Effects of elevated O₃ exposure on seed yield, N concentration and photosynthesis of nine soybean cultivars (*Glycine max* (L.) Merr.) in Northeast China

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ABSTRACT

Nine soybean cultivars widely cultivated in Northeast China were investigated in present study to assess their O₃ sensitivities on the basis of the response of photosynthesis and seed yield to ambient and future ozone (O₃) concentrations, and determine whether the effects of O₃ vary with the developmental stages (flowering and seed filling stages). Relative to charcoal-filtered air (CF), elevated O₃ concentration (E-O₃, ambient air + 40 ppb) significantly reduced soybean yields by 40%, with a range of 32–46% among cultivars. E-O₃ also induced significant decreases in pigment contents, net photosynthetic rate and chlorophyll *a* fluorescence at both flowering and seed filling stages in most cultivars. Except net photosynthetic rate and stomatal conductance (*g_s*) at seed filling stage, all variables showed no significant interaction between O₃ and cultivar, suggesting that all tested cultivars had similar sensitivities to O₃. The responses of seed N content to E-O₃ differed among cultivars. Ambient O₃ concentration (mean of daily concentration of 19 ppb) did not induce any change relative to CF. Significant positive relationship between endogenous *g_s* in CF and yield loss among cultivars was found only at seed filling stage. Positive correlation between effects of E-O₃ on leaf N content and effects on light saturated photosynthetic rate (*A_{sat}*) indicated that *g_s* and leaf N content at seed filling stage contributes to yield loss and decreased photosynthesis by E-O₃, respectively. It can be inferred that E-O₃ had a larger negative effects on seed filling stage than flowering stage of soybean.

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1. Introduction

As one of the most harmful air pollutants, ground-level ozone (O₃) is projected to increase by 40–60% by 2100 compared with the current background level ~40 ppb, particularly in Asia due to increased emissions of O₃ precursors [1]. Numerous studies have well documented the negative effects of O₃ on the growth, biomass and yield of staple food crops. Soybean (*Glycine max* (L.) Merr.), a most widely grown legume in the world, was most susceptible to O₃ among food crops [2,3]. A recent meta-analysis indicated that current O₃ concentration (31–50 ppb) reduced the yield of soybean

by 7.7%, and future O₃ concentration (51–75 ppb) drove a further 10% yield loss compared with yield loss at current O₃ concentration [4], suggesting that ambient O₃ concentration has threatened soybean production.

The yield, growth and physiological responses of soybean to O₃ have been widely studied in both open-top chambers and open air field condition [5–7]. Several studies in open-top field chambers indicated that soybean yield has already been under threat at current O₃ concentrations [3,8], with dramatic yield loss of 53% by ambient air in Pakistan [9]. An increase of O₃ concentration from 30 to 60 ppb for 7 h day^{−1} could decrease soybean yield by 16% [10], and greater yield loss under higher O₃ concentration [11]. Results from the open air field condition have also indicated that yield of American soybean was decreased by 20% when ambient O₃ level increased from 56 to 69 ppb (8-h daily average, 09:00–17:00 h) [5]. A relationship between AOT40 (the hourly mean ozone concentration accumulated over a threshold O₃ concentration of 40 ppb during daylight hours) and yield loss was built up from European

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and American cultivars [11]. However, limited information is available in Asia although current O₃ level potentially or actually posed a risk on local soybean production.

