Large variability in ambient ozone sensitivity across 19 ethylenediurea-treated Chinese cultivars of soybean is driven by total ascorbate

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A B S T R A C T

The sensitivity of Chinese soybean cultivars to ambient ozone (O3) in the field is unknown, although soybean is a major staple food in China. Using ethylenediurea (EDU) as an O3 protectant, we tested the gas exchange, pigments, antioxidants and biomass of 19 cultivars exposed to 28 ppm·hr AOT40 (accumulated O3 over an hourly concentration threshold of 40 ppb) over the growing season at a field site in China. By comparing the average biomass with and without EDU, we estimated the cultivar-specific sensitivity to O3 and ranked the cultivars from very tolerant (<10% change) to highly sensitive (>45% change), which helps in choosing the best-suited cultivars for local cultivation. Higher lipid peroxidation and activity of the ascorbate peroxidase enzyme were major responses to O3 damage, which eventually translated into lower biomass production. The constitutional level of total ascorbate in the leaves was the most important parameter explaining O3 sensitivity among these cultivars. Surprisingly, the role of stomatal conductance was insignificant. These results will guide future breeding efforts towards more O3-tolerant cultivars in China, while strategies for implementing control measures of regional O3 pollution are being implemented. Overall, these results suggest that present ambient O3 pollution is a serious concern for soybean in China, which highlights the urgent need for policy-making actions to protect this critical staple food.

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I N T R O D U C T I O N

Food security is a topical issue nowadays, especially in rapidly expanding China (Yin et al., 2009). China is the fourth largest world producer of soybean (Glycine max (L.) Merr.), with 12.2 million tons in 2014 (FAO, 2014). Soybean is a key source of vegetable protein for humans (Mateosaparicio et al., 2008). It is one of the most important agricultural crop species and the top legume species worldwide (FAO, 2013).

China is currently suffering from serious surface ozone (O3) pollution, with annual peak averages reaching as high as 60 ppb (Feng et al., 2015) and an increase of about 7% from...
Ozone is one of the most detrimental air pollutants for crops and natural ecosystems (Ainsworth et al., 2012). Soybean ranks among the most O₃-sensitive agricultural crops (Mills et al., 2007), and such current O₃ concentrations are high enough to cause significant yield losses (Morgan et al., 2003). Projected O₃-induced soybean yield losses were 9.5%–15% for the year 2030 at the global level (Avnery et al., 2011), and the financial losses for soybean were estimated as 2.0–5.8 billion US dollars annually based on the price in the year 2000 (Osborne et al., 2016). Many experiments in different parts of the world have been carried out to investigate the physiological, growth and yield responses of soybean to O₃ in open-top chambers and under ambient conditions (e.g., Sun et al., 2014; Zhang et al., 2014; Rai et al., 2015). Ozone exposure reduces photosynthesis, stomatal conductance and the leaf chlorophyll content of soybean (Morgan et al., 2003). A SoyFACE study showed a dose-dependent linear decrease in soybean yield and photosynthesis, and altered antioxidant capacity (Betzelberger et al., 2012).

Dose–response studies for a range of crops have revealed that O₃ sensitivity is a heritable trait (Reinert and Eason, 2000) and is highly variable among species and cultivars (Ariyaphanphitak et al., 2005; Mills et al., 2007). Studies on the response of soybean to O₃ in Asia have focused on the growth and yield of individual cultivars (Wahid et al., 2001; Singh et al., 2010; Singh and Agrawal, 2011; Rai et al., 2015). However, Zhang et al. (2014) demonstrated that O₃ sensitivity varied greatly across nine soybean cultivars widely cultivated in Northeast China, through elevated O₃ exposure experiments in open-top chambers. So far, however, there is no available data showing whether current ambient O₃ levels affect the growth and productivity of soybean in China.

The antiozonant ethylenediurea (N-[2-(2-oxo-1-imidazolidinyl)ethyl]-N'-phenyleurea, abbreviated as EDU, with formula C₁₄H₁₀N₄O₂), first described by Carnahan et al. (1978), is a well-known antiozonant chemical (Paoletti et al., 2009; Feng et al., 2010; Manning et al., 2011; Agathokleous et al., 2016a), able to prevent O₃ injury, especially visible foliar O₃ injury as well as growth reduction in agricultural and horticultural crops and forest trees, by stimulating the antioxidant defense (Tiwari et al., 2005; Elagöz and Manning, 2005; Szantoi et al., 2007; Paoletti et al., 2007; Feng et al., 2010; Rai et al., 2015). A meta-analysis suggested that the antiozonant activity of EDU is biochemical rather than biophysical (Feng et al., 2010), but conclusive evidence of the detailed basis for the protective action has not been confirmed. Recent results showed that EDU does not have side-effects on growth and is not toxic to plants at the concentrations required for O₃ protection (Agathokleous et al., 2016a). As a reliable, low-cost, and low-technology tool, EDU has great potential for assessing the effects of ambient O₃ on vegetation (Singh et al., 2014; Agathokleous et al., 2016b, 2016c).

We used EDU as a tool for assessing: (1) the relative sensitivity to ambient O₃ exposure in 19 soybean cultivars widely cultivated in China by using biomass as the response indicator, (2) whether these cultivars differ in their physiological and biochemical responses to O₃ (gas exchange, pigments, antioxidants), and (3) which parameters are the most important as predictors of O₃-sensitivity in these cultivars. This knowledge will help in cultivating the most O₃-tolerant cultivars in the areas at higher risk and breeding for more and more O₃-tolerant cultivars.

