1 kW, 1080 Pa, 19 m³ min⁻¹, CZR, Fengda, Shanghai, China). To continuously monitor the O₃ concentration within the OTCs, a UV-absorbed ozone analyzer (Model 49i, Thermo Scientific, Franklin, MA, USA) was employed. Before the experiment and once per month during the experiment, the calibration of the monitors was conducted using a 49i-PS calibrator (Thermo Scientific, Franklin, MA, USA). O₃ fumigation (9:00–17:00 daily, except on rainy days) was performed from July 26th to September 2nd. During the experimental period, the average 8-h O₃ concentrations for AO₃ and EO₃ were 41.71 ± 0.29 and 77.32 ± 0.71 nmol mol⁻¹, respectively. Moreover, the AOT40 values (accumulation over an hourly 40 nmol mol⁻¹ O₃ threshold) for AO₃ and EO₃ were 2.72 ± 0.06 and 11.45 ± 0.21 μ mol mol⁻¹ h, respectively.

Three EDU treatments had three EDU addition levels: spraying water (E0), 150 mg L⁻¹ EDU aqueous solution

(E150) and 300 mg L⁻¹ EDU aqueous solution (E300). The EDU was sprayed about every 10 days (Agathokleous, 2017). A total of 5 sprays were applied throughout the experimental period. The EDU solution was prepared and used immediately, with EDU being stirred thoroughly to ensure sufficient dissolution. Each plant's entire foliage was sprayed.

Alfalfa were inoculated with an AM fungus, *Rhizophagus irregularis* (Błaszk, Wubet, Renker & Buscot) C. Walker & A. Schüßler, CGMCC 12157 (+M) or were maintained as uninoculated controls (-M). AM inoculant was a mixture of growth substrate, fungal spores, and root segments. Before sowing, 60 g (about 3600 spores) of AM inoculum was mixed into one pot for +M treatment, and an equal amount of sterilized AM inoculant substrate was added for -M treatment.

The experiment consisted of three experimental factors, resulting in a total of 12 treatments and 48 pots arranged in a split-zone design. Within each OTC, there were 6 pots, representing a complete combination of various EDU and AM inoculation statuses.

2.3 Parameter measurements

2.3.1 Photosynthetic rate, stomatal parameters and leaf morphology

On September 2, 2021, the third fully mature leaf from the top of the alfalfa was chosen for the measurement of net photosynthetic rate, stomatal conductance and actual photochemical quantum efficiency (PhiPS2) (Yin et al., 2022b). A portable photosynthetic system (Li-COR 6800, LICOR Corp, Lincoln, USA) was used for the assessments. During the measurements, specific environmental conditions were maintained, including a light intensity of 1000 μmol (photons) m⁻² s⁻¹, relative humidity set at 65%, a CO₂ concentration of 400 μmol mol⁻¹, and the temperature fixed at 25°C (Yin et al., 2022b). The calculation formula of PhiPS2 was as follows (Li et al., 2023):

$$\text{PhiPS2} = \frac{F'_m - F_s}{F'_m} \quad (1)$$

where F'_m is the maximum fluorescence under the saturation flash, and F_s is the steady-state fluorescence under the measurement light.

After the measurement, the third leaf was removed and scanned with a scanner (V700 Photo, Epson, Nagano, Japan), and then Adobe Photoshop CS6 was used to obtain the leaf area. The leaf was dried in an oven to constant weight and weighed, the specific leaf weight was calculated by dividing the mass by the area of the leaf.

Nail polish was evenly applied to the abaxial and adaxial of the third mature leaf. After the nail polish film dries, it was

carefully peeled off to make slide samples. Three fields of view were randomly observed on each slide at 400× and then photographed using a microscope (Primostar 3, Zeiss, Oberkochen, Germany). Ando Analysis software (Andao Corp., Shunyi, Beijing, China) was used to count the number of stomas and measure the length and width of stomas (Yin et al., 2022a). Ten stomata were randomly measured in each image, and if there were less than 10 stomata in the image, all the stomata were measured. A total of 288 images were taken and 2820 stomata were measured. The stomatal density and stomatal aperture were calculated as follows (Zhu et al., 2018):

$$\text{Stomatal density} = \frac{\text{Number of stomas in image}}{\text{Area of image}} \quad (2)$$

$$\text{Stomatal aperture} = \frac{\pi \times \text{stomatal length} \times \text{stomatal width}}{4} \quad (3)$$

The stomatal density of each pot was the sum of the abaxial and adaxial stomata densities, and the stomatal aperture was calculated as the weighted average of abaxial and adaxial leaf stomatal apertures, considering the respective stomatal densities.

2.3.2 Ozone visible injury

Alfalfa's O₃ visible injury manifested as distinct yellow or brown spots on the upper leaf surface, positioned between the veins (Paoletti et al., 2009). Observe each pot of alfalfa daily until the first occurrence of O₃ visible injury symptoms. On September 2nd, the assessment of O₃ visible injury was conducted through visual observation. Visible injury severity was evaluated according to the following scale: 0, the whole leaf was free of disfigured spots; 1, 1%–10% of leaf area had disfigured spots; 2, 11%–25% of leaf area had disfigured spots; 3, 26%–50% of leaf area had disfigured spots; 4, over 50% of leaf area had disfigured spots (Wang et al., 2022). The visible injury per pot was calculated as follows (Yan et al., 2020; Yin et al., 2022b):

$$\text{Visible injury} = \frac{\sum_0^4 x_i y_i}{x_{\max} \sum y_i} 100\% \quad (4)$$

In the formula, x_i is rating score, x_{\max} is the maximum rating score, and y_i is the number of leaves corresponding to the rating score in a pot.

2.3.3 Plant harvest and biomass

On September 3, the potted plants were harvested. Shoot or root samples from multiple plants in the same pot were combined into a mixed sample. Fresh shoot samples were weighed and stored in a -80°C refrigerator for analyzing antioxidant enzyme activities and malondialdehyde (MDA)

concentration. Approximately 0.5 g of fresh root samples were used to determine AM root colonization. The remaining shoot and root samples were dried in an oven at 70°C until the weight became stable and weighed, and biomass was calculated (Wang et al., 2021). The dried samples were used to determine elemental concentrations.

2.3.4 AM root colonization

Fresh root samples were cut into 1 cm segments and then subjected to sequential treatments with 10% KOH at 90°C, 2% HCl, 0.05% trypan blue solution at 90°C, and lactate glycerin for 20 min, 5 min, 15 min, and over 180 min, respectively (Brundrett et al., 1984). Subsequently, 30 stained root segments were randomly selected from each sample and used to prepare microscopic slides. These slides were examined under a microscope to assess the AM colonization of each root segment. To determine the mycorrhizal colonization rate (M%) and arbuscular abundance (A%), MYCO-CALC software was employed (Trouvelot et al., 1986). The software calculated M% and A% using specific formulas as outlined in the Supplementary material (Methods S1).