From the perspective of crop productivity, O₃-induced significant reduction in plant biomass and yield is largely attributed to the negative impacts on primary metabolism and leaf longevity [12]. Both photosynthetic rate and carbon assimilation requires considerable N-derived enzymes e.g. Rubisco, to activate the photosynthetic metabolism [13]. Therefore, the variation of leaf N status over time is essential to understand the underlying mechanism in crop yield loss and photosynthetic processes after exposure to elevated O₃. Existing results demonstrated that elevated O₃ increased foliar N level in fen species [14], significantly decreased nitrate reductase activity in rice leaves [15], but had no significant effects on nutrient element concentrations in different tissues in wheat [16,17]. For soybean, the negative effects of O₃ on primary carbon metabolism have been well investigated, as indicated by stimulated aging and senescence, decreased photosynthesis and leaf chlorophyll content, impeded electron transport in the light reaction before senescence [18,19], a shorter development stage for carbon fixation [20] and relatively longer time for stomatal closure [21]. Generally, more than half of the N uptake by soybean is derived from N₂ fixation. Yield was positively related with N₂ fixation rates and dry matter production during seed filling period, and the increased biomass at the beginning of seed filling would provide a large N pool that was available for remobilization during seed filling [22]. However, the relationship between O₃-induced changes in leaf N content and photosynthesis rate response is scarce for those N fixation species. The underlying mechanisms responsible for decreased photosynthesis and C assimilation by O₃ varies among soybean cultivars (or genetic variation) [21,23]. Moreover, O₃ had larger effects on reproductive stage than vegetative stage for sensitive soybean cultivars [3], possibly due to larger O₃ accumulation and less N pool at reproductive stage [3,24].

Furthermore, many modern soybean cultivars with high stomatal conductance (g_s) are bred to gain high seed yield in China, particularly in Mollisol zone where it is a highly productive agricultural region with sown acreage and total yield accounting for 33% and 44% of the nation's total, respectively [25]. On the other hand, these high-yielding cultivars are also exposed to high O₃ flux, due to both larger g_s and its key development stages (flowering and seed filling stage) of soybean overlapping with the peak O₃ concentration from July to September, suggesting that the local soybean cultivars could be negatively affected by ground level O₃.

Therefore, the objectives of this study were: (1) to assess O₃ sensitivity in nine soybean cultivars widely cultivated in Mollisol zone of Northeast China on the basis of the response of photosynthesis, seed yield and its components, (2) to clarify the possible mechanism on the reduced photosynthesis rate caused by O₃, and (3) to determine whether the effects of O₃ vary with the developmental stages of soybean.

2. Materials and methods

2.1. Research site and experimental design

This research was conducted at the experimental field of Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences (45°73'N, 126°61'E), Harbin, Heilongjiang province, China. It has a continental climate with cold winters (mean January temperature of −19°C), warm summers (mean July temperature of 23°C), and mean annual precipitation of 569 mm with 65% in June–August.

Nine open-top chambers (OTCs, octagonal base, 8.5 m² of growth space and 2.7 m in height) were constructed with steel

frame, covered with polyethylene film. Meteorological parameters within the OTCs included photosynthetic photon flux density (PAR), air temperature and relative humidity. Three O₃ treatments were initiated, charcoal-filtered air (CF), non-filtered ambient air (NF) and ambient air added 40 ppb O₃ (E-O₃) with three chambers replicates, respectively. For E-O₃, O₃ was generated from pure oxygen by an electrical discharge O₃ generator (HY003, Chuangcheng Co. Jinan, China) and piped into OTCs, then mixed with ambient air to achieve the target O₃ concentration under the regulation of mass flow controllers. The air exchange velocity in the OTCs maintained within twice per minute. The O₃ concentration in the OTCs was continuously monitored at approximately 10 cm above plant canopy using a UV absorption O₃ analyzer (Model 49i-Thermo, USA).

2.2. Plant materials and ozone exposure

Nine soybean cultivars with similar growth periods (120–125 days) were selected considering their popularity in Black soil zone of Northeast China. They were: Hefeng25 (HF25), Hefeng35 (HF35), Hefeng55 (HF55), Heinong35 (HN35), Heinong37 (HN37), Heinong65 (HN65), Suinong22 (SN22), Suinong26 (SN26) and Suinong31 (SN31). The agronomic characteristics of these cultivars were listed in Table 1. On 20 May 2012, 9 cultivars were uniformly planted into circular plastic pots (diameter, 40 cm; height, 30 cm) filled with 15 kg soil in mixture with river sand at the ratio of 2:1 (v/v). The soil was typical Black soil with a pH of 6.3, soil organic C of 26.8 g kg^{−1}, and total N of 2.1 g kg^{−1}. Pure chemical fertilizer KH₂PO₄ of 3.29 g and urea of 3.26 g were applied at sowing. Nine seeds were planted in each pot and the plants were thinned to three per pot at vegetative growth stage (V₁). All plants were well watered with tap water to avoid drought stress during the whole growing season, with an increase in irrigation frequency in the hottest months (July and August).