### 1. Materials and methods

#### 1.1. Experimental conditions

The experiment was conducted under natural field conditions from June to October, 2015, at a suburban area of Beijing city, Changping District, 40°19'N, 116°13'E and 43.5 m a.s.l. (above sea level). The site is about 52 km from the city center. Mean monthly minimum and maximum temperatures were −3.1 °C (January) and 26.7 °C (July). The mean yearly precipitation was 550 mm and almost 60% of rain occurred in July and August.

Meteorological variables (air temperature and precipitation) were recorded by a portable automatic weather station (HOBO-U30, USA). The concentration of O₃ was continuously monitored using an ultraviolet (UV)-absorption O₃ analyzer (Model 49i, Thermo Scientific, USA) before the experiment and once a month during the experiment. Exceedances above 40 ppb were accumulated to calculate the exposure index AOT40 (accumulated O₃ over an hourly concentration threshold of 40 ppb) according to Mills et al. (2007). The distribution of hourly O₃ concentrations across 10 ppb classes of exposure and daily 8-hr means was calculated from 9:00 to 17:00 solar time.

The seeds of 19 soybean cultivars (Glycine max (L.) Merr.) were obtained from the Institute of Crop Science of Chinese Academy of Agricultural Sciences. The cultivars are widely planted in North China, have similar growing periods (110–130 days), and had not been tested for O₃ sensitivity previously. The agronomic characteristics of these cultivars are listed in Table 1. The soybean seeds were sown on the 10th of June and sprouted out of the earth on the 20th of June, 2015.

After measuring the physiological and biochemical parameters at two months after germination (23rd August), harvest was carried out at the very end of the growing season (8th October). Due to rainy days at the time of flowering (22nd July to 10th August) (Fig. 1), the plants did not produce seeds. No soybean yield occurred in the entire region in 2015. Therefore, the present paper shows only the results of biomass.

In this experiment, there were 7 plots and each plot occupied 65 m². For every plot, there were 19 lines (5 m in length for each line) i.e., one line per cultivar distributed at random. The basic physical and chemical properties of soil were as follows: organic C, 17.4 g/kg; total N, 0.9 mg/kg; available P, 38.1 mg/kg; available K, 102.1 mg/kg and pH of 8.3.

#### 1.2. EDU application

Among different concentrations of EDU, 450 ppm of EDU was used in this study as it was found to effectively protect different plant species from O₃ (Paoletti et al., 2009; Feng et al., 2010; Manning et al., 2011). For instance, foliar applications of EDU at 450 ppm significantly alleviated snap bean foliar injury, and increased the photosynthesis rate, seed and pod weights in O₃-sensitive genotypes (Yuan et al., 2015). EDU powder (100% available ingredient) was dissolved in warm water. Three plots were sprayed with water and four plots were sprayed with EDU. The entire foliage of each plant was sprayed until the drip point before sunrise each time. The EDU treatments started from the...
time of the first trifoliate leaf emergence. EDU was repeatedly applied at bi-weekly intervals until the end of the experiment. In total, EDU was applied 7 times during the growing period.

1.3. Gas exchange parameters

Photosynthesis at saturating light ($A_{sat}$) and stomatal conductance ($g_s$) were measured using a portable photosynthesis system (LI-6400, LI-COR Inc., Lincoln, NE, USA). For every cultivar, two fully expanded upper leaves per one plant in each plot were randomly selected on the 23rd of August. All measurements were conducted during 08:30–11:00 on clear days under the following conditions: saturating photosynthetic active radiation (PAR) of 1500 $\mu$mol/(m$^2$.sec), CO$_2$ at 400 ppm, leaf temperature at 28°C, and relative humidity in air between 50%–70%.

Table 1 – Characteristics of the 19 soybean cultivars used in the study.

