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Protecting the photosynthetic performance of snap bean under free air ozone exposure

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ABSTRACT

Tropospheric ozone (O₃) is a major air pollutant and causes serious injury to vegetation. To protect sensitive plants from O₃ damage, several agrochemicals have been assessed, including cytokinin (*e.g.*, kinetin, KIN) and ethylenediurea (EDU) with cytokinin-like activity. In higher plant, leaves are primarily injured by O₃ and protective agrochemicals are often applied by leaf spraying. To our knowledge, the mitigating abilities of EDU and KIN have not been compared directly in a realistic setup. In the present research, impacts of elevated O₃ (2× ambient O₃, 24 hr per day, for 8 days) on an O₃ sensitive line (S156) of snap bean (*Phaseolus vulgaris*), which is often used for biomonitoring O₃ pollution, were studied in a free air controlled exposure system. The day before starting the O₃ exposure, plants were sprayed with a solution of EDU (300 ppm), KIN (1 mmol/L) or distilled water, to compare their protective abilities. The results demonstrated that 2× ambient O₃ inhibited net photosynthetic rate and stomatal conductance, increased the minimal fluorescence yield of the dark-adapted state, decreased the maximal quantum yield of PSII photochemistry, and led to visible injury. KIN and EDU alleviated the reduction of the photosynthetic performance, and visible injury under O₃ fumigation. The plants sprayed with EDU showed greater ability to mitigate the O₃ damage than those sprayed with KIN. Chlorophyll fluorescence imaging may have detected more precisely the differences in O₃ response across the leaf than the conventional fluorometer.

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Introduction

Ground level ozone (O₃) is one of the major air pollutants and is a serious threat to crops, forests, and natural vegetation (Feng et al., 2003; Ainsworth et al., 2012; Matyssek et al., 2013; Feng et al., 2014; Agathokleous et al., 2016; Izuta, 2017). Ozone enters the leaf through the stomata and reacts with the apoplast of the mesophyll cells, generating reactive oxygen species (ROS) and provoking signaling cascades, which cause visible foliar injury, decrease stomatal conductance, and

inhibit net photosynthetic rate (Morgan et al., 2003; Tiwari et al., 2016).

In order to protect sensitive vegetation from O₃ pollution, several kinds of agrochemicals have been tested (Didyk and Blum, 2011). Among them, ethylenediurea (EDU) is the most frequently studied O₃-protectant chemical (Manning, 2000; Paoletti et al., 2009a; Manning et al., 2011). It is able to protect plants from O₃ injury by mitigating the loss of CO₂ assimilation and delaying O₃-induced accelerated senescence (Tiwari and Agrawal, 2010; Pandey et al., 2014). Nonetheless, in the

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last few years other types of agrochemicals are widely examined to find alternatives to EDU. It has been reported that cytokinin such as kinetin (KIN) could act as a protectant to cope with oxidative damage such as O₃ pollution (Rattan, 2002; Zhang et al., 2017). The antiozonant ability of EDU and KIN was compared by Lee and Chen (1982) using the tobacco callus bioassay. In higher plant, however, leaves are primarily injured by O₃ and protective agrochemicals are often applied by leaf spraying. This is the first study to compare the mitigating abilities of EDU and KIN in a realistic experimental setup.

It has been well known that some genotypes of bean (*Phaseolus vulgaris* L.) are sensitive to O₃ pollution, and have been widely used as model plants for bioindicating or biomonitoring O₃ pollution due to a typical symptomology and limited cultivation requirements (Sanders et al., 1992; UN/ECE, 1996; Burkey et al., 2005, 2012). Typical bronzing (red-brown pigmentation) is usually found in bean plants under elevated O₃ (Feng et al., 2014). In common bean, a decline of CO₂ assimilation was observed in a sensitive cultivar exposed to a short time and acute O₃ pollution (Guidi et al., 2009). Under chronic O₃, the maximum quantum yield for primary photochemistry (F_w/F_m) was inhibited in the sensitive genotype ('S156') of snap bean (Flowers et al., 2007). Moreover, it has been reported that higher O₃ sensitivity of bean cultivar 'Cannellino' could be partially attributed to higher stomatal conductance and lower ability to dissipate excess energy (Guidi et al., 2010). Although it has been proved that EDU is effective for protecting sensitive genotypes of bean under O₃ stress (Elagöz and Manning, 2005), a comparative study on the ameliorating effects of EDU and KIN on O₃ stressed bean plant has not been reported.