All plants were pre-adapted to OTCs condition for 10 days before O₃ fumigation. O₃ fumigation started from 20 June to 11 September with a daily maximum of 8 h (from 09:00 to 17:00), with a total of 68 effective fumigation days when there was no rain, fog, mist or dew. For each treatment, four pots with one cultivar were randomly arranged in each of three chambers. Pots were sufficiently spaced to avoid mutual shading and the position of pots was changed every 3–5 days within each OTC to eliminate positional effects during the fumigation. The mean air temperature and relative air humidity within and out of OTCs were 22.4°C and 21.8°C, 83.1% and 73.8%, respectively, and averaged 8 h PAR was 624 μmol m^{−2} s^{−1} during O₃ fumigation.

2.3. Growth measurements

Harvests were performed on 22 September (124 days after planting) when the pods were ripe but not yet burst. For any cultivar, total twelve plants of four pots per chamber were harvested. The pod number, node numbers on main axis, seed yield per plant and 100-seed mass were determined.

2.4. N concentration analysis

Seeds and upper leaves were dried at 80°C for 48 h, and then milled to a fine powder before analysis. The concentration of N in the seed and leaf powder was determined by an elemental analyzer (Virio EL III, Elementar Inc., Germany).

2.5. Gas exchange and chlorophyll *a* fluorescence measurement

Gas exchange and chlorophyll *a* fluorescence parameters were measured using a portable photosynthesis system fitted with a 6400-40 leaf chamber fluorometer (LCF) (LI-6400, LI-COR Inc.,

Table 1
The characteristics of nine soybean cultivars used in the study.

Cultivars	Year of release	Maturity days	Male parent	Female parent	Leaf shape	Podding habit
HF25	1984	115–120	Kejiao4430-20	Hefeng 23	Lanceolate	Semi-determinate
HF35	1994	115–120	Sui81-272	Hejiao8009-1612	Lanceolate	Semi-determinate
HF55	2008	117	Suinong4	Beifeng11	Lanceolate	Indeterminate
HN35	1990	115	Heinong16	Shishengchangye	Lanceolate	Semi-determinate
HN37	1992	125	Ha78-8391	Heinong28	Ovoid	Semi-determinate
HN65	2010	115	Heinong40	Kenjian7	Lanceolate	Semi-determinate
SN22	2005	118	Sui96-81029	Suinong15 (F5)	Lanceolate	Indeterminate
SN26	2008	120	Sui96-81029	Suinong15	Lanceolate	Indeterminate
SN31	2009	121	Nongda05687*Suinong4	Suinong4	Lanceolate	Indeterminate

Lincoln, NE, USA). For any cultivar, two fully expanded upper leaves per plant and three plants were randomly selected from different pots at each chamber and were measured at flowering stage and seed filling stage from Node 8 to Node 10, and from Node 13 to Node 15, respectively. All measurements were conducted during 09:00–11:00 on clear days under saturated PPFD (photosynthetic photon flux density) of $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$, ambient temperature of 33°C , CO_2 concentration of $380 \mu\text{mol mol}^{-1}$, and relative humidity (RH) at 45–60%. During gas exchange measurements, chlorophyll *a* fluorescence parameters were recorded as well. All gas exchange and fluorescence measurements were recorded when the stability or steady state indicated by total coefficient of variation was $\leq 3\%$.

2.6. Leaf pigments content

Leaf pigment contents were examined on trifoliate leaves used for gas exchange and fluorescence measurement. Two leaf discs per plant from three plants in each chamber were extracted with 4 ml 95% ethanol in the dark for 48 h at 4°C . The leaf pigment was then assayed for chlorophyll (Chl) and carotenoid (Car) content according to the specific absorption coefficients provided by Lichtenthaler [26].