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Cultivars (abbreviation)</th>
<th>Year of release</th>
<th>Maturity (days)</th>
<th>Male parent</th>
<th>Female parent</th>
<th>Leaf shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jidou12</td>
<td>J12</td>
<td>2002</td>
<td>100</td>
<td>Oil 83-14</td>
<td>Jinda7826</td>
<td>Ovoid</td>
</tr>
<tr>
<td>ZhongHuang13</td>
<td>ZH13</td>
<td>2001</td>
<td>105</td>
<td>Yudou8</td>
<td>Zhongguo90052-76</td>
<td>Ovoid</td>
</tr>
<tr>
<td>ZhongHuang20</td>
<td>ZH20</td>
<td>2003</td>
<td>100</td>
<td>Yi-2</td>
<td>Hobbit</td>
<td>Ovoid</td>
</tr>
<tr>
<td>ZhongHuang39</td>
<td>ZH39</td>
<td>2006</td>
<td>100</td>
<td>Zhongpin661</td>
<td>Zhonghuang14</td>
<td>Ovoid</td>
</tr>
<tr>
<td>ZhongHuang40</td>
<td>ZH40</td>
<td>2007</td>
<td>104</td>
<td>Jindou6</td>
<td>Yudou12</td>
<td>Ovoid</td>
</tr>
<tr>
<td>ZhongHuang41</td>
<td>ZH41</td>
<td>2009</td>
<td>108</td>
<td>Kefeng14</td>
<td>Kexin3</td>
<td>Ovoid</td>
</tr>
<tr>
<td>ZhongHuang42</td>
<td>ZH42</td>
<td>2007</td>
<td>116</td>
<td>Youchu4</td>
<td>Jindou33</td>
<td>Ovoid</td>
</tr>
<tr>
<td>ZhongHuang43</td>
<td>ZH43</td>
<td>2006</td>
<td>101</td>
<td>Jidou7</td>
<td>Xinke3</td>
<td>Ovoid</td>
</tr>
<tr>
<td>ZhongHuang44</td>
<td>ZH44</td>
<td>2009</td>
<td>107</td>
<td>Kefeng14</td>
<td>Kexin3</td>
<td>Ovoid</td>
</tr>
<tr>
<td>ZhongHuang49</td>
<td>ZH49</td>
<td>2009</td>
<td>106</td>
<td>Kefeng14</td>
<td>Kexin3</td>
<td>Ovoid</td>
</tr>
<tr>
<td>ZhongHuang50</td>
<td>ZH50</td>
<td>2010</td>
<td>106</td>
<td>Zhonghuang13</td>
<td>Zhongpin661</td>
<td>Ovoid</td>
</tr>
<tr>
<td>ZhongHuang62</td>
<td>ZH62</td>
<td>2011</td>
<td>100</td>
<td>Zhonghuang25</td>
<td>Xindou1</td>
<td>Lanceolate</td>
</tr>
<tr>
<td>ZhongHuang66</td>
<td>ZH66</td>
<td>2014</td>
<td>112</td>
<td>Zhongpin661</td>
<td>Cheng9039-2-4-3-1</td>
<td>Ovoid</td>
</tr>
<tr>
<td>ZhongHuang69</td>
<td>ZH69</td>
<td>2012</td>
<td>121</td>
<td>Kefeng14</td>
<td>Kexin3</td>
<td>Lanceolate</td>
</tr>
<tr>
<td>ZhongHuang70</td>
<td>ZH70</td>
<td>2013</td>
<td>102</td>
<td>Zhonghuang13</td>
<td>Ludou11</td>
<td>Ovoid</td>
</tr>
<tr>
<td>ZhongHuang74</td>
<td>ZH74</td>
<td>2013</td>
<td>109</td>
<td>Zhongdou27</td>
<td>Zhonghuang3</td>
<td>Ovoid</td>
</tr>
<tr>
<td>ZhongHuang75</td>
<td>ZH75</td>
<td>2014</td>
<td>131</td>
<td>NFS58</td>
<td>Teifeng31</td>
<td>Ovoid</td>
</tr>
<tr>
<td>ZhongHuang79</td>
<td>ZH79</td>
<td>2015</td>
<td>131</td>
<td>Zhongpin 661</td>
<td>Yudou25</td>
<td>Ovoid</td>
</tr>
</tbody>
</table>

1.4. Photosynthetic pigment

After the photosynthesis measurement, the leaf was sampled for photosynthetic pigment. For every cultivar, two leaflets from two fully expanded leaves per plant were randomly punched, and treated with 2 mL 95% ethanol in the dark for 48 hr at 4°C. Assays for chlorophyll (Chl) $a$ and $b$ and carotenoid (Car) content were carried out by ultraviolet–visible (UV–VIS) spectrophotometry (Alpha-1506, Lab-Spectrum Instruments Co., Ltd., China) according to the specific absorption coefficients provided by Lichtenthaler (1987).  

1.5. Antioxidant parameters

Leaves for antioxidant analyses were collected immediately after the photosynthesis measurement. Two fully expanded

Fig. 1 – The 8-hr (9:00–17:00) mean O$_3$ concentrations and AOT 40 during the study period from the 20th of June to the 8th of October, 2015. AOT 40: accumulated O$_3$ over an hourly concentration threshold of 40 ppb.
upper leaves were randomly sampled from two plants per each cultivar in each plot, frozen immediately in liquid nitrogen, and stored at −80 °C until analysis. Malondialdehyde (MDA), which is related with the level of lipid peroxidation (Feng et al., 2011), was assessed for estimation of lipid peroxidation by 2-thiobarbituric acid-reactive metabolite (TBA) according to the method of Heath and Parker (1968). The optical density (OD) values were obtained in a 96-well plate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA) and the MDA content (C_{MDA}, mmol/L) was calculated by the equation C_{MDA} = 6.45 \times (A532–A600) – 0.56 \times A450 (A532, A600 and A450 represent extinction under 450, 532 and 600 nm wavelength, respectively) in order to rule out the disturbances from non-specific (A600) and sugar (A450) absorbance. Total ascorbate (AsA), which is the main antioxidant metabolite in the mesophyll (Noctor, 2006), was determined using an α-α′-bipyridyl-based colorimetric assay for approximately 30 mg of ground leaf tissue in a 96-well plate reader SpectraMax 190 (Gillespie and Ainsworth, 2007). Samples (~100 mg) for total antioxidant capacity (TAC), which is often used as a synthetic index of the antioxidant pool in the leaves (Gillespie and Ainsworth, 2007), were added to 2 mL cold 70% (v/v) ethanol and homogenized in darkness, according to Benzie and Strain (1996). The mixture was incubated for 20 min in darkness at 4 °C, then centrifuged at 3000 r/min for 20 min. One-hundred milliliters of supernatant was reached.

3000 r/min for 20 min. One-hundred milliliters of supernatant incubated for 20 min in darkness at 4 °C, then centrifuged at 3000 r/min for 20 min. One-hundred milliliters of supernatant was reached.

was taken for the ferric reducing antioxidant power (FRAP) assay to express TAC as Fe3+ equivalents (mmol Fe2+/g fresh mass), according to Benzie and Strain (1996). The mixture was reached.