2.3.5 Element concentrations

After drying in oven, the shoot and root samples underwent grinding in a ball mill (GT200, Grinder Co., Ltd., Beijing, China) at 1600 r min⁻¹ for 2 min (Li et al., 2022). Carbon (C) and N concentrations were directly measured using an elemental analyzer (Vario Max Cube, Elementar, Frankfurt, Germany). Plant samples were digested with nitric acid in a microwave digestion system (Mars 5, CEM Co., Ltd., Matthews, NC, USA), and P concentration was determined using an inductively coupled plasma optical emission spectrometer (ICP-OES, Prodigy, Leeman, USA). The recoveries of P were 97.3%–101.7% for standard sample.

2.3.6 MDA concentration and antioxidant enzyme activities

About 0.1 g of frozen shoot sample was ground mixed with 5 mL of 5% trichloroacetic acid in a pre-cooled mortar and the resulting mixture was centrifuged to obtain a supernatant. One milliliter supernatant was taken and combined with 2 mL of 0.67% 2-thiobarbituric acid. The resulting mixture was heated in a boiling water bath for 30 min. Subsequently, the optical densities (ODs) at 450 nm, 532 nm, and 600 nm were measured. The MDA concentration was calculated as follows (Xing et al., 2018):

$$\text{MDA concentration (mmol L}^{-1}\text{)} = 6.45 \times (\text{OD}_{532} - \text{OD}_{600}) - 0.56 \times \text{OD}_{450} \quad (5)$$

The resulting MDA concentration was presented as nmol g⁻¹ fresh weight (FW).

Likewise, about 0.1 g of the frozen shoot samples were placed in a pre-cooled mortar and ground with 5 mL of phosphate buffer (0.2 M, pH = 7.8). The supernatant was collected by centrifugation. The SOD activity was measured using the nitrogen blue tetrazolium (NBT) photoreduction method (Li et al., 2000). One SOD activity unit was defined as the enzyme amount that inhibited NBT photoreduction by 50% per minute in the reaction system. POD activity was determined by the guaiacol method. The activity of POD enzyme was calculated by measuring the change of the absorbance value at 470 nm of the reaction system (Li et al., 2000). CAT enzyme catalyzes the decomposition of hydrogen peroxide into oxygen and water. The amount of enzyme that reduced the absorption at 240 nm by 0.1 within one min was regarded as one CAT activity unit (McKee et al., 1997). The APX activity was determined using the ultraviolet absorption method (Knorzer et al., 1996), and the enzyme activity was calculated according to the decrease of the absorbance value at 290 nm per unit time (Li et al., 2000).

2.3.7 Statistical analysis

A statistical significance threshold of $\alpha = 0.05$ was chosen. The normality and homogeneity of the data were assessed using the Shapiro-Wilk and Levene's tests, respectively. Data that did not meet the requirements underwent Box-Cox transformation (Box and Cox, 1964). Utilizing OTCs as a random variable, a mixed linear model analysis was conducted to achieve a split-zone variance analysis for O₃, EDU, AM inoculation, and their interactions (Yin et al., 2022a). Tukey's HSD post-hoc test was employed for conducting multiple comparisons across all treatments. Split-plot ANOVA and multiple comparisons were conducted using SPSS Statistics 24 (IBM Corp., Armonk, NY, USA). Graphing was performed using Origin 2018 (Origin-Laboratory Corp., Northampton, MA, USA).

3 Results

3.1 Mycorrhizal colonization

No mycorrhizal colonization was detected in -M plant roots. Under AO₃ treatment, the mycorrhizal colonization rates of plants under E0, E150, and E300 treatments were 32.89%, 38.94%, and 47.83%, and the arbuscular abundances were 18.35%, 22.37%, and 27.88%, respectively. Under EO₃ treatment, the mycorrhizal colonization rates of plants under E0, E150, and E300 treatments were 31.26%, 32.68%, and 29.17%, and the arbuscular abundances were 18.42%, 17.14%, and 16.54%, respectively. O₃, EDU, and their interaction had no significant effect on the mycorrhizal colonization rate and arbuscular abundance of plants (Table S1).

3.2 Biomass

In general, O₃ enrichment significantly decreased shoot biomass, root biomass, and total biomass by 23.57%, 29.50%, and 26.24%, respectively, when averaged across all EDU and AM treatments. EDU had no significant effect on plant biomass. Regardless of O₃ and EDU treatments, AM inoculation significantly increased the shoot biomass, root biomass and total biomass of plants (Fig. 1A, 1B, 1C; Table S2).

In addition, O₃, EDU and AM inoculation and their interaction had no significant effect on root/shoot ratio (Fig. 1D, Table S2).

3.3 Photosynthetic parameters

O₃ and EDU treatments had no significant effect on the net photosynthetic rate and stomatal conductance. AM inoculation had a significant effect on the net photosynthetic rate ($P < 0.01$) and stomatal conductance ($P < 0.01$), the net

photosynthetic rate and stomatal conductance of +M plants were higher than that of -M plants regardless of O₃ and EDU status (Fig. 2A, 2B; Table S2).

In general, O₃ enrichment significantly decreased PhiPS2 ($P < 0.05$). Spraying different concentrations of EDU had no significant effect on PhiPS2. AM inoculation significantly increased PhiPS2 under each EDU and O₃ treatment combination (Fig. 2C, Table S2).

3.4 Leaf morphology and stomatal parameters

O₃ and EDU did not significantly affect the area of third leaf and specific leaf weight, while AM inoculation significantly increased the area of the third leaf ($P < 0.01$) and significantly decreased specific leaf weight ($P < 0.05$) (Fig. 3A, 3B; Table S2).

For stomatal density, the interaction between O₃ and AM inoculation was significant ($P < 0.05$): AM inoculation increased the stomatal density by 29.44% under EO₃ treatment, but had no significant effect on the stomatal density

