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Effects of Reactive Oxygen Species Metabolic System on Soybean (*Glycine max*) Under Exogenous Chitosan to Ozone Stress

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Abstract In order to reveal effect mechanism of crop reactive oxygen species metabolic system under exogenous chitosan to ozone stress, open top chambers were utilized to investigate the change of reactive oxygen species production rate, lipid peroxidation extent, anti-oxidative enzymes activities and antioxidant content in soybean (*Glycine max*) leaves. Exogenous chitosan treatment relieves the aggravation of reactive oxygen species damage through ozone stress, which represents protective efficacy to soybean, the superoxide anion production rate, hydrogen peroxide content, malondialdehyde content and membrane permeability decreased, while anti-oxidative enzymes activity and anti-oxidative substances increased. But the alleviation of exogenous chitosan to ozone is limited.

Keywords Reactive oxygen species · Soybean · Exogenous chitosan · Ozone stress

The concentration of tropospheric ozone has been rising due to human activity since the Industrial Revolution (IPCC 2007). Tropospheric ozone concentration in the clean atmosphere was about 40 nL L^{-1}, but the concentration was as high as 100–200 nL L^{-1} in the pollution air. Models predict that ozone concentrations in the atmospheric boundary layer will increase globally by 33–100% during the period 2000–2100 (UNEP 2006). Adverse ozone effects on plants include foliar injuries, premature leaf loss, reduced growth (Matyssek and Sandermann 2003), and limited belowground carbon allocation (Andersen 2003). Thus, the impact of near ground ozone concentration to plant has become the focus of scholars.

Free radicals and reactive oxygen species, entities containing one or more reactive oxygen atoms including hydroxyl radical ($^{\bullet}OH$), superoxide anion radical ($O_2^{-\bullet}$), and hydrogen peroxide (H_2O_2). Whilst excess reactive oxygen species can damage cellular components, ozone rapidly degrades into various reactive oxygen species at the cell wall interface (Schraudner et al. 1998). A biochemical marker for plant sensitivity to ozone is an apoplastic reactive oxygen species burst after ozone treatment, which is absent or reduced in ozone-resistant plants (Overmyer et al. 2002).

Chitosan is a non-toxic and biodegradable cationic polymer derived by deacetylation of chitin, a homopolymer of β-(1-4)-linked N-acetyl-D-glucosamine. Owing to its biocompatibility, biodegradability, and biological activities, chitosan has been used in a variety of applications (Rinaudo 2006). With the progresses in intensive study of chitosan, it was also found that chitosan can promote germination obviously, improve secondary metabolism and resistance-related enzyme activity of plant, increase crop yields and agricultural quality (Bol et al. 1990).

Soybean (*Glycine max*) is one of main agrotype and its reactive oxygen species metabolic system under exogenous chitosan were investigated in open-top chambers under elevated ozone concentration in this paper, such as the content of reactive oxygen species, lipid peroxidation extent, anti-oxidative enzymes activities and antioxidant content. The effects of different treatments on reactive oxygen species metabolic parameters of soybean were studied to analyze its reactive oxygen species metabolic response mechanisms to elevated ozone concentration and predict the feasibility of soybean as one of composing crop under future climate change.

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Materials and Methods

The study was conducted on soybean (*Glycine max*) plants grown in six open-top chambers at the Shenyang Ecological Experiment Station of Chinese Academy of Sciences. The open-top chambers were 1.15 m in diameter and 2.4 m in height with a 45° sloping frustum and the minimum distance between any two chambers is 4 m. Ozone was produced from pure oxygen with an ozone generator (GP-5J, China). Ozone concentration were continuously monitored by ozone analyzers (S-900 Aeroqual, New Zealand), and were controlled by computers, using a professional program for ozone dispensing and monitoring.

The experimental site consists of five rings each of control: CK (ambient air; ozone concentration about 45 nmol mol^{-1}), elevated O_3 (ozone concentration of 110±10 nmol mol^{-1}), H_2O (exogenous spraying with distilled water to the plants in elevated ozone in branching), CTS-1 (exogenous spraying with chitosan to the plants in elevated ozone in branching, concentration of 50 mg/L), and CTS-2 (exogenous spraying with chitosan to the plants in elevated ozone in branching, concentration of 150 mg/L), and there were three replications for each treatment. At this facility, plants were grown in the field form seed in 30 April 2008, and exposed to ozone for 8 h (09:00–17:00) from 10 June to 30 August. Field water or chitosan treatments of intact plants were made on 19 June, with 50 mL each plant. Full developed leaves were sampled form all individual soybeans in all the chambers at 9 A.M. on branching (26 June), flowering (17 July), podding (10 August), filling (19 August), and were transported in liquid nitrogen, then stored at −40°C until processed.