2.7. Statistical analysis

Values in each chamber were used as an independent experimental unit for any treatment throughout the statistics. The original data were tested for normality and transformed using square roots, natural logs and/or arcsine square roots where necessary to normalize variances between O_3 treatments. Main effects of O_3 , cultivars and their interaction were tested with analysis of generalized linear model. Student's *t*-test was used to compare the difference of means between CF and E-O_3 . Treatment effects were considered significant if $P < 0.05$. Data shown in figures were mean $\pm$ S.E. ($n = 3$).

3. Results

3.1. Ozone exposure

Daily mean O_3 concentration (M8) in NF averaged 19.1 ppb with a maximum of 35.2 ppb, and E-O_3 averaged 58.6 ppb with a maximum of 75.2 ppb (Fig. 1). AOT40 in E-O_3 was 10.6 ppm h.

Due to low O_3 concentration in NF, no significant difference between CF and NF was found in all variables tested. Here, only results of CF and E-O_3 treatments were shown.

3.2. Seed yield and yield components

E-O_3 significantly decreased the seed yield by $\sim 40\%$ in all cultivars, with a range from 32% in HF55 to 46% in HN35 (Table 2). Relative to CF, E-O_3 significantly reduced the seed yield components including 100-seed mass, pod number and node number on

main axis. However, no significant interaction between O_3 and cultivars was found due to similar response to E-O_3 across all cultivars, despite significant difference among cultivars (Table 2). Overall, E-O_3 significantly decreased 100-seed mass by 21% compared with CF. Similar decrease was also found in pod number, with a range from 14% to 38%, but only two cultivars HN65 and SN26 showed significant decrease by E-O_3 . Within these two cultivars, only HN65

Fig. 1. Environmental conditions within the open-top chamber including daily O_3 concentration from 09:00 to 17:00 h in three treatments (charcoal-filtered air, CF; non-filtered air, NF; ambient air + 40 ppb, E-O_3), daily air temperature within and out of open-top chambers, and daily photosynthetic active radiation (PAR).

Table 2

Seed yield and its components in 9 soybean cultivars exposed to charcoal-filtered air (CF) and elevated O₃ (E-O₃). Values are mean ± S.E. (n = 3). Significant differences between O₃ treatments for each cultivar are noted with an asterisk at P < 0.05.

	Seed yield (g/plant)			Mass per 100-seed (g)			Pod number			Node number on main axis		
	CF	E-O ₃	%	CF	E-O ₃	%	CF	E-O ₃	%	CF	E-O ₃	%
HF25	19.0 ± 0.9	11.6 ± 0.6	−39*	21.39 ± 1.16	16.04 ± 0.59	−25*	43.5 ± 4.3	28.6 ± 3.3	−34	17.7 ± 0.4	15.9 ± 0.7	−10
HF35	17.4 ± 0.7	11.7 ± 0.6	−33*	21.07 ± 1.23	15.74 ± 0.55	−25*	32.6 ± 1.8	27.9 ± 1.1	−14	16.6 ± 0.2	16.0 ± 0.5	−3
HF55	16.9 ± 0.9	11.4 ± 0.9	−32*	22.90 ± 0.59	19.93 ± 1.08	−13	33.2 ± 0.6	27.7 ± 1.4	−16	18.4 ± 0.1	18.1 ± 0.3	−1
HN35	12.8 ± 1.2	6.9 ± 0.6	−46*	19.23 ± 0.23	15.67 ± 0.35	−18*	24.4 ± 3.0	18.7 ± 2.8	−24	14.4 ± 0.5	13.2 ± 0.5	−9
HN37	20.4 ± 1.3	12.4 ± 1.0	−39*	20.89 ± 0.08	17.29 ± 0.85	−17*	48.9 ± 3.3	36.6 ± 4.8	−25	15.4 ± 0.4	14.9 ± 0.5	−3
HN65	17.0 ± 0.9	9.9 ± 0.3	−42*	19.59 ± 0.31	17.01 ± 1.40	−13	35.0 ± 1.1	22.9 ± 1.3	−35*	16.8 ± 0.3	15.7 ± 0.2	−7*
SN22	18.7 ± 0.8	10.4 ± 0.5	−44*	23.03 ± 0.68	15.67 ± 0.46	−32*	40.5 ± 7.8	28.0 ± 2.6	−31	18.1 ± 0.3	17.8 ± 0.3	−2
SN26	16.4 ± 0.8	9.5 ± 0.5	−42*	22.05 ± 0.23	16.60 ± 0.67	−25*	32.1 ± 1.4	19.8 ± 3.5	−38*	17.1 ± 0.4	15.8 ± 0.5	−8
SN31	19.5 ± 0.8	11.9 ± 0.6	−39*	21.89 ± 0.45	17.58 ± 1.53	−20	47.7 ± 3.8	37.4 ± 1.6	−21	16.5 ± 0.2	16.2 ± 0.2	−2
ANOVA (P-values)												
O ₃		0.000			0.000			0.000			0.000	
Cultivars		0.000			0.003			0.000			0.000	
O ₃ × cultivars		0.843			0.123			0.736			0.470	