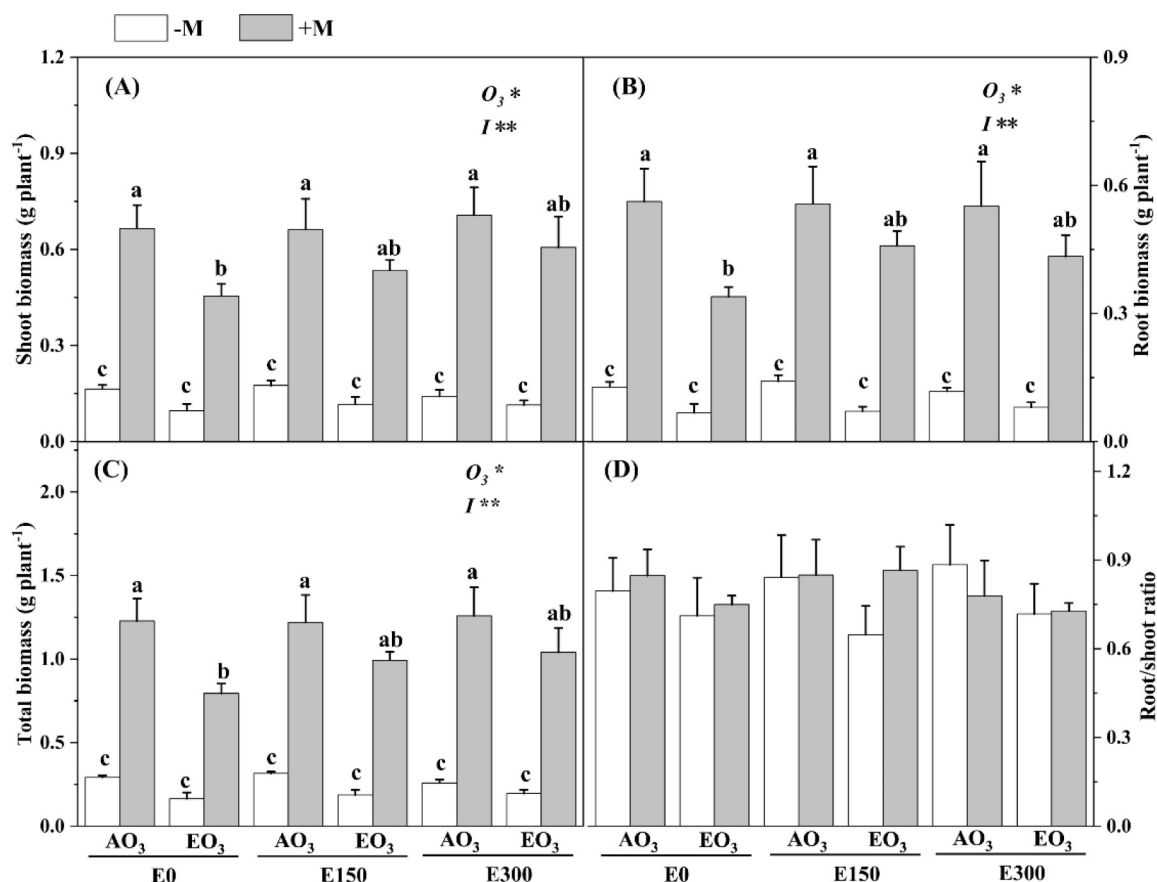


Fig. 1 Effects of ozone (O₃), EDU (E) and AM inoculation (I) on shoot biomass (A), root biomass (B), total biomass (C) and root/shoot ratio (D). AO₃: ambient air O₃, EO₃: elevated O₃ with 40 nmol mol⁻¹ O₃ enrichment; E0: spray water, E150: spray 150 mg L⁻¹ EDU aqueous solution, E300: spray 300 mg L⁻¹ EDU aqueous solution; -M and +M represent non-AM and AM inoculation with *Rhizophagus irregularis*, respectively. Only significant results of ANOVA are shown. *, $P < 0.05$, **, $P < 0.01$. Different letters above the columns denote significant differences ($P < 0.05$) among corresponding treatments by Tukey's HSD tests. Data are presented as means \pm standard error (SE) ($n = 4$).

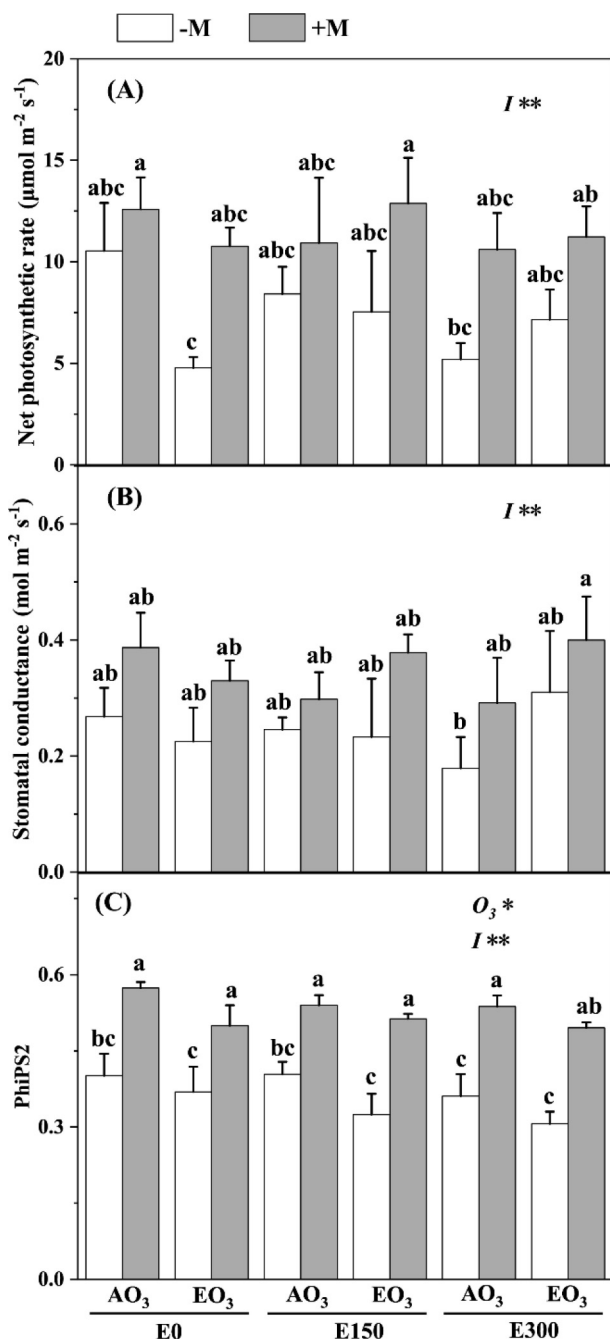


Fig. 2 Effects of ozone (O_3), EDU (E) and AM inoculation (I) on net photosynthetic rate (A), stomatal conductance (B), and PhiPS2 (C). AO₃: ambient air O_3 , EO₃: elevated O_3 with 40 nmol mol⁻¹ O_3 enrichment; E0: spray water, E150: spray 150 mg L⁻¹ EDU aqueous solution, E300: spray 300 mg L⁻¹ EDU aqueous solution; -M and +M represent non-AM and AM inoculation with *Rhizophagus irregularis*, respectively. Only significant results of ANOVA are shown. *, $P < 0.05$, **, $P < 0.01$. Different letters above the columns denote significant differences ($P < 0.05$) among corresponding treatments by Tukey's HSD tests. Data are presented as means \pm standard error (SE) ($n = 4$).

under AO₃ treatment (Fig. 3C). In addition, O₃, EDU and AM inoculation and their interaction had no significant effect on stomatal aperture (Fig. 3D).