The objective of this research therefore was to compare the mitigating effects of EDU and KIN on leaf visible injury, gas exchange, and chlorophyll fluorescence parameters of snap bean under O₃ free air controlled exposure (FACE). The results of this study can provide hints to the protection of crops in O₃-polluted areas.

1. Materials and methods

1.1. Plant material

Snap beans (*P. vulgaris* 'S156', source K.O. Burkey, USDA, USA) were planted in plastic pots (17 cm in diameter, 2 L), filled with a mixture of peat and vermiculite (3:1, V/V). After they had at least two fully expanded trifoliolate leaves (4-week old), the seedlings were moved from the experimental garden to the O₃ FACE facility for the treatments as described below. Plants were irrigated every day until maximum soil water storage capacity to avoid drought stress. The hourly mean temperature, relative humidity, and photosynthetic photon flux density (PPFD) at the plant height were (25.5 ± 0.4)°C, (49.3 ± 1.4)%, and (616.7 ± 53.5) μmol photon/(m²·sec), respectively. The precipitation was 2.7 mm during the treatment period.

1.2. Treatments

Two O₃ treatments were applied: ambient air and 2× ambient O₃. Spraying treatments included two agrochemicals: EDU

(Source W.J. Manning, University of Massachusetts, USA) and KIN (Source Duchefa, Haarlem, the Netherlands). Distilled water was selected as control. EDU (300 ppm) was used because this concentration is effective in ameliorating O₃ injury in this genotype as reported before (Paoletti et al., 2014). KIN (1 mmol/L) was selected as in our previous study (Zhang et al., 2017). This concentration was able to mitigate O₃-caused leaf visible injury better than 0.1 or 0.01 mmol/L as per our preliminary experiment (unpublished). The EDU formulation was a 100% wettable powder. The day before O₃ exposure, EDU was dissolved in warm distilled water, and then applied when it was back to ambient temperature. Due to insoluble in water, KIN (0.5 mmol) was dissolved in 1 mol/L NaOH (10 mL) according to the instruction and the solution was diluted into 500 mL using distilled water. Leaves were sprayed to dripping point with distilled water or 300 ppm EDU solution, or 1 mmol/L KIN solution. Exposure to ambient air or 2× ambient O₃ was carried out for eight consecutive days from 13th to 20th August 2016, 24 hr per day, in a FACE facility (for a description of this facility, see Paoletti et al., 2017). Ozone concentration was continuously monitored by O₃ analyzers (Model 202, 2B Technologies Inc., Boulder, Colorado, USA). Ozone concentration was expressed as AOT40, i.e., the sum of the differences between hourly O₃ concentrations and 40 ppb for each hour when the concentration is above 40 ppb during daylight hours (short wave radiation >50 W/m²) according to CLRTP (2015). In ambient air, the daily mean O₃ concentration was (34.8 ± 1.3) ppb, the maximum hourly O₃ concentration was 60.3 ppb, and AOT40 was 0.61 ppm·hr. Under elevated O₃ exposure, the daily mean O₃ concentration was (66.8 ± 1.9) ppb, the maximum hourly O₃ concentration was 120.5 ppb, and AOT40 was 4.17 ppm·hr.

1.3. Visible injury assessment

On the first day after the end of O₃ treatments, the percentage of injured surface per symptomatic leaflet (IA) and the percentage of injured leaflets per plant (LA) were visually assessed by two surveyors with the help of photoguides (Innes et al., 2001; Paoletti et al., 2009b). A plant injury index (PII) was calculated as (LA × IA) / 100 according to Paoletti et al. (2014).