showed significant decrease in node numbers on main axis by E-O₃ compared with CF (Table 2).

3.3. N concentration in leaves and seeds

The effects of E-O₃ on leaf N concentration depend on development stage or accumulated O₃ concentration. At flowering stage, the main effect of O₃ on leaf N concentration was significant, resulting in an average decrease by 6% compared with CF (Table 3). At seed filling stage, however, significant effects of O₃, cultivars and the interaction on leaf N concentration were found. HF25 in E-O₃ showed significant decrease by 16%. In contrast, E-O₃ induced significant increases by 36%, 28% and 48% in cultivars HN65, SN26 and SN31, respectively, relative to CF.

Significant interaction of O₃ and cultivars was found in seed N concentration, as indicated that E-O₃ induced significant increase in SN22 by 8%, while a significant decrease in SN26 by 16% relative to CF (Table 3).

3.4. Pigment contents, gas exchange and chlorophyll a fluorescence

At both flowering and seed filling stages, E-O₃ significantly reduced total Chl and Car contents, while no significant interaction between O₃ and cultivars was found (Table 4). At flowering stage, only HN35 showed significant reductions by E-O₃. At seed filling stage, E-O₃ induced significant decreases in all cultivars (with an exception of Car content in SN31), as indicated by an averaged decrease by 65% and 45% in total Chl and Car contents, respectively

(Table 4, Fig. 2). In addition, Chl *a/b* ratio was not affected by E-O₃ or cultivars or interaction of O₃ and cultivars.

For the response of gas exchange at flowering stage, E-O₃ significantly decreased *A*_{sat} by 45% and increased *C*_i by 12% compared with CF across all cultivars, but had no significant effects on *g*_s. No interaction between O₃ and cultivar was found (Table 4, Fig. 3a, c and e). A significant decrease in water use efficiency (WUE) by E-O₃ was observed in all cultivars with an exception of SN22, ranging from 30% to 55% (Table 4, Fig. 3g). Similar to the response of *A*_{sat}, E-O₃ caused a significant decrease in Φ_{PSII} and *qP*, with on average decreases of 27% and 19%, respectively, although all cultivars had a similar response, as indicated by no significant interaction (Table 4, Fig. 4a, c and e).

At seed filling stage, the main effects of O₃ and cultivars on both gas exchange and chlorophyll a fluorescence were statistically significant, with significant interaction between O₃ and cultivars on *A*_{sat}, *g*_s, and *qP* (Figs. 3 and 4). Compared with CF, E-O₃ significantly decreased *A*_{sat} and *g*_s by 41% and 21%, respectively, whereas increased *C*_i by 9% for all cultivars. Notably, only HF25 showed significant increases in *g*_s and *C*_i by 50% and 23%, respectively. In addition, E-O₃ induced significant reductions in *F*_v'/*F*_m', Φ_{PSII} , and *qP* by 10%, 34%, and 27%, respectively, which was much greater than that at flowering stage.