The superoxide anion ($O_2^{-\bullet}$) assay was modified from the method of Wang and Luo (1990). The specific absorption maximum at 530 nm was determined. Hydrogen peroxide (H_2O_2) accumulation was analyzed according to Zou (2000). Supernatant was read at 415 nm against a reagent blank.

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content and membrane permeability according to the method of Zou (2000) and Xue (1985). Malondialdehyde quantity was determined by thiobarbituric acid reaction, and membrane permeability was measured by extravasation conductivity.

Superoxide dismutase (SOD) activity was determined using nitroblue tetrazolium (NBT) salt (Beyer and Fridovich 1987). One unit of SOD was defined as the amount of enzyme that yielded a 50% inhibition of the reduction of NBT. The activity of SOD was expressed as activity per mg protein. Catalase (CAT) activity was performed as described by Li et al. (2004). One unit of CAT activity was defined as the amount of enzyme required for oxidize 1 μmol of H_2O_2 per minute. Peroxidase (POD) activity was determined using guaiacol as described by Li et al. (2004). The rate of POD reaction was measured as an absorbance increase at 470 nm.

Ascorbic acid (AsA) was assayed following the method of Zou (2000). The absorbance was read at 534 nm using distilled water as reference using a spectrophotometer. Glutathione (GSH) concentrations were measured by 5, 5′-dithio-bis-2-nitrobenzoic acid (DTNB)-glutathione disulfide reductase recycling assay, as described by Anderson (1985). The specific absorption maximum at 412 nm was determined.

All data were subjected to statistical analysis of variance (ANOVA) using SPSS 11.5 for Windows statistical software.

Results and Discussion

In the whole growth period, $O_2^{-\bullet}$ production rate and H_2O_2 content in soybean leaves for all the five treatments are different (Fig. 1). Elevated ozone increased the rate of O_2 production, and compared to controls, there were significant increases of 10.5%–109.6% ($p < 0.01$) in growing stages (except flowering). After exogenous spraying with different concentration of chitosan, the rate of O_2 production in soybean leaves decreased compared to elevated ozone, it decreased significantly by 3.6%–24.7% under CTS-1 treatment. While under CTS-2 treatment, it had a 31.7% ($p < 0.05$) decrease compared to elevated ozone treatment only in podding. As shown in Fig. 2, H_2O_2 content in the leaves of soybean under elevated ozone had a significant increase of 15.4%–89.3% than control. In contrast to the elevated ozone, H_2O_2 content decreased after spraying with different concentration of chitosan. Hydrogen peroxide content had a maximum reduction of 16.4% under CTS-1 and 16.9% under CTS-2, respectively, compared with ozone treatment.

For each growing stage analyzed separately, we found significant treatment differences of MDA content (Fig. 3) and relative conductivity (Fig. 4). Elevated ozone

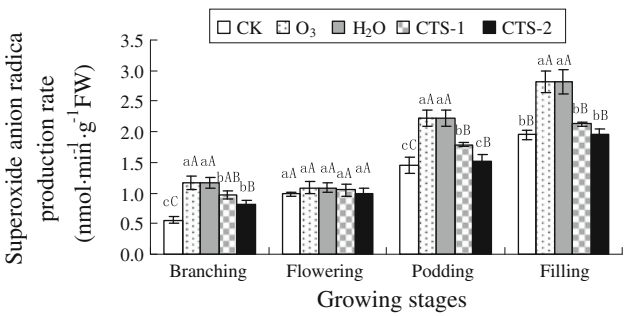

Fig. 1 Effects of superoxide anion ($O_2^{-\bullet}$) production rate in soybean leaves under exogenous chitosan to ozone stress in branching

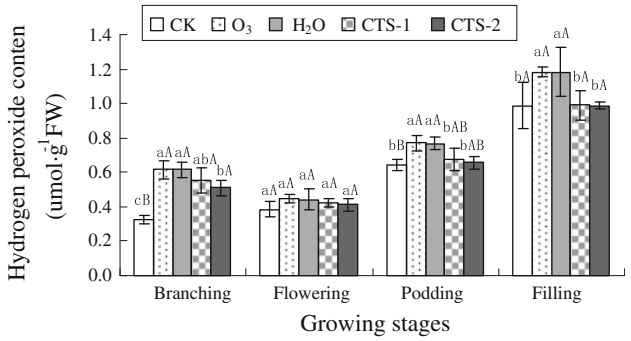

Fig. 2 Effects of hydrogen peroxide (H_2O_2) content in soybean leaves under exogenous chitosan to ozone stress in branching