1.4. Gas exchange measurement

Instantaneous gas exchange was measured on the top leaflet of the second fully expanded trifoliolate leaf from 09:00 AM to 12:30 PM on the first day after the end of O₃ exposure using a portable system (Li-6400, Li-Cor, USA). Six plants per treatment were used for measurement. The second fully expanded trifoliolate leaf under 2× O₃ treatment showed visible injury and those in ambient air were asymptomatic. According to Yuan et al. (2015), light intensity inside the leaf chamber and was set to 1500 μmol photon/(m²·sec) PPFD to achieve saturation light. Leaf temperature inside the leaf chamber was set to (25 ± 0.5)°C in accordance with the hourly mean temperature during experiment. CO₂ concentration (400 ± 1 μmol/mol) was used as reference (Yuan et al., 2015). Net photosynthetic rate under saturating PPFD (P_N), stomatal conductance (g_s),

transpiration rate (E), and intercellular CO_2 concentration (C_i) were recorded after the values were stable.

1.5. Chlorophyll *a* (Chl-*a*) fluorescence measurement

In the early morning of the second day after the end of O_3 exposure, Chl-*a* fluorescence was measured on the top leaflet of the second fully expanded trifoliate leaf using a direct fluorometer (Handy PEA, Plant Efficiency Analyser, Hansatech Instruments, UK). Six plants per treatment were used. The selected leaves were adapted in the dark for 30 min using leaf clips (Villányi et al., 2014). The rising transient was induced by saturating red-actinic-radiation ($1500 \mu\text{mol photon}/(\text{m}^2\cdot\text{sec})$, peak at 650 nm, duration 1 sec). Data acquisition was recorded from 10 μs to 1 sec after the onset of irradiation (Contran et al., 2009). The values of the minimal fluorescence yield in the dark-adapted state (F_o) and the maximal fluorescence yield of the dark-adapted state (F_m) were recorded. F_v/F_m was calculated as $(F_m - F_o) / F_m$ (Kitajima and Butler, 1975).

The imaging technique was performed by using an IMAGING-PAM Chl fluorometer (Walz, Effeltrich, Germany) on the second day immediately after the measurement by the direct fluorometer, and the measurement lasts for two days due to the fact that it was time consuming. Three plants per treatment were used. The second fully expanded trifoliate leaf was cut in the water using a sharp knife and the petiole was immediately immersed into a centrifuge tube to avoid water loss during the measurement. Then the trifoliate leaf was placed in the darkness for 30 min prior to measurement (Simko et al., 2015). The current fluorescence yield (F_t) was measured continuously and the F_o images were recorded in a quasi-dark state. F_m was determined with a saturating pulse of $2400 \mu\text{mol photon}/(\text{m}^2\cdot\text{sec})$ PPFD. The images of F_o and F_m were subtracted and divided by F_m to generate the images of the F_v/F_m .

Actinic illumination ($701 \mu\text{mol photon}/(\text{m}^2\cdot\text{sec})$) was then switched on to determine the momentary fluorescence yield of an illuminated sample shortly before application of a saturation pulse (F) and then the quantum yield of nonregulated heat dissipation and fluorescence emission (Y_{NO}) was computed as F/F_m . Images of the fluorescence parameters were displayed by means of a false color code ranging from black (0.0) to purple (ending at 1.0) via red, yellow, green, and blue.

1.6. Statistical analysis

The experiment was a completely randomized block design. Each combination of O_3 treatment and agrochemical application had three blocks and each block had two plants. Mean values of visible injury, gas exchange, and Chl-*a* fluorescence parameters of the plants in each block were used for statistical analysis ($n = 3$). Data were checked for normal distribution by the Kolmogorov–Smirnov test. If the variables (such as X) were not normal distribution, data were transformed to $\log(X + 1)$ so that the normal distribution was achieved. Effects of agrochemical (distilled water, 300 ppm EDU, and 1 mmol/L KIN), O_3 (ambient air and $2 \times$ ambient O_3) and their interaction ($\text{O}_3 \times$ agrochemical) were evaluated by a two-way analysis of variance (ANOVA). Means of each parameter among different treatments were compared by post-hoc Duncan's test.