The cultivar-specific sensitivity to O3 (B_{EFF}) was estimated by comparing the average biomass (B) with and without EDU protection, i.e., B_{EFF} = (B_{EDU} – B_{Water}) \times 100/B_{EDU} (B_{EDU} means biomass treated with EDU, B_{Water} means biomass treated with water). The same approach was used to estimate the effect of O3 on each variable X.

1.8. Statistical analyses

The statistical unit was the single plot, with four plots for EDU and three plots for water treatment. Data were checked for normal distribution and homogeneity of variance. Non-normally distributed data i.e., biomass, AsA, POD, and APX, were log transformed prior to analysis. Data in the figures and tables are not transformed, but rather original data means ± SD. Data were subject to two-way analysis of variance (ANOVA) including the effects of EDU, cultivar and their interaction using Statistica 10.0 software (StatSoft, Italy). Student’s t test was used to analyze the effect of EDU within each cultivar. Simple linear correlations were applied to test the relationship of all variables X (X_{EFF}, X_{EDU} and X_{Water}) with B_{EFF}. Effects were considered significant if p < 0.05.

2. Results

2.1. Ozone pollution

The AOT40 index calculated for the whole experimental period was 28.04 ppm-hr (Fig. 1). Daily 8-hr mean O3 concentrations varied from 22 to 140 ppb. The daily average along the growing season was 64.9 ppb. There were 141 hr with concentrations higher than 100 ppb, which happened mostly in July (Table 2).

<table>
<thead>
<tr>
<th>Concentration (ppb)</th>
<th>June (20th–30th)</th>
<th>July</th>
<th>August</th>
<th>September</th>
<th>October (1st–8th)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;30</td>
<td>4</td>
<td>31</td>
<td>14</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>31–40</td>
<td>16</td>
<td>16</td>
<td>20</td>
<td>31</td>
<td>13</td>
</tr>
<tr>
<td>41–50</td>
<td>10</td>
<td>35</td>
<td>48</td>
<td>44</td>
<td>17</td>
</tr>
<tr>
<td>51–60</td>
<td>7</td>
<td>18</td>
<td>57</td>
<td>56</td>
<td>12</td>
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<tr>
<td>61–70</td>
<td>5</td>
<td>23</td>
<td>34</td>
<td>27</td>
<td>1</td>
</tr>
<tr>
<td>71–80</td>
<td>9</td>
<td>26</td>
<td>17</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>81–90</td>
<td>5</td>
<td>22</td>
<td>13</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>91–100</td>
<td>11</td>
<td>17</td>
<td>11</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>101–110</td>
<td>5</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>111–120</td>
<td>2</td>
<td>6</td>
<td>12</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>121–130</td>
<td>6</td>
<td>11</td>
<td>7</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>131–140</td>
<td>5</td>
<td>12</td>
<td>1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>141–150</td>
<td>1</td>
<td>11</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>&gt;150</td>
<td>2</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Sum of hours</td>
<td>88</td>
<td>248</td>
<td>248</td>
<td>240</td>
<td>64</td>
</tr>
</tbody>
</table>

1.7. Definition of O3 stress effects

Plants were harvested on the 8th of October. The above-ground parts of two plants for each cultivar were collected in each plot. Samples were dried in an oven at 80°C until a constant weight was reached.
2.2. Gas exchange parameters

\( A_{\text{sat}} \) of cultivars protected by EDU showed a significant variability and was significantly increased by 40% across all cultivars, relative to non-protected plants (Table 3). EDU significantly increased \( A_{\text{sat}} \) in Zhonghuang13 (ZH13), Zhonghuang20 (ZH20), Zhonghuang48 (ZH48) and Zhonghuang49 (ZH49). Interestingly, constitutional \( g_s \) did not significantly differ across the EDU-treated cultivars. Also, the effect of EDU on \( g_s \) was not significant.

The cultivars differed in their foliar content of photosynthetic pigments, as indicated by the higher contents in Zhonghuang74 (ZH74) and Zhonghuang66 (ZH66), and lower content in Zhonghuang39 (ZH39), Zhonghuang44 (ZH44) and Zhonghuang75 (ZH75) (Table 4). EDU significantly increased Chl \( a \), Chl \( b \), total Chl and Car contents by 26%, 52%, 30% and 23% across all cultivars, respectively. There was significant interaction between EDU and cultivar, as indicated by a significant EDU-induced increase in some cultivars but not in others.

2.3. Antioxidant parameters

The foliar content of antioxidant metabolites was significantly affected by the cultivar, the EDU treatment and their interaction (Table 5). Across all cultivars, MDA and AsA were decreased by 9% and 27%, respectively, while TAC was increased by 31% due to the EDU treatment.

Also, the activity of antioxidant enzymes was significantly affected by the cultivar, the EDU treatment and their interaction (Table 6). Across all cultivars, SOD showed a negligible 3% reduction due to the EDU treatment, while APX, POD and CAT increased by 28%, 15% and 8%, respectively.

2.4. Biomass and ozone sensitivity of the cultivars

Biomass at harvest showed a significant variation with the cultivar, the EDU treatment and their interaction (Fig. 2a). EDU significantly increased the biomass in 15 out of 19 cultivars (i.e., with the exception of Zhonghuang69 (ZH69), Zhonghuang43 (ZH43), Zhonghuang49 (ZH49) and Jidou12 (J12)), thus a significant interaction between EDU and cultivars was found. On average, the biomass of EDU-treated cultivars was 36% higher than that of water-treated cultivars.