3.5 Ozone visible injury and malondialdehyde concentration

There was no O₃ visible injury of alfalfa under AO₃ treatment. All alfalfa treated with EO₃ showed visible injury. EDU significantly decreased the O₃ visible injury ($P < 0.05$). On the average of AM inoculation treatment, spraying 150 mg L⁻¹ EDU solution reduced the visible injury by 28.67%, and spraying 300 mg L⁻¹ EDU solution reduced the visible injury of alfalfa by 68.47% (Table 1). AM inoculation had a significant effect on visible injury ($P < 0.05$): AM plants had higher visible injury than -M plants regardless of EDU treatment. However, it is worth noting that there was no significant difference in visible injury between +M plants and -M plants under E300 treatment (Table 1). EDU spraying significantly delayed the date of first appearance of O₃ injury symptoms ($P < 0.01$), while AM inoculation significantly advanced the date of first appearance of O₃ injury symptoms ($P < 0.01$) (Table 1).

For MDA concentration, the interaction between O₃ and EDU was significant ($P < 0.05$): O₃ enrichment significantly increased MDA concentration by 70.66% under E0 treatment, but had no significant effect on MDA concentration under E150 and E300 treatments. EDU spraying significantly decreased MDA concentration, while AM inoculation had no significant effect on MDA concentration (Fig. 4, Table S2).

3.6 Element concentrations

O₃ and EDU had no significant effect on shoot and root C concentrations, but AM inoculation significantly increased shoot C concentration ($P < 0.01$) by 6.84% and root C concentration ($P < 0.01$) by 1.84% (Fig. 5A, 5B; Table S2).

There were no significant effects of O₃ and AM inoculation on shoot N concentration. EDU had a significant effect on shoot N concentration ($P < 0.05$): compared with E0, spraying 150 mg L⁻¹ EDU and 300 mg L⁻¹ EDU increased shoot N concentration by 0.29% and 9.60%, respectively (Fig. 5C, Table S2). O₃ enrichment and EDU spraying had no significant effect on root N concentrations, while AM inoculation significantly decreased root N concentration ($P < 0.01$) (Fig. 5D, Table S2).

In general, the shoot and root P concentrations of plants were not significantly affected by O₃ and EDU treatments. However, AM inoculation resulted in a significant increase of 48.72% in shoot P concentration and 108.12% in root P concentration (Fig. 5E, 5F; Table S2). In addition, the interaction of O₃, EDU and AM inoculation had a significant effect on shoot P concentration (Fig. 5E).

3.7 Antioxidant enzyme activities

In general, O₃ enrichment led to a significant decrease in SOD activity ($P < 0.05$), while EDU spraying resulted in a significant increase in SOD activity ($P < 0.05$). The SOD

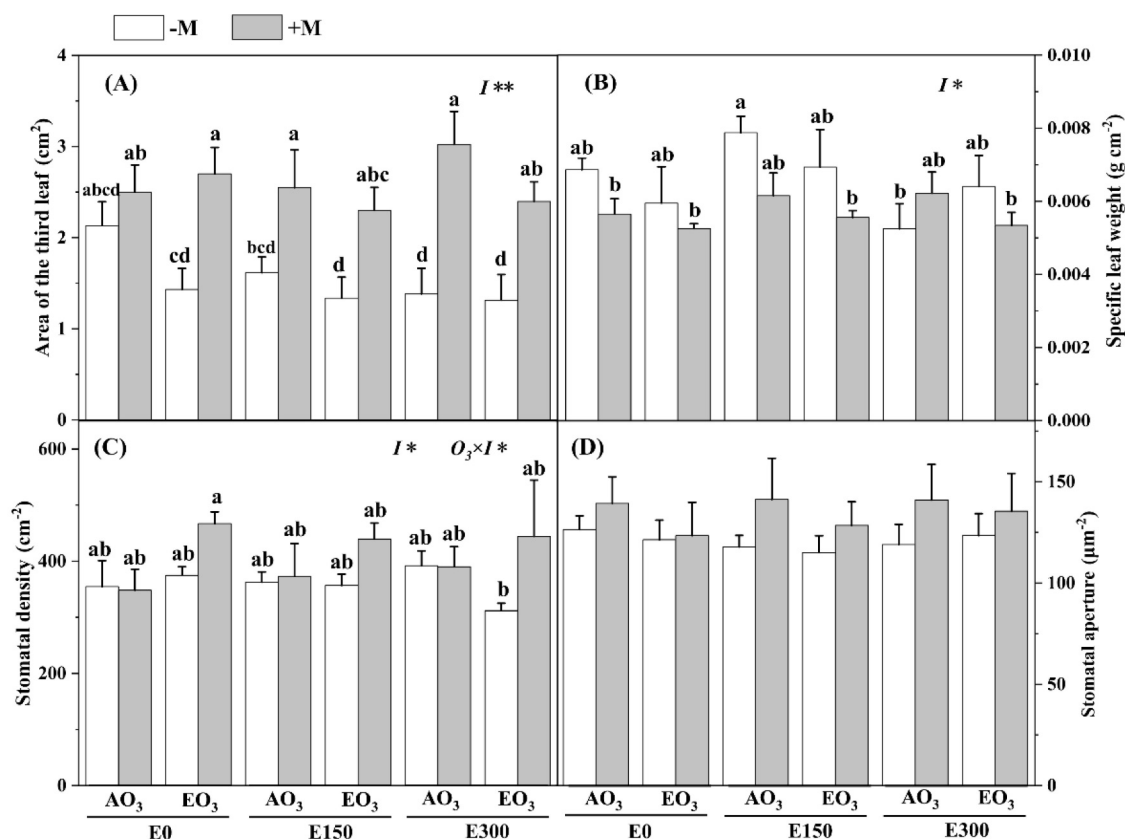


Fig. 3 Effects of ozone (O₃), EDU (E) and AM inoculation (I) on area of the third leaf (A), specific leaf weight (B), stomatal density (C) and stomatal aperture (D). AO₃: ambient air O₃, EO₃: elevated O₃ with 40 nmol mol⁻¹ O₃ enrichment; E0: spray water; E150: spray 150 mg L⁻¹ EDU aqueous solution, E300: spray 300 mg L⁻¹ EDU aqueous solution; -M and +M represent non-AM and AM inoculation with *Rhizopagus irregularis*, respectively. Only significant results of ANOVA are shown. *, *P* < 0.05, **, *P* < 0.01. Different letters above the columns denote significant differences (*P* < 0.05) among corresponding treatments by Tukey's HSD tests. Data are presented as means ± standard error (SE) (*n* = 4).