2. Results

2.1. Visible injury

Under $2 \times$ ambient O_3 concentration, bronzing occurred in the interveinal regions of adaxial surface of fully expanded leaves

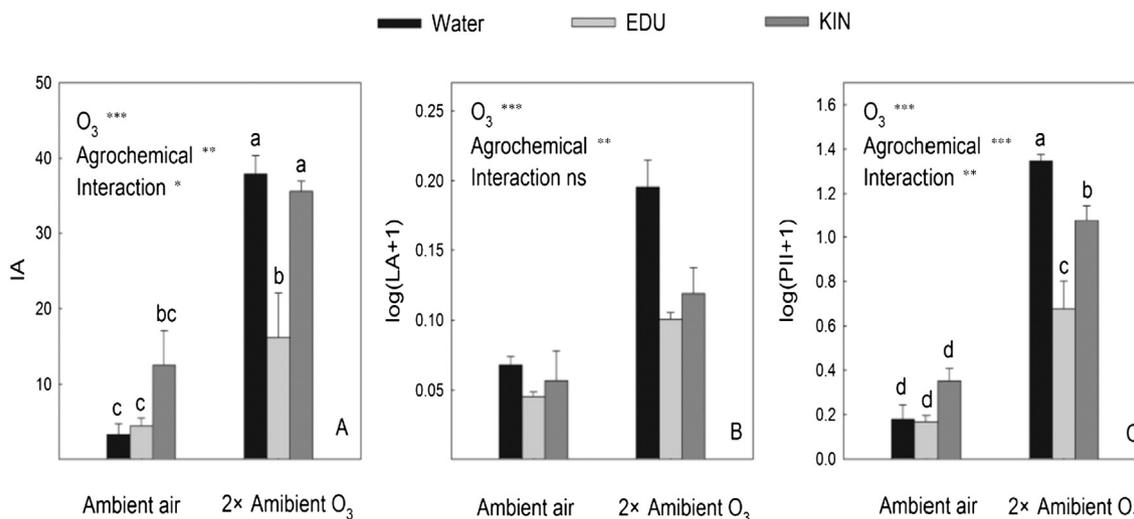


Fig. 1 – Visible foliar injury. Visible foliar ozone (O_3) injury expressed as percentage of injured surface per symptomatic leaflet (IA), percentage of injured leaflet per plant (LA), plant injury index (PII), in snap bean plants exposed to ambient air (AOT40 = 0.61 ppm·hr) or $2 \times$ ambient O_3 for eight days (AOT40 = 4.17 ppm·hr) and sprayed with distilled water, 300 ppm EDU solution or 1 mmol/L KIN solution before the exposure. Different letters show significant differences among the bars in each graph (Duncan's test, $p < 0.05$, $n = 3$). Results of two-way ANOVA for the effects of O_3 , agrochemical, and interaction are showed in the inset. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns $p > 0.05$ (not significant). EDU: ethylenediurea; KIN: kinetin; ANOVA: analysis of variance.

and no visible injury were observed in the abaxial surface (not shown). Plants in ambient air showed very limited visible foliar injury (Fig. 1), but the symptom was similar to that under 2× ambient O₃ (not shown). IA of leaves that sprayed with different agrochemicals was similar in ambient air. Compared with those in ambient air, IA was increased significantly under 2× ambient O₃ exposure (Fig. 1A). Plants sprayed with EDU showed the lowest IA, which was 45% of KIN and 43% of distilled water, respectively (Fig. 1A). The data of LA were not normally distributed and thus were transformed. There were no significant differences of log(LA + 1) among plants sprayed with different agrochemicals

in ambient air (Fig. 1B). log(LA + 1) of plants under 2× ambient O₃ exposure had significantly higher values than those in ambient air (Fig. 1B). Plant sprayed with distilled water had 1.9 or 1.6 fold higher log(LA + 1) value than those sprayed with EDU or KIN (Fig. 1B). The data of PII were also transformed into log(PII + 1) because of not normal distribution. log(PII + 1) of plants upon 2× ambient O₃ exposure had significantly higher values than those in ambient air. Under elevated O₃, the plants sprayed with distilled water had the highest log(PII + 1) value (1.35), followed by those sprayed with KIN (1.08), and those sprayed with EDU (0.68) (Fig. 1C).