The cultivar-specific O3 sensitivity, expressed as percent variation of biomass when plants were protected by ethylenediurea (\( B_{\text{EFF}} \)), showed a remarkable variation among cultivars, with the most tolerant cultivars ZH43, ZH49 and ZH69 showing insignificant changes (<10%) and the most sensitive cultivars ZH42, ZH50 and ZH70 showing large variations (>45%) (Fig. 2b).

When linear correlations were applied to test the effects of all variables (\( X_{\text{EDU}}, X_{\text{Water}} \) and \( X_{\text{EFF}} \)) on \( B_{\text{EFF}}, MDA_{\text{Water}} \) and \( APX_{\text{EFF}} \) increased significantly with increasing O3 sensitivity of cultivars.
Table 4 – Photosynthetic pigments (chlorophyll a, b (Chl a, b)) and total carotenoids (Car)) (mean ± SD) of 19 soybean cultivars exposed to ambient O₃ concentrations (water) or protected by ethylenediurea (EDU), levels of significance showing differences between EDU and water for any cultivar, and two-way ANOVA (EDU × cultivar) results.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Chl a (mg/m²)</th>
<th>Chl b (mg/m²)</th>
<th>Chl a + b (mg/m²)</th>
<th>Car (mg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EDU</td>
<td>Water</td>
<td>Significance</td>
<td>EDU</td>
</tr>
<tr>
<td>J12</td>
<td>35.7 ± 7.41</td>
<td>37.7 ± 2.6</td>
<td>ns</td>
<td>7.42 ± 1.69</td>
</tr>
<tr>
<td>ZH13</td>
<td>35.1 ± 3.23</td>
<td>28.5 ± 4.83</td>
<td>ns</td>
<td>7.17 ± 1.22</td>
</tr>
<tr>
<td>ZH20</td>
<td>34.5 ± 4.55</td>
<td>16.3 ± 1.46</td>
<td>**</td>
<td>9.39 ± 2.42</td>
</tr>
<tr>
<td>ZH39</td>
<td>22.9 ± 3.38</td>
<td>23.2 ± 4.85</td>
<td>ns</td>
<td>5.08 ± 0.68</td>
</tr>
<tr>
<td>ZH40</td>
<td>28.7 ± 2.52</td>
<td>26.1 ± 2.92</td>
<td>ns</td>
<td>6.04 ± 3.12</td>
</tr>
<tr>
<td>ZH41</td>
<td>24.1 ± 5.98</td>
<td>20 ± 5.39</td>
<td>ns</td>
<td>5.8 ± 1.55</td>
</tr>
<tr>
<td>ZH42</td>
<td>37.8 ± 4.12</td>
<td>20.8 ± 3.59</td>
<td>**</td>
<td>9.51 ± 1.23</td>
</tr>
<tr>
<td>ZH39</td>
<td>29.5 ± 1.6</td>
<td>22.7 ± 2.04</td>
<td>ns</td>
<td>13.39 ± 5.97</td>
</tr>
<tr>
<td>ZH44</td>
<td>21 ± 6.34</td>
<td>20.2 ± 4.76</td>
<td>ns</td>
<td>8.2 ± 5.47</td>
</tr>
<tr>
<td>ZH48</td>
<td>34.4 ± 3.49</td>
<td>18.5 ± 2.27</td>
<td>**</td>
<td>7.79 ± 0.44</td>
</tr>
<tr>
<td>ZH49</td>
<td>27 ± 5.23</td>
<td>23 ± 5.28</td>
<td>ns</td>
<td>6.07 ± 2.33</td>
</tr>
<tr>
<td>ZH50</td>
<td>29.4 ± 2.5</td>
<td>28.4 ± 1.55</td>
<td>ns</td>
<td>6.45 ± 1.42</td>
</tr>
<tr>
<td>ZH62</td>
<td>26.3 ± 5.04</td>
<td>21.9 ± 1.15</td>
<td>ns</td>
<td>4.35 ± 1.51</td>
</tr>
<tr>
<td>ZH66</td>
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<td>27.6 ± 4.52</td>
<td>ns</td>
<td>10.86 ± 2.39</td>
</tr>
<tr>
<td>ZH69</td>
<td>26.5 ± 4.64</td>
<td>24.9 ± 4.17</td>
<td>ns</td>
<td>4.03 ± 0.61</td>
</tr>
<tr>
<td>ZH70</td>
<td>36.4 ± 2.45</td>
<td>30.1 ± 5.88</td>
<td>*</td>
<td>9.78 ± 1.67</td>
</tr>
<tr>
<td>ZH74</td>
<td>41 ± 3.54</td>
<td>29.5 ± 1.84</td>
<td>**</td>
<td>8.93 ± 1.29</td>
</tr>
<tr>
<td>ZH75</td>
<td>28.3 ± 5.8</td>
<td>23.4 ± 5.65</td>
<td>ns</td>
<td>8.42 ± 4.37</td>
</tr>
<tr>
<td>ZH79</td>
<td>30.9 ± 3.67</td>
<td>24.4 ± 6.76</td>
<td>ns</td>
<td>11.74 ± 1.79</td>
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<td>EDU &lt;0.0001a</td>
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<tr>
<td>Cultivar &lt;0.0001a</td>
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<tr>
<td>EDU × Cultivar &lt;0.0001a</td>
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<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

ns: non-significant.  
⁎ p ≤ 0.05.  
** p ≤ 0.01.  
a p ≤ 0.001.
the cultivars, while $R_{\text{Water}}$, MDA$_{\text{EFF}}$ and AsA$_{\text{EDU}}$ significantly decreased (Table 7).