3.5. Photosynthesis-N and seed yield –*g*_s relationship among cultivars

There was a significant positive correlation (*r* = 0.710, *P* = 0.032) between effects of E-O₃ on leaf N concentration and effects of

Table 3

Seed N concentration and Leaf N concentration at both flowering and seed filling stages in 9 soybean cultivars exposed to charcoal-filtered air (CF) and elevated O₃ (E-O₃). Values are mean ± S.E. (n = 3). Significant differences between O₃ treatments for each cultivar are noted with an asterisk at P < 0.05.

	Seed N concentration (%)			Leaf N concentration at flowering stage (%)			Leaf N concentration at seed filling stage (%)		
	CF	E-O ₃	%	CF	E-O ₃	%	CF	E-O ₃	%
HF25	7.21 ± 0.13	7.01 ± 0.09	−3	4.56 ± 0.07	4.50 ± 0.14	−1	2.50 ± 0.08	2.09 ± 0.02	−16*
HF35	6.56 ± 0.14	6.76 ± 0.07	3	4.88 ± 0.25	4.25 ± 0.11	−13	2.01 ± 0.05	1.90 ± 0.24	−6
HF55	6.86 ± 0.02	6.71 ± 0.21	−2	4.88 ± 0.15	4.93 ± 0.10	1	2.48 ± 0.18	2.88 ± 0.22	16
HN35	6.70 ± 0.05	6.71 ± 0.20	0	4.67 ± 0.14	4.64 ± 0.40	−1	2.34 ± 0.28	1.96 ± 0.18	−16
HN37	6.02 ± 0.16	6.05 ± 0.02	1	4.52 ± 0.22	4.14 ± 0.08	−8	2.29 ± 0.29	2.68 ± 0.12	17
HN65	6.29 ± 0.02	6.37 ± 0.05	1	4.70 ± 0.26	4.40 ± 0.12	−6	1.85 ± 0.12	2.52 ± 0.04	36*
SN22	6.31 ± 0.08	6.80 ± 0.04	8*	4.68 ± 0.31	4.36 ± 0.23	−7	2.75 ± 0.43	3.38 ± 0.21	23
SN26	6.81 ± 0.09	5.75 ± 0.01	−16*	4.95 ± 0.18	3.98 ± 0.50	−19	2.65 ± 0.08	3.38 ± 0.06	28*
SN31	6.99 ± 0.02	6.93 ± 0.19	−1	5.02 ± 0.14	4.83 ± 0.19	−4	2.17 ± 0.12	3.20 ± 0.05	48*
ANOVA (P-values)									
O ₃		0.146			0.006			0.001	
Cultivars		0.000			0.191			0.000	
O ₃ × cultivars		0.000			0.461			0.003	

Table 4
Analysis of variance of the effects of O₃, cultivars and their interactions on pigments content, gas exchange and chlorophyll *a* fluorescence in 9 soybean cultivars. *P*-values were calculated from mixed linear model analysis.

	Chl	Car	Chl <i>a</i> / <i>b</i>	<i>A</i> _{sat}	<i>g</i> _s	<i>C</i> _i	WUE	<i>F</i> _v '/ <i>F</i> _m '	Φ _{PSII}	<i>qP</i>
Flowering stage										
O ₃	0.000	0.000	0.840	0.000	0.297	0.000	0.000	0.056	0.000	0.000
Cultivars	0.044	0.166	0.598	0.343	0.026	0.516	0.478	0.374	0.548	0.430
O ₃ × cultivars	0.918	0.903	0.948	0.953	0.108	0.859	0.748	0.752	0.502	0.556
Seed filling stage										
O ₃	0.000	0.000	0.054	0.000	0.000	0.000	0.000	0.001	0.000	0.000
Cultivars	0.004	0.016	0.286	0.000	0.000	0.000	0.000	0.001	0.000	0.000
O ₃ × cultivars	0.589	0.574	0.665	0.000	0.000	0.131	0.379	0.552	0.194	0.044

Fig. 2. Chlorophyll (Chl), carotenoid (Car) content and the ratio of chlorophyll *a*/*b* (Chl *a*/*b*) in leaves of soybean cultivars at both flowering (a, c, e) and seed filling stage (b, d, f) grown in CF and E-O₃. Values are mean ± S.E. (*n* = 3). Significant differences between O₃ treatments for each cultivar are noted with an asterisk at *P* < 0.05.