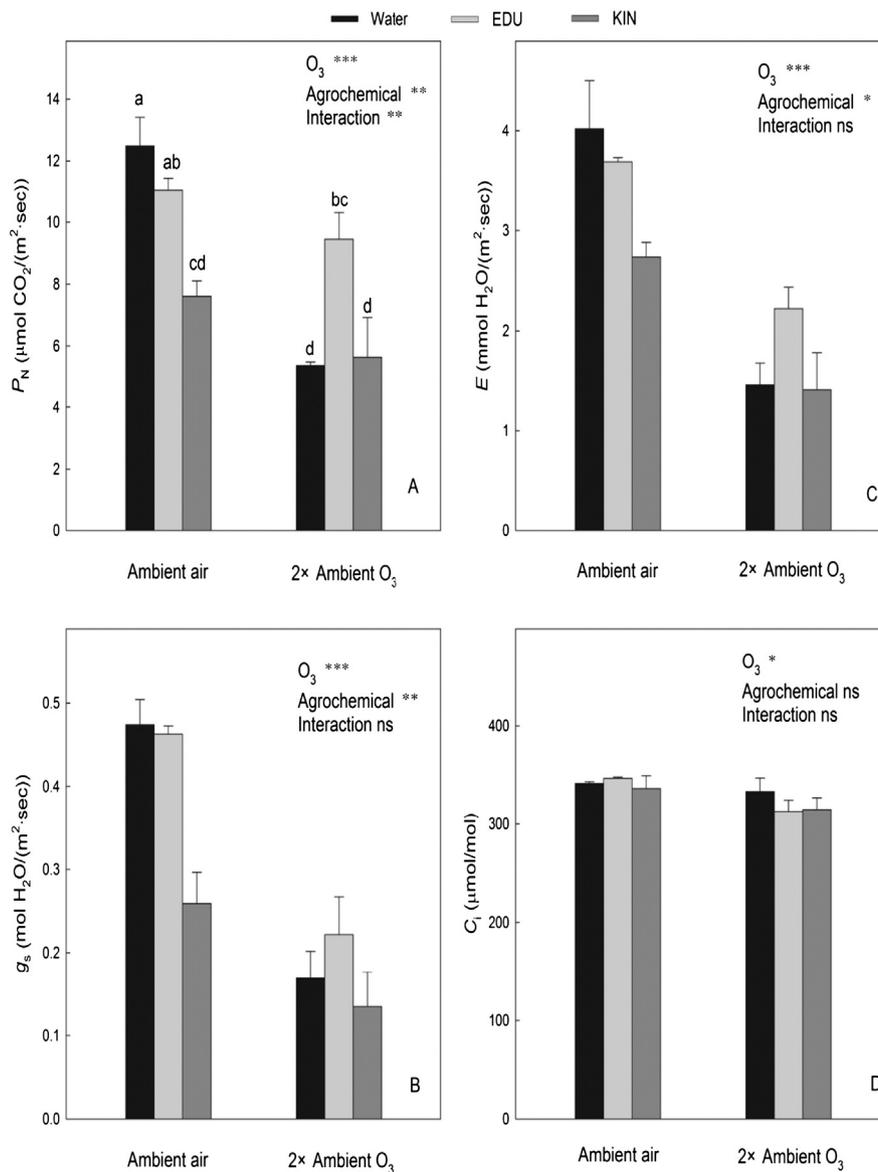


Fig. 2 – Gas exchange parameters. Gas exchange parameters (P_N : net photosynthetic rate under saturating PPFD; g_s : stomatal conductance; E : transpiration rate; C_i : intercellular CO₂ concentration) in snap bean plants exposed to ambient air (AOT40 = 0.61 ppm·hr) or 2× ambient ozone (O₃) for eight days (AOT40 = 4.17 ppm·hr) and sprayed with distilled water, 300 ppm EDU solution or 1 mmol/L KIN solution before the exposure. Different letters show significant differences among the bars in each graph (Duncan's test, $p < 0.05$, $n = 3$). Results of two-way ANOVA for the effects of O₃, agrochemical, and interaction are showed in the inset. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns $p > 0.05$ (not significant). PPFD: photosynthetic photon flux density; KIN: kinetin.