### 3. Discussion

During the growing season of soybean, the daily average ambient O$_3$ concentration was 65 ppb and AOT$_{40}$ was 28 ppm-hr. This level exceeds by far the existing critical level of 3 ppm-hr AOT$_{40}$ over 3 months recommended in Europe for the protection of agricultural crops (CLRTAP, 2015). Modeling studies confirm that AOT$_{40}$ levels over China may exceed 15 ppm-hr and are projected to further increase until 2020 (Tang et al., 2015). The ozone concentration in Beijing, the capital of China, has been continuously increasing, e.g., the daily maximum 8-hr average O$_3$ concentration increased by 1.14 ppb/year from 2004 to 2015 (Cheng et al., 2016). Overall, O$_3$ pollution is a serious concern for many Asian countries, for example India (Singh and Agrawal, 2011; Pandey et al., 2014), Pakistan (Ahmad et al., 2013) and Japan (Hoshika et al., 2011). Beijing and its surroundings, where our experiment was carried out, are a hot-spot of O$_3$ pollution (Yuan et al., 2015; Zhu et al., 2015).

Even though O$_3$ pollution is such a pressing issue for food security in China (Yin et al., 2009; Miao et al., 2018) and soybean is a major staple legume crop for Chinese population (FAO, 2014), previous studies were carried out by simulated O$_3$ exposure under controlled conditions, i.e., in open-top chambers (Zhang et al., 2014; Zhao et al., 2015). While chambers are well suited for mechanistic studies on O$_3$ impacts, risk assessment and cultivar screening may be affected by artifacts due to modification of the environmental variables (Paoletti et al., 2007), as demonstrated in the case of soybean (Howell et al., 1979). EDU has been verified as a useful tool to protect crops from O$_3$ and assess the effects of O$_3$ on plants under ambient conditions (Singh et al., 2009; Paoletti et al., 2007; Feng et al., 2011; Hoshika et al., 2013a; Carriero et al., 2015; Yuan et al., 2015; Pandey et al., 2015). Our results confirm that EDU is a valid and easy approach for field assessment of ambient O$_3$ injury to vegetation.