Fig. 3. Light-saturated photosynthetic rate (A_{sat}), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and water use efficiency (WUE) in leaves of soybean cultivars at both flowering (a, c, e, g) and seed filling stage (b, d, f, h) grown in CF and E- O_3 . Values are mean $\pm$ S.E. ($n=3$). Significant differences between O_3 treatments for each cultivar are noted with an asterisk at $P < 0.05$.

Fig. 4. Photochemical efficiency of PSII (F_v/F_m') in the light, light-adapted apparent quantum yield of PSII (Φ_{PSII}) and quenching of photochemical efficiency of PSII (qP) in leaves of soybean cultivars at both flowering (a, c, e) and seed filling stage (b, d, f) grown in CF and E- O_3 . Values are mean $\pm$ S.E. ($n = 3$). Significant differences between O_3 treatments for each cultivar are noted with an asterisk at $P < 0.05$.

Fig. 5. Correlation between effects of E-O₃ on leaf N concentration per mass (E-O₃ N_m) relative to CF (CF N_m) and effects of E-O₃ on A_{sat} (E-O₃A_{sat}/CF A_{sat}) (a) and Correlation between seed yield loss and g_s in CF (b), at both flowering and seed filling stages. Each datapoint represents a cultivar.

E-O₃ on A_{sat} measured at seed filling stage but not flowering stage (Fig. 5a). Also, g_s in CF at seed filling stage rather than flowering stage showed significant correlation with seed yield loss among cultivars (r = 0.770, P = 0.015) (Fig. 5b), suggesting that those most sensitive cultivars usually have larger g_s, and thus higher O₃ uptake.

4. Discussion

Ambient O₃ concentration at experimental site was much lower than the concentration in mid-latitudes of Northern Hemisphere [27] and many regions in eastern China, e.g. Yangtze River delta [28,29]. In E-O₃, however, the peak and average of daily mean O₃ concentration (75.2 and 58.6 ppb, respectively) are close to ambient O₃ concentration in many other regions throughout the world [5,30]. Therefore, elevated O₃ treatment in this study could represent ambient O₃ in many regions of China or future O₃ level at experimental site.

The present study clearly demonstrated that O₃ concentration between 40 and 60 ppb is sufficient high enough to cause significant yield losses of 9 soybean cultivars in Northeast China. Similar results were also found in OTCs and SoyFACE [5,10]. Compared with CF, E-O₃ induced a reduction of 40% in seed yield across all cultivars (Table 2), which is an unexpected greater than that in a meta-analysis result of 24% from 53 peer-reviewed studies exposed to an average O₃ concentration of 70 ppb [3]. One explanation for the larger unexpected yield losses might be the photosynthesis of these cultivars in the current experiment much more sensitive to O₃ stress, compared with those 10 Mid-western cultivars in USA [23]. Furthermore, the greater yield loss might be ascribed to non-limiting growth conditions, especially the high humidity of approximately 80% in chambers. In this case, stomata were wide open allowing a high flux of O₃ into leaves even at low O₃ concentrations, supported by the hypothesis that increasing humidity around the leaf in an enclosure system may increase stomatal opening and O₃ uptake [31].

The yield loss caused by E-O₃ due to the decreases in 100-seed mass (21%) and pod number (19%), supported the findings that reduced carbon availability and small plant size usually produce fewer pods, and less weight of those seeds [3]. Consistent with this study, Jaoudé et al. found a 47% yield loss at high O₃ concentration (AOT40 of 9 ppm h) in comparison with the control (AOT40 of 0 ppm h), due to a lower numbers of pod per plant and less 100-seed mass [32]. However, Christ et al. found that the increased pods prevented any yield loss when soybean was exposed to 1.2 times ambient O₃ concentration [33].