2.2. Gas exchange

There were significant interactions between O₃ treatment and agrochemical spraying on P_N, which suggests that the plants with different agrochemicals responded to elevated O₃ differently. Under elevated O₃, P_N was significantly inhibited in plants sprayed with distilled water, while values of plants treated with EDU and KIN were not significantly different from those in ambient air (Fig. 2A). The interactions between O₃ treatment and agrochemical spraying on g_s, E and C_i were not significant. Increased O₃ significantly decreased g_s, E and C_i (Fig. 2B, C, D).

2.3. Chl-a fluorescence

F_o increased upon 2× ambient O₃ but only in the plants sprayed with distilled water (26%) (Fig. 3A). The interaction between O₃ treatment and agrochemical spraying, in fact, was significant. There was no significant difference of F_m between different O₃ treatments or among different agrochemicals (Fig. 3B). Elevated O₃ significantly decreased F_v/F_m (Fig. 3C). The interaction between O₃ treatment and agrochemical spraying was significant but only the plants sprayed with water had significantly lower F_v/F_m (–15%) under 2× ambient O₃ than those in ambient air (Fig. 3C).

In the present study, the images of efficient quantum yield of PSII (Y_{PSII}) were similar to those of F_v/F_m and the images of Y_{NPQ} were almost black due to the very lower values. Therefore, only images of F_v/F_m and Y_{NO} were shown (Figs. 4, 5). Plants sprayed with distilled water, EDU or KIN had similar images of F_v/F_m in ambient air (Fig. 4A,C, E). Under 2× ambient O₃, heterogeneous distributions of F_v/F_m over the screened leaf area were observed especially in leaves sprayed with distilled water or KIN (Fig. 4B, F). Elevated O₃ decreased F_v/F_m of plants sprayed with distilled water and the interveinal parts of

leaves showed lower values than other parts (Fig. 4B). The marginal parts of leaves sprayed with KIN showed lower F_v/F_m than other parts, but the extent of injured leaf area was lower than in plants treated with distilled water (Fig. 4F). Compared with ambient air, plants sprayed with EDU did not show significant difference under elevated O₃ (Fig. 4D). Y_{NO} is a good index for photodamage of photosynthetic apparatus and a high value means that both protective regulatory mechanisms and photochemical energy conversion are inefficient (Huang et al., 2010). The area with higher Y_{NO} value was that with lower F_v/F_m ratio (Fig. 5). Plants with different spraying treatments had similar Y_{NO} in ambient air (Fig. 5A, C, E). Under 2× ambient O₃, leaves sprayed with distilled water had higher Y_{NO} than those in ambient air. Moreover, interveinal leaf areas had higher values than other parts (Fig. 5B). Leaves treated with KIN had highest Y_{NO} values in the marginal areas, but the extent was lower than in leaves sprayed with distilled water (Fig. 5F). Plants sprayed with EDU had similar Y_{NO} under different O₃ concentrations (Fig. 5D).

3. Discussion

Different types of visible foliar injury such as chlorosis, bleaching, flecking, bronzing, and stippling, may be observed after exposure to elevated O₃ (Long and Naidu, 2002). In this study, interveinal bronzing areas were found on leaves sprayed with distilled water or KIN upon 2× ambient O₃. The result indicates that the leaf area near the veins was less sensitive to O₃ than the interveinal areas, in agreement with other reports (Omasa et al., 2002; Agathokleous et al., 2017). This difference could partly be ascribable to the difference in stomatal density between interveinal areas and across veins of the abaxial surface of this genotype (Agathokleous et al.,