By using the biomass of EDU-protected plants as a proxy of the biomass in a non-O$_3$-polluted environment, we were able to rank the relative O$_3$-sensitivity of 19 soybean cultivars widely cultivated in China. The most tolerant cultivars showed insignificant changes (<10% variation of biomass when plants were protected by EDU), while the most sensitive cultivars showed large deviations (>45%). It is well known, in fact, that sensitive plants show significant responses to O$_3$ when treated with EDU, while tolerant plants show limited responses to O$_3$ (Szantoi et al., 2007; Singh et al., 2009). Such serious variability in the cultivar-specific O$_3$ sensitivity has been already shown in other species, e.g., wheat (Biswas et al., 2008; Singh et al., 2009), rice (Akhtar et al., 2010) and tomato...
### Table 6 – The activity of antioxidative enzymes (APX, SOD, POD and CAT) (mean ± SD) of 19 soybean cultivars exposed to ambient O₃ concentrations (water) or protected by ethylenediurea (EDU), levels of significance showing differences between EDU and water treatments for any cultivar, and two-way ANOVA (EDU × cultivar) results.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>SOD (U/(g FW))</th>
<th>APX (U/(g FW))</th>
<th>POD (U/(g FW))</th>
<th>CAT (U/(g FW))</th>
<th>EDU</th>
<th>Water</th>
<th>Significance</th>
<th>EDU</th>
<th>Water</th>
<th>Significance</th>
<th>EDU</th>
<th>Water</th>
<th>Significance</th>
<th>EDU</th>
<th>Water</th>
<th>Significance</th>
</tr>
</thead>
</table>
| J12      | 305 ± 54.3     | 372 ± 60.4    | NS             | 333 ± 31.9     | 159 ± 9.7 | NS    | 9.94 ± 0.54 | 8.52 ± 0.87 | NS | 1.70 ± 0.34 | 3.07 ± 0.129 | **  
| ZH13     | 347 ± 64.5     | 364 ± 69.5    | NS             | 154 ± 10.4     | 286 ± 59.3 | NS    | 6.17 ± 0.96 | 6.18 ± 0.34 | NS | 1.82 ± 0.35 | 2.18 ± 0.19 | NS  
| ZH20     | 376 ± 96.5     | 294 ± 103.9   | NS             | 233 ± 6.5      | 131 ± 7.2  | NS    | 13.7 ± 0.88 | 9.98 ± 1.22 | NS | 2.04 ± 0.07 | 1.37 ± 0.22 | NS  
| ZH39     | 401 ± 89.7     | 395 ± 13.1    | NS             | 212 ± 38       | 162 ± 59.2 | NS    | 7.10 ± 1.02 | 6.41 ± 0.49 | NS | 2.34 ± 0.15 | 2.70 ± 0.16 | NS  
| ZH40     | 412 ± 26.3     | 396 ± 68.1    | NS             | 382 ± 84       | 242 ± 18.8 | NS    | 8.70 ± 0.49 | 8.37 ± 0.087 | NS | 4.16 ± 0.18 | 3.13 ± 0.25 | NS  
| ZH41     | 231 ± 50.3     | 216 ± 31.2    | NS             | 321 ± 36.8     | 141 ± 40.5 | NS    | 9.10 ± 0.52 | 6.26 ± 0.12 | NS | 3.38 ± 0.34 | 1.63 ± 0.36 | NS  
| ZH42     | 272 ± 65.2     | 258 ± 20.4    | NS             | 538 ± 117.9    | 253 ± 19.4 | NS    | 7.37 ± 0.26 | 9.89 ± 18.39 | NS | 2.42 ± 0.38 | 2.26 ± 0.43 | NS  
| ZH43     | 248 ± 55.6     | 299 ± 68.7    | NS             | 110 ± 5.3      | 280 ± 16.6 | NS    | 5.83 ± 0.51 | 8.47 ± 1.27 | NS | 1.66 ± 0.22 | 1.89 ± 0.22 | NS  
| ZH44     | 436 ± 36.4     | 397 ± 78.6    | NS             | 330 ± 38.3     | 199 ± 5.8  | NS    | 8.44 ± 1.3  | 5.93 ± 1.02 | NS | 3.36 ± 0.26 | 1.52 ± 0.06 | **  
| ZH48     | 425 ± 34.2     | 355 ± 51.6    | NS             | 148 ± 38.4     | 154 ± 21.6 | NS    | 8.33 ± 0.24 | 5.85 ± 0.82 | NS | 1.56 ± 0.16 | 2.87 ± 0.12 | **  
| ZH49     | 249 ± 43.9     | 281 ± 15.8    | NS             | 128 ± 16.3     | 100 ± 15.9 | NS    | 13.1 ± 0.46 | 7.85 ± 0.54 | NS | 2.54 ± 0.14 | 2.13 ± 0.12 | **  
| ZH50     | 354 ± 56.1     | 302 ± 56.9    | NS             | 109 ± 6.1      | 102 ± 4.3  | NS    | 11.3 ± 0.61 | 4.46 ± 0.49 | NS | 1.57 ± 0.17 | 2.23 ± 0.20 | **  
| ZH60     | 338 ± 47.1     | 371 ± 52.3    | NS             | 376 ± 6.6      | 131 ± 21.7 | NS    | 8.29 ± 0.38 | 7.51 ± 0.29 | NS | 1.90 ± 0.21 | 1.24 ± 0.24 | NS  
| ZH66     | 386 ± 40.9     | 349 ± 48      | NS             | 533 ± 52.9     | 163 ± 2.6  | NS    | 13.7 ± 0.21 | 10.7 ± 0.95 | NS | 3.36 ± 0.19 | 3.34 ± 0.15 | **  
| ZH69     | 416 ± 71.3     | 393 ± 37.8    | NS             | 162 ± 57       | 400 ± 73.1 | NS    | 6.80 ± 0.29 | 7.91 ± 1.40 | NS | 1.90 ± 0.069 | 2.46 ± 0.13 | *   
| ZH70     | 371 ± 78.8     | 331 ± 65.7    | NS             | 764 ± 15       | 235 ± 19.6 | NS    | 6.78 ± 0.60 | 7.01 ± 1.14 | NS | 2.52 ± 0.28 | 1.69 ± 0.30 | **  
| ZH74     | 313 ± 117.8    | 378 ± 30.7    | NS             | 217 ± 62.4     | 320 ± 3.2  | *     | 12.1 ± 0.44 | 6.93 ± 0.77 | NS | 3.34 ± 0.31 | 3.42 ± 0.21 | NS  
| ZH75     | 225 ± 145.1    | 390 ± 27.7    | NS             | 155 ± 50.1     | 203 ± 4.4  | NS    | 10.3 ± 0.165 | 6.25 ± 0.58 | NS | 2.48 ± 0.25 | 2.98 ± 0.099 | *   
| ZH79     | 250 ± 38.1     | 381 ± 46.6    | NS             | 210 ± 31.3     | 228 ± 23  | NS    | 5.70 ± 0.016 | 11.2 ± 1.45 | NS | 1.60 ± 0.17 | 2.36 ± 0.19 | **  
| EDU      | 0.341 ns       | <0.0001 a     | NS             | <0.0001 a      | <0.0001 a | NS    | <0.0001 a | <0.0001 a | NS | <0.0001 a | <0.0001 a | NS  
| Cultivar | <0.001 a       | <0.001 a      | NS             | <0.001 a       | <0.001 a | NS    | <0.001 a | <0.001 a | NS | <0.001 a | <0.001 a | NS  
| EDU × Cultivar | 0.049 a | <0.001 a | NS | <0.001 a | <0.001 a | NS    | <0.001 a | <0.001 a | NS | <0.001 a | <0.001 a | NS  

ns: non-significant; APX: ascorbate peroxidase; SOD: superoxide dismutase; POD: peroxidase; CAT: Catalase; FW: fresh weight; ANOVA: analysis of variance.

*  \( p \leq 0.05 \).

**  \( p \leq 0.01 \).

a  \( p \leq 0.001 \).
(Calvo et al., 2007), as well as in North American soybean (Burkey and Carter, 2009). This knowledge may be used for breeding novel O₃-tolerant cultivars as an adaptive strategy to O₃ pollution (Teixeira et al., 2011). As yield is a major plant trait for soybean, further studies including yield are recommended. Current conventional genetic improvement efforts screen for high-yielding cultivars and have indirectly selected genotypes with very high sensitivity to O₃, as demonstrated in wheat (Biswas et al., 2008). Another short-term adaptive strategy in hot-spot areas may thus be the cultivation of soybean cultivars that are both high-yielding and O₃-tolerant (Teixeira et al., 2011), such as our cultivar ZH69, while the most O₃-sensitive cultivars should be excluded.