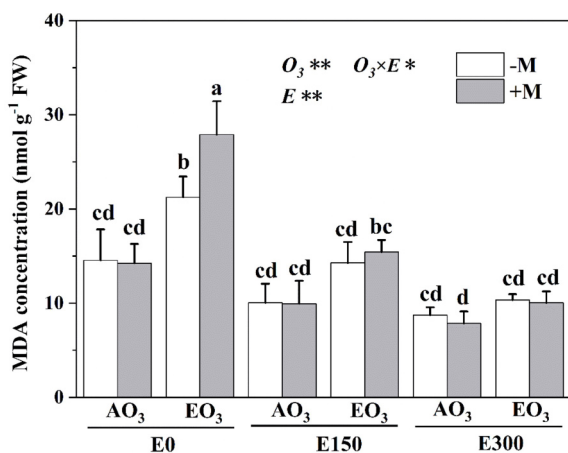


Fig. 4 Effects of ozone (O₃), EDU (E) and AM inoculation (I) on MDA concentration. AO₃: ambient air O₃, EO₃: elevated O₃ with 40 nmol mol⁻¹ O₃ enrichment; E0: spray water, E150: spray 150 mg L⁻¹ EDU aqueous solution, E300: spray 300 mg L⁻¹ EDU aqueous solution; -M and +M represent non-AM and AM inoculation with *Rhizopagus irregularis*, respectively. Only significant results of ANOVA are shown. *, *P* < 0.05, **, *P* < 0.01. Different letters above the columns denote significant differences (*P* < 0.05) among corresponding treatments by Tukey's HSD tests. Data are presented as means ± standard error (SE) (*n* = 4).

activities of plants treated with E150 and E300 were 31.64% and 21.14% higher than that of E0 plants, respectively. Furthermore, AM inoculation significantly increased SOD activity (*P* < 0.05) (Fig. 6A, Table S2).

The POD activity was significantly increased by O₃ enrichment (*P* < 0.01), and EDU also resulted in a significant increase in POD activity (*P* < 0.05). The SOD activities of E300 plants were 12.74% higher than that of E0 plants. There was a significant interaction between EDU and AM inoculation (*P* < 0.01): AM inoculation tended to reduce POD activity at E0 and E150, while the POD activity of AM plants was 36.82% higher than that of -M plants under E300 treatment (Fig. 6B, Table S2).

O₃ and EDU treatments showed no significant impact on APX and CAT activities, whereas AM inoculation significantly increased APX and CAT activities by 39.53% and 211.23%, respectively (Fig. 6C, D; Table S2).

4 Discussion

Generally, O₃ enters the plant leaf cells through the stomata

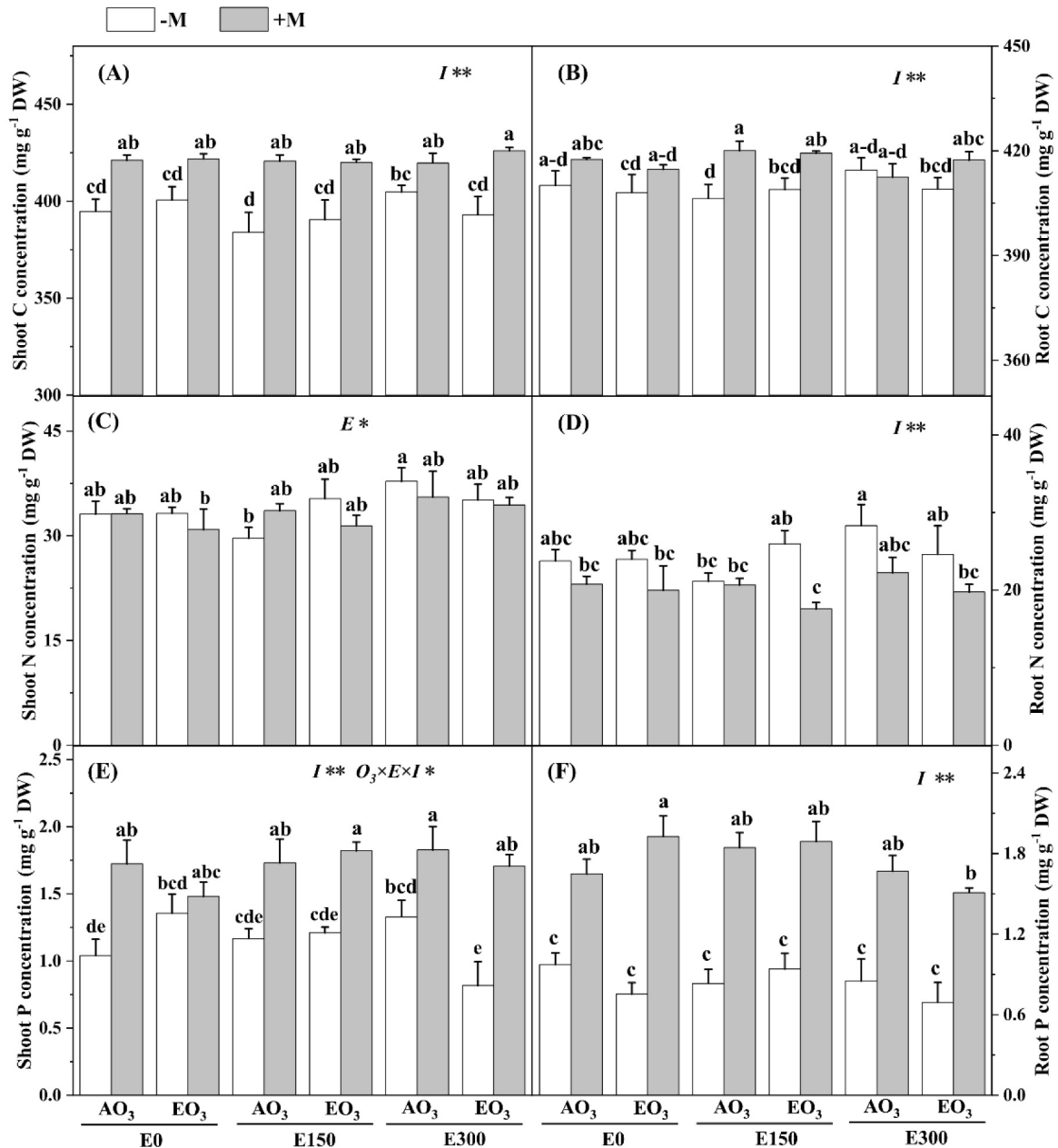


Fig. 5 Effects of ozone (O_3), EDU (E) and AM inoculation (I) on shoot C (A), root C (B), shoot N (C), root C (D), shoot P (E) and root P (F) concentrations. AO₃: ambient air O_3 , EO₃: elevated O_3 with 40 nmol mol⁻¹ O_3 enrichment; E0: spray water, E150: spray 150 mg L⁻¹ EDU aqueous solution, E300: spray 300 mg L⁻¹ EDU aqueous solution; -M and +M represent non-AM and AM inoculation with *Rhizopagus irregularis*, respectively. Only significant results of ANOVA are shown. *, $P < 0.05$, **, $P < 0.01$. Different letters above the columns denote significant differences ($P < 0.05$) among corresponding treatments by Tukey's HSD tests. Data are presented as means \pm standard error (SE) ($n = 4$).