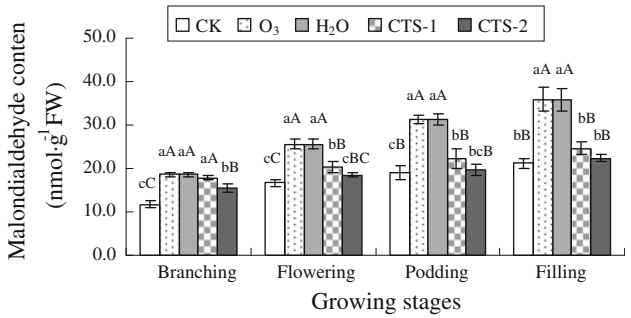

Fig. 3 Effects of malondialdehyde (MDA) content in soybean leaves under exogenous chitosan to ozone stress in branching

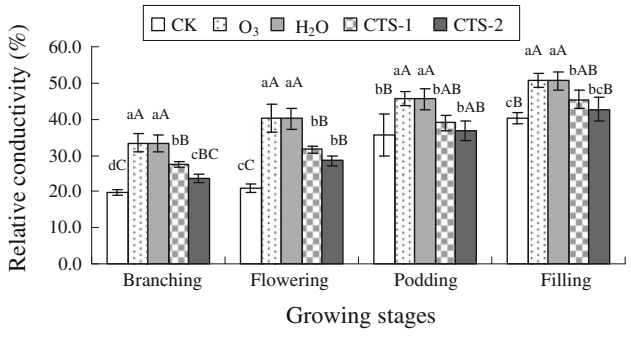

Fig. 4 Effects of relative conductivity in soybean leaves under exogenous chitosan to ozone stress in branching

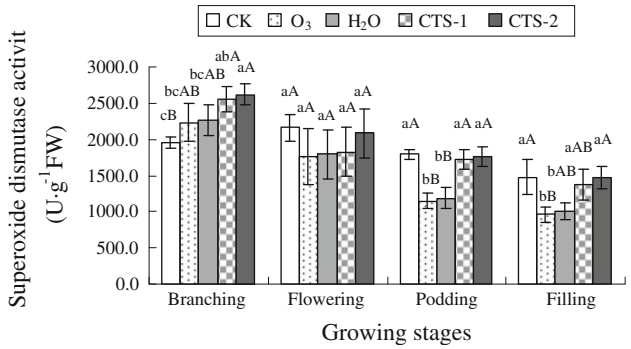

Fig. 5 Effects of superoxide dismutase (SOD) activity in soybean leaves under exogenous chitosan to ozone stress in branching

increased the content of MDA significantly ($p < 0.01$) compared to control in the whole growth period, ranging from 53.6% to 69.8%. Malondialdehyde content was lower in chitosan treatment than elevated ozone treatment, chitosan decreased MDA content by 31.5% ($p < 0.01$) under CTS-1 and by 37.9% ($p < 0.01$) under CTS-2. Elevated ozone statistically significantly affect relative conductivity of soybean leaves, the increasing ratio ranged from 25.5% to 91.6% ($p < 0.01$). Though relative conductivity was lower in chitosan treatment than ozone fumigation treatment, it still higher than control. CTS-1 decreased significantly by 10.2%–21.6%, while CTS-2 decreased significantly by 15.6%–29.5% in the whole growth period.

Changes in anti-oxidative enzymes activity caused by the experimental treatments during the growing period had visible impacts on soybean leaves. Elevated ozone gradually decreased SOD activity during growing expect branching (Fig. 5), it had a significant decrease of 36.1% ($p < 0.01$) than control in podding and filling. Relative to elevated ozone treatment, exogenous chitosan treatment increased SOD activity, but it still lower than control (expect branching). CTS-1 and CTS-2 increased SOD activity by 3.6%–50.2% and 17.3%–54.3% under elevated ozone, respectively. Catalase activity in the elevated ozone treatment continued to decrease each growing stage than control but at an increasing rate first and then decreasing, its maximum decrease was 58.8% (Fig. 6). Exogenous chitosan increased CAT activity under elevated ozone. Both CTS-1 and CTS-2 had a significant increase in the growing period except flowering, CAT activity was increased 2.4%–135.7% and 13.5%–137.8%, respectively. Peroxidase activity was modified markedly by any of the treatments (Fig. 7); it tended to be lower (−23.0%) in elevated ozone treatment during each growing stage. There was a significant reducing effect on POD activity in the whole growth period. Peroxidase activity under CTS-1 and CTS-2 was 10.0% and 22.7% greater than under elevated ozone, respectively.