Fig. 2 – Above-ground biomass (mean ± SD) of 19 soybean cultivars exposed to ambient O₃ concentrations (water) or protected by ethylenediurea (EDU), levels of significance showing differences between EDU and water treatments for any cultivar, and two-way ANOVA (EDU × cultivar) results. Cultivars are sorted (a) according to decreasing biomass and (b) according to increasing ozone sensitivity, expressed as percent variation when protected by ethylenediurea. The inset shows the result of a two-way analysis of variance (ANOVA) (cultivar × EDU treatment). *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ns: non-significant; SD: standard deviation; BEFF: the cultivar-specific sensitivity to O₃; ZH: Zhonghuang; J12: Jidou12. 

(2008; Feng et al., 2010; Inada et al., 2012; Su et al., 2017). We
assessed the main physiological and biochemical responses to understand which parameters were the most important as predictors of O$_3$-sensitivity in these cultivars and whether the sensitivity mechanisms were similar in the different cultivars. All gas exchange, pigment and biomass responses were consistent in a meta-analysis on EDU effects on 15 crop species exposed to O$_3$ (Feng et al., 2010), even though the magnitude of changes was higher in our experiment likely due to exposure to higher O$_3$ concentrations. Also, the responses of antioxidant metabolites and enzymes were consistent with results in the literature for other species treated by EDU (Hassan, 2006; Paolletti et al., 2008; Singh et al., 2009; Pandey et al., 2015). Interestingly, our cultivars differed in both constitutional and O$_3$-induced levels of all variables but gs. Ozone responses may have been affected by the high variability following the stomatal sluggishness that is a typical response to O$_3$ (Hoshika et al., 2013b, 2014), although a decline of gs is a common response to O$_3$ (Booiker et al., 2009). However, no difference in gs among cultivars means that this trait cannot be used for selecting O$_3$-tolerant cultivars.

An analysis on how the different variables were related to O$_3$ sensitivity (B$_{EFF}$) across the cultivars showed significant correlations with: MDA in plants exposed to ambient O$_3$ (MDA$_{Water}$); percent variation of MDA and APX when plants were protected by EDU (MDA$_{EFF}$ and APX$_{EFF}$); biomass in plants exposed to ambient O$_3$ (B$_{Water}$); and constitutional content of AsA in plants protected by EDU (AsA$_{EDU}$). MDA is a marker of O$_3$-induced lipid peroxidation in the plant membranes (Feng et al., 2011). This is why MDA$_{Water}$ was higher in the most sensitive cultivars, which did not possess efficient mechanisms of membrane protection from O$_3$ injury. As a consequence, MDA$_{EFF}$, i.e., the difference in lipid peroxidation between EDU-protected and non-protected plants, was higher in the most sensitive cultivars. A higher APX$_{EFF}$ in the most sensitive cultivars was likely due to an excess of H$_2$O$_2$, as APX catalyzes the reduction of H$_2$O$_2$ by AsA (Chernikova et al., 2000). Such elevated oxidative stress translated into lower B$_{Water}$ in the sensitive cultivars, which is a well-known impact of O$_3$ (Feng and Kobayashi, 2009). The only constitutional factor that explained the cultivar tolerance well was AsA$_{EDU}$, as it was lower in the most sensitive cultivars when protected by EDU. While it is well known that the direct reaction of O$_3$ with cell wall ascorbate is a central mechanism of plant tolerance to this pollutant (Plöchl et al., 2000), this is the first proof linking higher intra-specific O$_3$ tolerance with higher total ascorbate in soybean. This knowledge will help in breeding for more and more O$_3$-tolerant cultivars.

4. Conclusions

This was the first experimental study to show that ambient O$_3$ is able to threaten the growth, physiological and biochemical responses of soybean in China. The results suggested that current O$_3$ pollution is a serious concern for soybean, which highlights the urgent need for policy-making actions protecting this critical staple legume species for food security in China. Fortunately, cultivars showed a considerable variability in their sensitivity to O$_3$, which gives guidance to farmers in choosing the best-suited cultivars for local cultivation. Among the most tolerant cultivars, ZH69 also showed excellent biomass productivity and should be tested for the quantity and quality of yield production.

These important results were obtained by applying the antioxidant EDU as a tool for evaluating the O$_3$ sensitivity of plants. Although preliminary results are encouraging (Agathokleous et al., 2016a), toxic side effects on the food chain by this synthetic chemical cannot yet be excluded, and thus EDU can be recommended only as a scientific tool and not as an O$_3$ protectant in common agricultural practice.

The cultivars showed some interesting similarities in their responses to O$_3$. We thus conclude that higher lipid peroxidation (MDA$_{Water}$ and MDA$_{EFF}$) and activity of the ascorbate peroxidase enzyme (APX$_{EFF}$) were responses to O$_3$ injury, which eventually translated into lower biomass production (B$_{Water}$). Rather than these factors, the constitutional level of total ascorbate (AsA$_{EDU}$) in the leaves was the major parameter explaining soybean cultivar sensitivity to O$_3$. This result will guide future breeding efforts towards more O$_3$-tolerant soybean cultivars in China, while strategies for implementing control measures of regional O$_3$ pollution are being implemented.

Acknowledgments

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