(Harmens et al., 2018), and then undergoes a series of chemical reactions to form ROS (Feng et al., 2021). ROS can destroy the membrane structure in plant cells and cause damage to leaves (Fiscus et al., 2005; Feng et al., 2019a). O_3 damage is evident through visible injury symptoms, characterized by yellow or brown spots along the veins of leaves (Yin et al., 2022b). Furthermore, this damage is also reflected at the physiologic level. MDA serves as an indicator of lipid peroxidation (Draper and Hadley, 1990), and O_3

enrichment leads to the accumulation of MDA in plants (Feng et al., 2018b; Yin et al., 2022b). Additionally, leaf damage can result in reduced photosynthetic capacity and biomass (Li et al., 2016; Yin et al., 2023). The results of this study showed that O_3 enrichment could significantly decrease the light use efficiency and biomass of alfalfa (Figs. 1, 2), and significantly increased plant O_3 visible injury and MDA concentrations (Fig. 4, Table 1). However, it was worth noting that O_3 enrichment did not significantly

Table 1 Effects of EDU (*E*) and AM inoculation (*I*) on visible injury and number of days of fumigation with first appearance of O₃ injury symptoms.

<i>I</i>	<i>E</i>	Visible injury%	Number of days of fumigation with first appearance of O ₃ injury symptoms (days)
Treatment			
E0	-M	4.03 ± 0.39bc	20.50 ± 0.96ab
	+M	8.56 ± 1.99a	15.50 ± 0.65c
E150	-M	2.44 ± 0.66c	21.00 ± 1.68ab
	+M	6.54 ± 1.82ab	17.75 ± 1.11bc
E300	-M	1.95 ± 1.13c	22.75 ± 0.48a
	+M	2.02 ± 0.50c	21.75 ± 1.25a
ANOVA			
<i>E</i>		0.0100	0.0046
<i>I</i>		0.0109	0.0036
<i>E</i> × <i>I</i>		0.1728	0.2199

E0: spray water, E150: spray 150 mg L⁻¹ EDU aqueous solution, E300: spray 300 mg L⁻¹ EDU aqueous solution; -M and +M represent non-AM and AM inoculation with *Rhizophagus irregularis*, respectively. Different letters following the means in the same column denote significant differences ($P < 0.05$) among corresponding treatments by Tukey's HSD tests. A split-plot ANOVA was performed on the data, with significant effects highlighted in bold ($P < 0.05$). Data are presented as means ± standard error (SE) ($n = 4$).

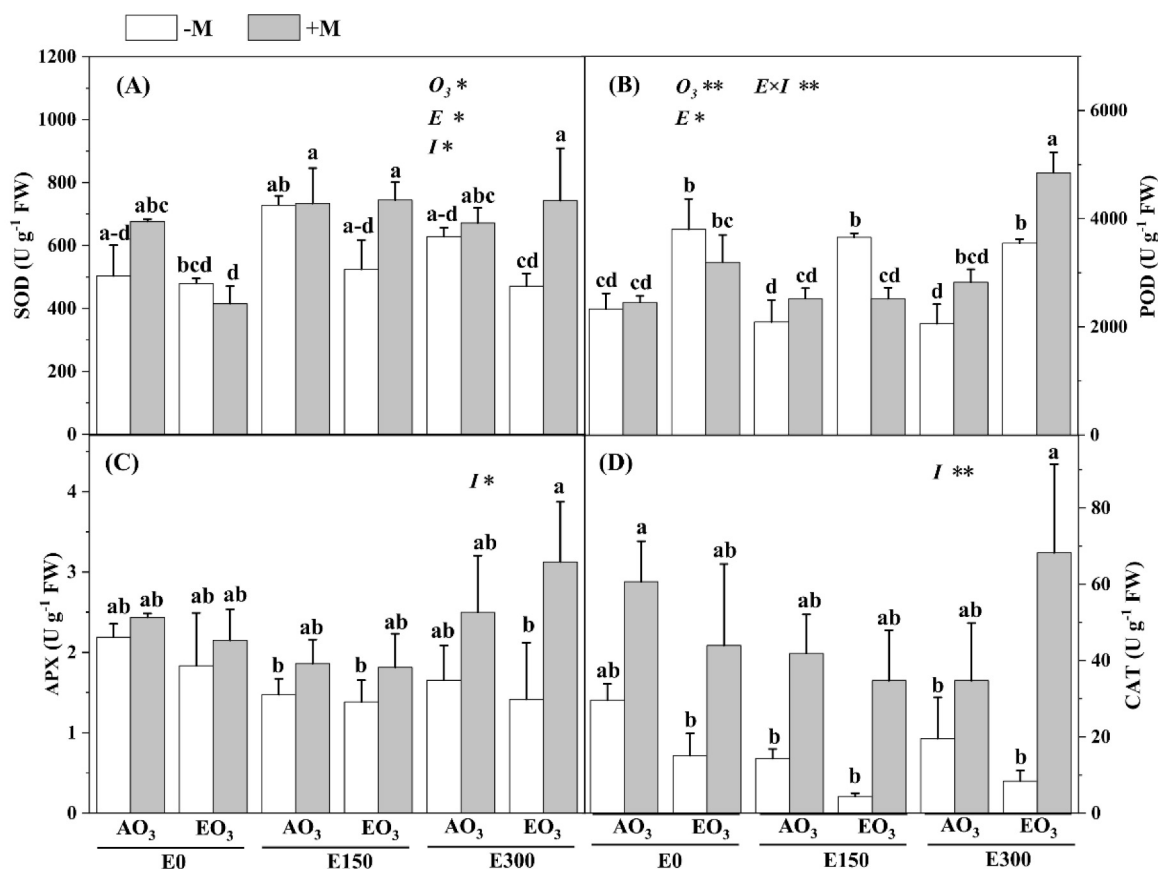


Fig. 6 Effects of ozone (O₃), EDU (*E*) and AM inoculation (*I*) on SOD (A), POD (B), APX (C) and CAT (D) activities. AO₃: ambient air O₃, EO₃: elevated O₃ with 40 nmol mol⁻¹ O₃ enrichment; E0: spray water, E150: spray 150 mg L⁻¹ EDU aqueous solution, E300: spray 300 mg L⁻¹ EDU aqueous solution; -M and +M represent non-AM and AM inoculation with *Rhizophagus irregularis*, respectively. Only significant results of ANOVA are shown. *, $P < 0.05$, **, $P < 0.01$. Different letters above the columns denote significant differences ($P < 0.05$) among corresponding treatments by Tukey's HSD tests. Data are presented as means ± standard error (SE) ($n = 4$).

decrease the net photosynthetic rate and stomatal conductance (Fig. 2 A, 2B), which was inconsistent with most previous studies (Wang et al., 2023; Yin et al., 2023). This might be because the intensity of O₃ effect depended on the plant's cumulative O₃ absorption (Anav et al., 2016; Xu et al., 2018; Yuan et al., 2020). In this study, the third mature leaf, which was used for measuring photosynthetic parameters, had a shorter duration of O₃ exposure compared to the older low-leaves, possibly resulting in a relatively weaker O₃ effect. Previous studies on the photosynthesis measurement of the same leaf position of alfalfa at the end of the growing season also showed that O₃ enrichment had no significant effect on photosynthetic rate and stomatal conductance (Yin et al., 2022a; 2022b), which also supported the results from present study (Fig. 2A and 2B).