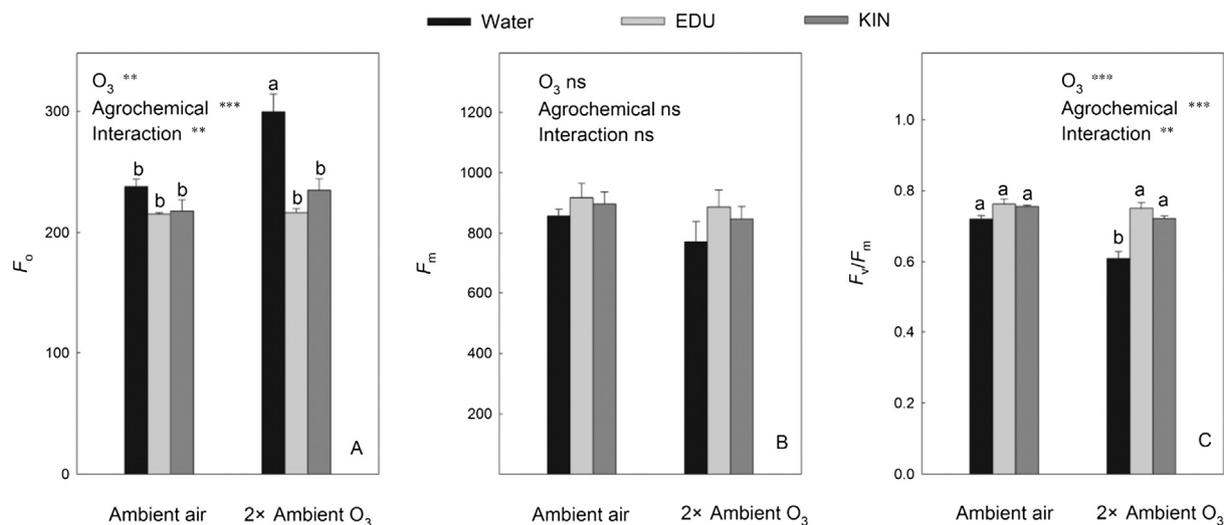


Fig. 3 – Chlorophyll fluorescence parameters. Chlorophyll fluorescence parameters (F_o: the minimal fluorescence yield of the dark-adapted state; F_m: the maximal fluorescence yield of the dark-adapted state; F_v/F_m: the maximal quantum yield of PSII photochemistry), in snap bean plants exposed to ambient air (AOT40 = 0.61 ppm·hr) or 2× ambient ozone (O₃) for eight days (AOT40 = 4.17 ppm·hr) and sprayed with distilled water, 300 ppm EDU solution or 1 mmol/L KIN solution before the exposure. Different letters show significant differences among the bars in each graph (Duncan’s test, p < 0.05, n = 3). Results of two-way ANOVA for the effects of O₃, agrochemical, and interaction are showed in the inset. *p < 0.05, **p < 0.01, ***p < 0.001, ns p > 0.05 (not significant).