In addition to seed yield loss, the N concentration in seeds was also significantly influenced by E-O₃. Limited information was

available on crop quality traits after O₃ exposure [34,35]. We found that SN22 and SN26 from the same parents but different breeding generations performed different responses to E-O₃ in seed N concentration despite similar response in seed yield and its component, and eco-physiological characteristics of leaves. The findings from cultivar SN22 supported the results from winter wheat that elevated O₃ increased grain N concentration [36]. But seed N concentration in SN26 showed an opposite tendency toward E-O₃. It suggests that the cumulative effects of E-O₃ on plant N metabolism might be much complicated in some key processes, e.g. the N pool size stored in vegetative tissues, which appears to determine the amount of available N during seed filling [17].

Accelerated leaf senescence was the most typical symptoms by elevated O₃ [2,37], which is regulated by processes involving chlorophyll degradation and leaf nutrient status [23,38,39]. In this study, E-O₃ significantly decreased net photosynthetic rate (45%) in all cultivars at flowering stage after about 15 consecutive exposure days, suggesting that the photosynthesis unit of soybean is very sensitive to O₃. With the elongation of O₃ exposure, the decrease by E-O₃ in chlorophyll content changed from 22% at flowering stage to 65% at seed filling stage, indicating that E-O₃ significantly accelerated senescence. We found a significant linear relationship between yield loss and g_s in CF at seed filling stage rather than flowering stage (Fig. 5b), suggesting that the cultivars with higher endogenous g_s at seed filling stage are most affected by O₃ although the g_s of all cultivars were lower at seed filling stage than flowering stage. The cultivars with higher endogenous g_s easily take up more O₃, thus causing large damage to chloroplast of plants. g_s decreases with leaf aging, indicating less O₃ uptake in seed filling stage than flowering stage. It could be inferred that accumulative effects of O₃ contribute to the larger effects of E-O₃ during seed filling stage because of similar O₃ exposure concentration at both developmental stages.

Generally, the decrease in net photosynthesis by O₃ was attributed to reduced pigment contents and g_s, less assimilatory area and assimilation capacity per unit of leaf area, weak chlorophyll *a* fluorescence, and short leaf lifespan. g_s was reduced at higher O₃ concentration as an avoidance mechanism of plants under air pollution stress [40,41]. In this study, g_s was not significantly influenced by E-O₃ at flowering stage but at seed filling stage, as indicated by significant decrease in most cultivars at seed filling stage (Fig. 4). The prolonged stomatal closure in E-O₃ also reduces C fixation, and there by the amount of assimilate available for seeds/pods/leaves [42]. Furthermore, E-O₃ significantly decreased total Chl and Car contents, *qP* and effective quantum yield of PSII photochemistry at both flowering and seed filling stages. All these results suggested that both dark and light reactions of

photosynthesis were negatively affected by E-O₃ in most cultivars. Similar results were also found in winter wheat [38] and rice [43].

It was acknowledged that O₃-induced reduction in net photosynthesis was usually caused by non-stomatal factors [4,44], particularly Rubisco activity which is highly correlated with leaf nitrogen (N) content in many higher plants [45]. Previous study reported that nitrogen metabolism changes from assimilation to remobilization at the onset of senescence. A study by Pausch et al. found that O₃ significantly decreased seed yield and affected both short- and long-term N uptake/partitioning dynamics in soybean [46]. Similarly, in this study, we found a significant positive correlation between effects of E-O₃ on leaf N content and effects of E-O₃ on A_{sat} at seed filling stage rather than flowering stage, supporting the hypothesis that the mechanism of chronic O₃ action involves an inhibition of carbon translocation from leaves to other organs. From the response of all variables related with photosynthesis, it can be inferred that O₃-induced reduction in net photosynthesis is mainly driven by chlorophyll *a* fluorescence at flowering stage, and by stomata, chlorophyll *a* fluorescence and leaf N status at seed filling stage.

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