Previous studies have shown that spraying EDU can alleviate the adverse effects of O₃ on plants (Feng et al., 2010; Manning et al., 2011; Yuan et al., 2015). The results of this study demonstrated that the application of EDU markedly decreased O₃ visible injury, delayed the appearance of initial O₃ injury symptoms (Table 1), and significantly decreased the shoot MDA concentration (Fig. 4). Taken together, these findings indicated that the application of EDU had a mitigating effect on O₃ damage. However, it was worth noting that even with the application of 300 mg L⁻¹ of EDU, alfalfa leaves still exhibited O₃ injury, although only at around 2% (Table 1), indicating that the O₃ protective effect of EDU might be difficult to achieve at 100%. Previous studies have also showed similar results, for example, some studies on rice indicated that although the application of EDU played a role in protecting yield under O₃ enrichment, it unable to offer complete protection (Zhang et al., 2021; Shang et al., 2022).

Mechanistically, all possible pathways for EDU mitigating O₃-induced damage to plants, including decreasing stomatal conductance to reduce O₃ intake (Darrall, 1989), supplementing N to enhance plant stress resistance (Agathokleous et al., 2016), and bolstering plant antioxidant capacity for more effective removal of ROS (Brunschonharti et al., 1995; Singh et al., 2009), were considered in this study. Our results showed that EDU spraying did not significantly affect plant stomatal conductance (Fig. 2B). When O₃ pollution occurs, plants exhibit stress avoidance responses leading to a decrease in stomatal conductance (Wang et al., 2023; Yin et al., 2023). The influence of EDU on stomatal conductance might be indirectly achieved by altering the intensity of this stress avoidance response. In this study, O₃ enrichment did not have a significant effect on stomatal conductance (Fig. 2B). Consequently, the indirect impact of EDU was weaker compared to the direct effect of O₃, resulting in the insignificance of EDU's influence on stomatal conductance. Our results suggested that EDU did not mediate stomatal

conductance to affect plant O₃ sensitivity.

Furthermore, our results showed that EDU spraying significantly increased shoot N concentration (Fig. 5C). Due to N element in EDU molecules accounts for 22.56% (Manning et al., 2011), EDU partially serves as a N fertilizer. Some studies have proposed that the N element in EDU contributes to the protection against O₃-induced leaf injury (Shang et al., 2018; Yin et al., 2022b), but this remains speculative and has not been experimentally validated (Manning et al., 2011). On the contrary, a study involving the application of urea solution with the same N concentration as EDU solution to tobacco (*Nicotiana tabacum* L.) leaves found no reduction in O₃-induced leaf injury compared to the control (Godzik and Manning, 1998). Additionally, research on poplar trees indicated that applying an equivalent amount of N fertilizer as EDU treatment did not provide O₃ injury protection (Agathokleous et al., 2023). These pieces of evidence collectively suggest that the N addition effect resulting from EDU application might not be the underlying mechanism for alleviating O₃ damage.

Of particular note, our findings indicated that the application of EDU significantly increased shoot SOD and POD activities (Fig. 6 A, 6B). Surabhi et al. (2022) sprayed EDU on the leaves of wheat in the field and found that EDU significantly increased SOD activity in plants. Similarly, investigations involving rice demonstrated that the application of EDU led to a marked increase in the antioxidant enzyme activities across diverse rice varieties, while also reducing membrane lipid peroxidation in plants (Singh et al., 2022). SOD can catalyze the dismutation of O₂⁻ into hydrogen peroxide (H₂O₂) and oxygen, while POD can remove H₂O₂ by oxidizing other substrates with H₂O₂ (Li et al., 2000). The increase of antioxidant enzyme activities helps to remove the ROS in the plant due to O₃ enrichment, so as to achieve detoxification (Yin et al., 2023). Taken together, our results suggest that EDU does not affect stomatal conductance, but mediates antioxidant enzyme activities to reduce O₃-induced plant damage. This confirms hypothesis (1).

In this study, the growth-promoting effect of AM inoculation was indeed impressive. Due to AM fungi can help alfalfa to effectively uptake P (Fig. 5E, 5F), AM plants have higher photosynthetic rate (Fig. 2B) and larger biomass (Fig. 1) than -M plants. Whether AM symbiosis can alleviate the damage of O₃ to plants is another focus of our attention. Our study showed AM inoculation significantly increased O₃ visible injury and advanced the date of first appearance of O₃ injury symptoms (Table 1), which revealed that AM inoculation instead increased O₃ sensitivity of alfalfa. Notably, the interaction between O₃ and AM inoculation was not significant on biomass (Fig. 1), suggesting that visible injury is a more sensitive indicator of plant O₃ sensitivity than biomass. The study by Yin et al. (2023) indicated that the influence of AM

symbiosis on plant O₃ sensitivity was species-dependent, so we compared the previous research on alfalfa with the results of this study. In an O₃ fumigation test, where an elevated O₃ with 60 nmol mol⁻¹ O₃ enrichment was introduced to alfalfa under similar conditions, contrary to the findings of this study, AM inoculation did not impact the O₃ sensitivity of plants (Yin et al., 2022b). This divergence might be attributed to the varying O₃ concentrations. Alfalfa, being highly sensitive to O₃, tended to rapidly exhibit O₃ injury under severe O₃ stress, which might mask the effect of AM inoculation on O₃ sensitivity. In another study using the same elevated O₃ concentration as in this study, it was shown that AM inoculation increased the O₃ sensitivity of alfalfa, which supports the results of this study (Yin et al., 2022a).

Mechanistically, AM symbiosis may affect plant O₃ sensitivity by affecting stomatal conductance, antioxidant enzyme activities, nutrient status, and leaf morphology. Our results showed that AM inoculation significantly increased stomatal conductance (Fig. 2B). This was consistent with the vast majority of research outcomes; for instance, experiments involving AM inoculation in licorice (*Glycyrrhiza uralensis* Fisch.) (Xie et al., 2018,2019) and barley (*Hordeum vulgare* L. cv. Pallas) (Li et al., 2014) demonstrated a significant enhancing effect of AM inoculation on plant stomatal conductance. Compared with non-inoculated plants, AM plants had better water status and higher leaf water content, which might have led to greater stomatal conductance of plants (Auge, 2001), and AM fungi might accelerate the transfer of C from aboveground to belowground (Auge, 2001), a decrease in leaf photosynthate might stimulate an increase in stomatal conductance (Jarvis and Davies, 1998; Auge et al., 2015). Due to there existed a strong linear correlation between stomatal conductance and the plant's intake of O₃ (Yuan et al., 2017; Yuan et al., 2020), an increase in stomatal conductance undoubtedly amplified the risk of plants experiencing O₃-induced injury. Previous studies had indicated that stomatal conductance was jointly influenced by stomatal density and stomatal aperture (Israel et al., 2022). To delve further, we verified how AM inoculation impacted stomatal conductance at an anatomical level. We observed a significant increase in stomatal density due to AM inoculation (Fig. 3C), rather than an increase in stomatal aperture (Fig. 3D). This aligned with findings from studies on wheat and alfalfa (Chitarra et al., 2016; Zhu et al., 2018; Yin et al., 2022a). AM symbiosis had the potential to trigger the upregulation of genes responsible for controlling the development of stomata (such as LeEPFL9, LeEPF1/2), resulting in an augmentation of stomatal density (Chitarra et al., 2016).