Across all treatments, there were significant differences in AsA content (Fig. 8) and GSH content (Fig. 9). Ascorbic acid content in the elevated ozone treatment was approximately 29.1% lower than control during the peak of the growing stage (except flowering). CTS-1 and CTS-2 increased AsA content of soybean leaves under the elevated ozone by 25.0% and 36.7%, respectively. Exogenous chitosan increased GSH content of soybean leaves in elevated ozone (Fig. 9). Exposure to elevated ozone resulted in GSH content 34.4%–47.6% lower than control in all growing stages. Glutathione content in soybean leaves

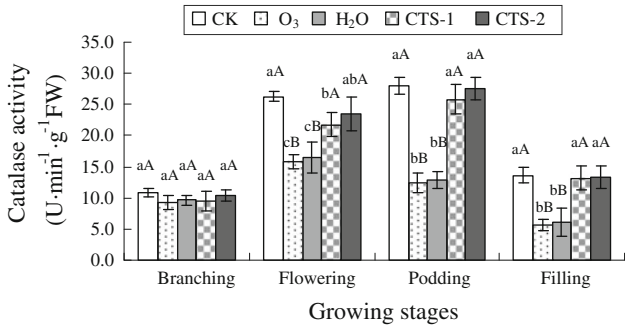

Fig. 6 Effects of catalase (CAT) activity in soybean leaves under exogenous chitosan to ozone stress in branching

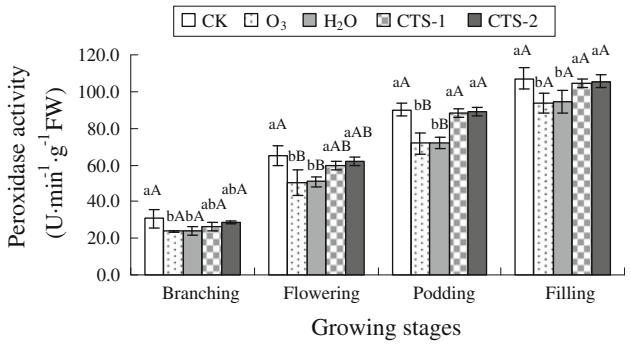

Fig. 7 Effects of peroxidase (POD) activity in soybean leaves under exogenous chitosan to ozone stress in branching

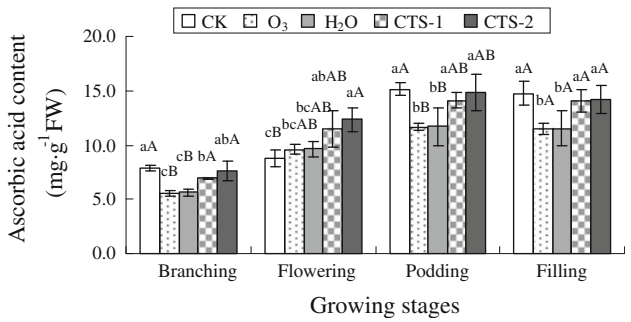

Fig. 8 Effects of ascorbic acid (AsA) content in soybean leaves under exogenous chitosan to ozone stress in branching

grown under CTS-1 and CTS-2 was 76.4% and 81.3% greater than that soybean growing elevated ozone, respectively.

In conclusion, compared to control, elevated ozone significantly increased $O_2^{-\bullet}$ production rate, H_2O_2 content, MDA content and membrane permeability, whereas decreased the activity of SOD, CAT, POD and the content of AsA, GSH. The negative effects of elevated ozone largely destroyed the reactive oxygen species metabolism and anti-oxidative system, consistently induced oxidative damage, damaged membrane, and decreased the capability to resist ozone stress. Exogenous spraying with chitosan in

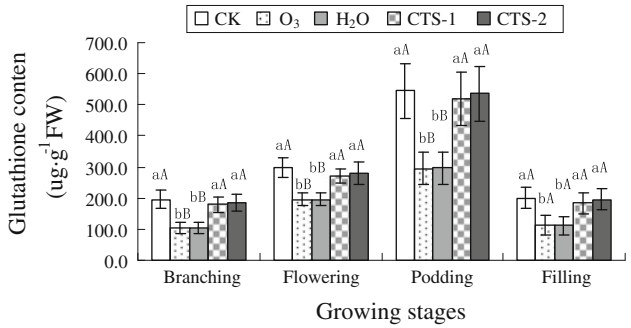

Fig. 9 Effects of glutathione (GSH) content in soybean leaves under exogenous chitosan to ozone stress in branching

elevated ozone in soybean branching, contrary to elevated ozone, $O_2^{-\bullet}$ production rate, H_2O_2 content, MDA content and membrane permeability decreased, but still higher than control, while the activity of SOD, CAT, POD and the content of AsA, GSH increased, but still lower than control. Results form this study has shown that, under interacting chitosan and ozone, chitosan can suppress the toxic effects of ozone to soybean; it ameliorated ozone-related oxidative damage and protected anti-oxidative system from ozone stress, but this function was limited. Reactive oxygen species responses play a key role in plant growth. Understanding the potential interactive impacts of elevated ozone and exogenous chitosan on reactive oxygen species metabolic system of soybean is crucial for crop yield in the future changing global climate.

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