Furthermore, our results showed that AM inoculation significantly increased the activities of SOD, APX and CAT

in plants (Fig. 6). APX is an enzyme that utilizes ascorbate as a substrate to catalyze the decomposition of H₂O₂ into water, whereas CAT directly catalyzes the breakdown of H₂O₂ into water and oxygen (Maruta et al., 2016). Similar to SOD, these two enzymes play a vital role in removing ROS within the plant, protecting it from O₃-induced damage (Rao et al., 1995; Hassan, 2006). Although a large number of researches confirmed that AM symbiosis could significantly increase the antioxidant capacity of plants (Zhu et al., 2010; Yin et al., 2022a, 2023), and molecularly proved that AM symbiosis could significantly upregulate the expression of antioxidant enzyme genes, such as *PtFe-SOD*, *PtCu /Zn-SOD*, and *PtMn-SOD* (Ding et al., 2022; Han et al., 2022), but this still could not explain why AM fungi helped plants to develop such high antioxidant levels regardless of O₃ stress. We speculate that this may not be strongly related to P nutrition, even though the provision of P by AM fungi to plants remains potent, regardless of O₃ stress. The majority of studies have indicated that P addition results in the alleviation of P limitation, which in turn tends to lead to a reduction in plant antioxidant enzyme activities (Sayantan and Shandendu, 2013; Desai et al., 2014). We believe this might be an indication of the functional redundancy within the plant-AM fungi system. AM symbiosis enhances the plant's antioxidant capacity against the sudden occurrence of environmental stress, thereby bolstering the plant's survival capabilities in dynamic environments (Bennett and Groten, 2022). Additionally, our results showed that AM inoculation significantly increased the P concentration in plants (Fig. 5E, 5F). Previous studies suggested that P is the main component of rRNA (Agren, 2008), and the increase of shoot P may be beneficial for plants to synthesize structural proteins to resist O₃ stress (Shang et al., 2018; Yin et al., 2022a). However, there is still a lack of direct experimental evidence that P affects the O₃ sensitivity of plants. The study of spruce (*Picea abies* L. (Karst.)) seedlings showed that there was no significant interaction between P addition and O₃ (Utraiainen and Holopainen, 2001).

Interestingly, we found that AM significantly increased the area of the third leaf (Fig. 3A), and more importantly, significantly decreased plant specific leaf weight (Fig. 3B). Specific leaf weight is an important functional trait to measure the O₃ sensitivity of plants (Li et al., 2016; Feng et al., 2018a), and low specific leaf weight may mean that the plant has a large O₃ load per unit mass and thus makes plants more susceptible to O₃ damage (Plochl et al., 2000; Dai et al., 2017). The majority of research findings indicated that AM inoculation resulted in a reduction of specific leaf weight (Chen et al., 2020; Yin et al., 2022a). This could potentially be attributed to the improved nutritional status of AM plants compared to -M plants, causing shifts in the plants' survival strategy. As a result, the leaf morphology

tended to become thin and large (Fig. 3A, 3B), optimizing the utilization of light energy to facilitate plant growth. In summary, our study reveals that AM symbiosis mediates an increase in stomatal density, leading to enhanced stomatal conductance of alfalfa. Simultaneously, it decreases the specific leaf weight of alfalfa. Although AM inoculation increases antioxidant enzyme activities, the negative effects of O₃ sensitivity triggered by stomatal conductance and specific leaf weight are greater than the positive effects induced by antioxidant enzyme activity. As a result, AM inoculation amplified the O₃ sensitivity of alfalfa. This partly validates hypothesis (2).

In this study, there was no significant interaction between EDU spraying and AM inoculation for almost all indicators. EDU is sprayed on leaves and acts on plant leaves, while AM fungi acts on plant roots. It is reasonable that there is no direct interaction between the two. The effects of EDU and AM inoculation on the O₃ sensitivity of alfalfa were opposite. The results showed that the AM plants sprayed with 300 mg L⁻¹ of EDU had lower visible injury than -M plants sprayed with water, and the first visible injury symptoms appeared later (Table 1). This indicated that the effect of spraying 300 mg L⁻¹ EDU solution on O₃ sensitivity reduction overwhelmed the intensifying effect of AM inoculation on O₃ sensitivity. The results of this study showed that the combined application of EDU and AM inoculation could promote the growth of plants without affecting the O₃ sensitivity of plants, or even reduced plants O₃ sensitivity, thereby alleviating O₃ damage to plants. Therefore, the combined application of EDU and AM inoculation can be used as a powerful approach of O₃ risk prevention and control. This confirms hypothesis (3).

5 Conclusions

In this study, we found that EDU mediated antioxidant enzyme activities, rather than stomatal conductance, in reducing O₃ sensitivity of alfalfa plants. While AM inoculation significantly increased antioxidant enzyme activities (which was beneficial for scavenging ROS), it also significantly increased stomatal conductance and decreased specific leaf weight (both of which increased the risk of O₃-induced damage to plants). The negative impact of the latter outweighed the positive effects of the former, so AM inoculation increased O₃ sensitivity in alfalfa. Furthermore, our research indicated that AM inoculation increased plant stomatal conductance through stomatal density rather than stomatal aperture. Moreover, we demonstrated that the application of EDU at 300 mg L⁻¹ was sufficient to counteract the adverse effects of AM inoculation on O₃ sensitivity. The combined application of EDU and AM inoculation could

significantly improve plant growth performance while reducing O₃ sensitivity, greatly alleviating the adverse effects of O₃ on plants. However, this study utilized only one AM strain and plant species. The generalizability of these conclusions to other single or composite AM strains, as well as to other plant species, requires further verification. Additionally, considering real-world situations, it is essential to conduct field experiments to validate the conclusions.

Author contributions

Conceptualization, B.C. and Z.H.; methodology, R.Y., X.Y., X.Z., S.G., X.H. and L.W.; data analysis, R.Y. and X.Y.; writing—original draft preparation, R.Y.; writing—review and editing, B.C.; funding acquisition, B.C. All authors have read and agreed to the published version of the manuscript.

Data availability statement

The data sets generated during the current study are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Electronic supplementary material

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