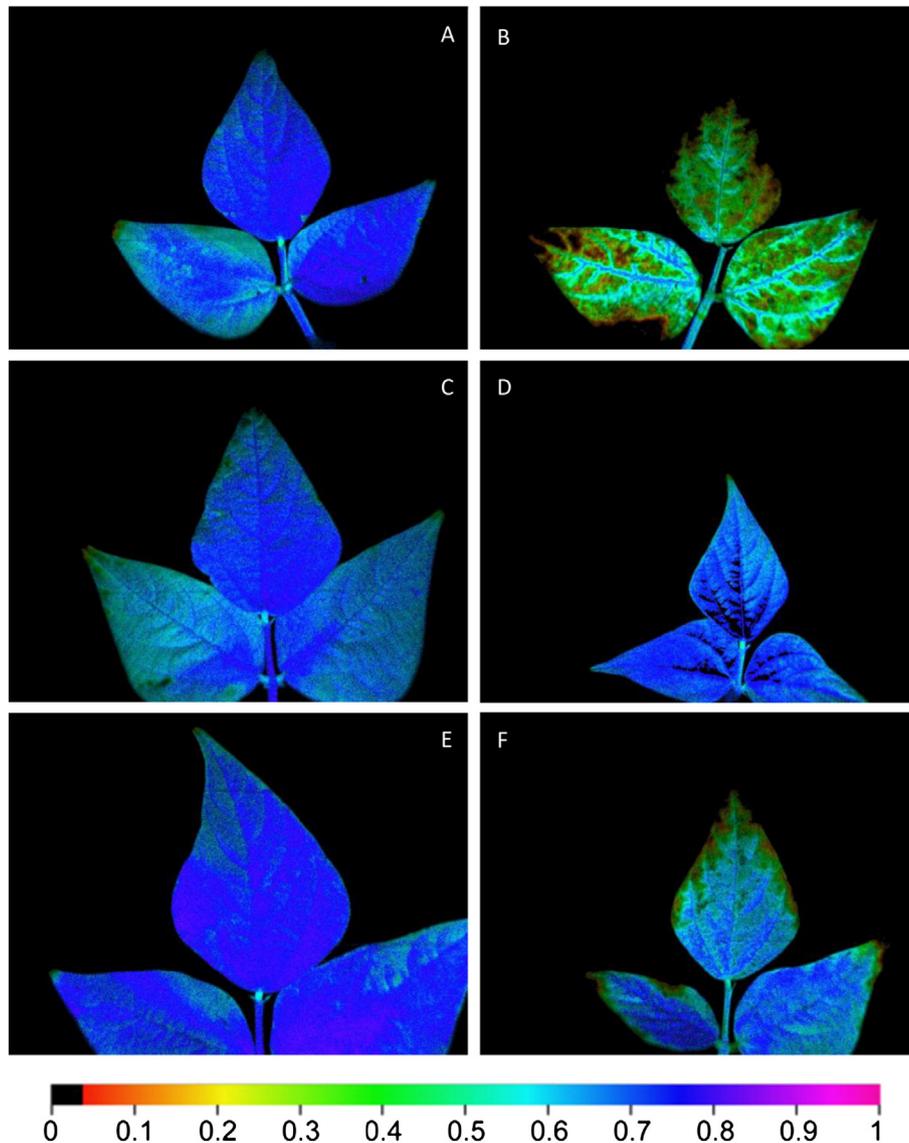


Fig. 4 – Chlorophyll fluorescence imaging of maximal quantum yield of PSII photochemistry. Chlorophyll fluorescence imaging of the maximal quantum yield of PSII photochemistry (F_v/F_m) in snap bean plants exposed to ambient air for eight days ($AOT_{40} = 0.61$ ppm-hr) and sprayed with distilled water (A), 300 ppm EDU solution (C) or 1 mmol/L KIN solution (E) before the exposure or exposed to $2\times$ ambient ozone for eight days ($AOT_{40} = 4.17$ ppm-hr) and sprayed with distilled water (B), 300 ppm EDU solution (D) or 1 mmol/L KIN solution (F) before the exposure. Images were taken from a single leaf per treatment and normalized to the false color bar provided. The false color code depicted at the bottom ranges from black (0.0) to (ending at 1.0) via red, yellow, green, blue to purple.

2017). Under elevated O_3 , plants sprayed with KIN or EDU had significantly lower LA and PII than those sprayed with distilled water, which confirmed the mitigating effects of EDU or KIN to O_3 . This result is in concert with other findings (Paoletti et al., 2014; Zhang et al., 2017). In addition, the lower IA and PII further demonstrated that EDU had higher ability to eliminate O_3 induced visible injury than KIN.

Gas exchange parameters are reconsidered as very important indices when assessing O_3 sensitivity (Fiscus et al., 2005). In ambient air, the lower P_N of plants sprayed with KIN compared with other agrochemical treatments suggests that 1 mmol/L KIN damaged the photosynthetic processes to some extent. This result is not consistent with some reports (Shah,

2011) in which KIN with different concentrations (10^{-6} – 10^{-4} mol/L) increased the photosynthesis of black cumin, but confirms similar results from our previous study (Zhang et al., 2017). The possible reason might be due to the slight damage of NaOH, which is the solvent of KIN or the higher concentration of KIN although damage was not serious in the chlorophyll fluorescence images (Figs. 4E and 5E). Inhibition of CO_2 assimilation is a common response to O_3 exposure (Zhang et al., 2010, 2017). In this study, $2\times$ ambient O_3 caused a significant decrease of P_N . Although the KIN damaged the photosynthesis in ambient air, however, the plants sprayed with KIN had a relative lower loss than the plants sprayed with water between O_3 treatments. Both EDU and KIN were

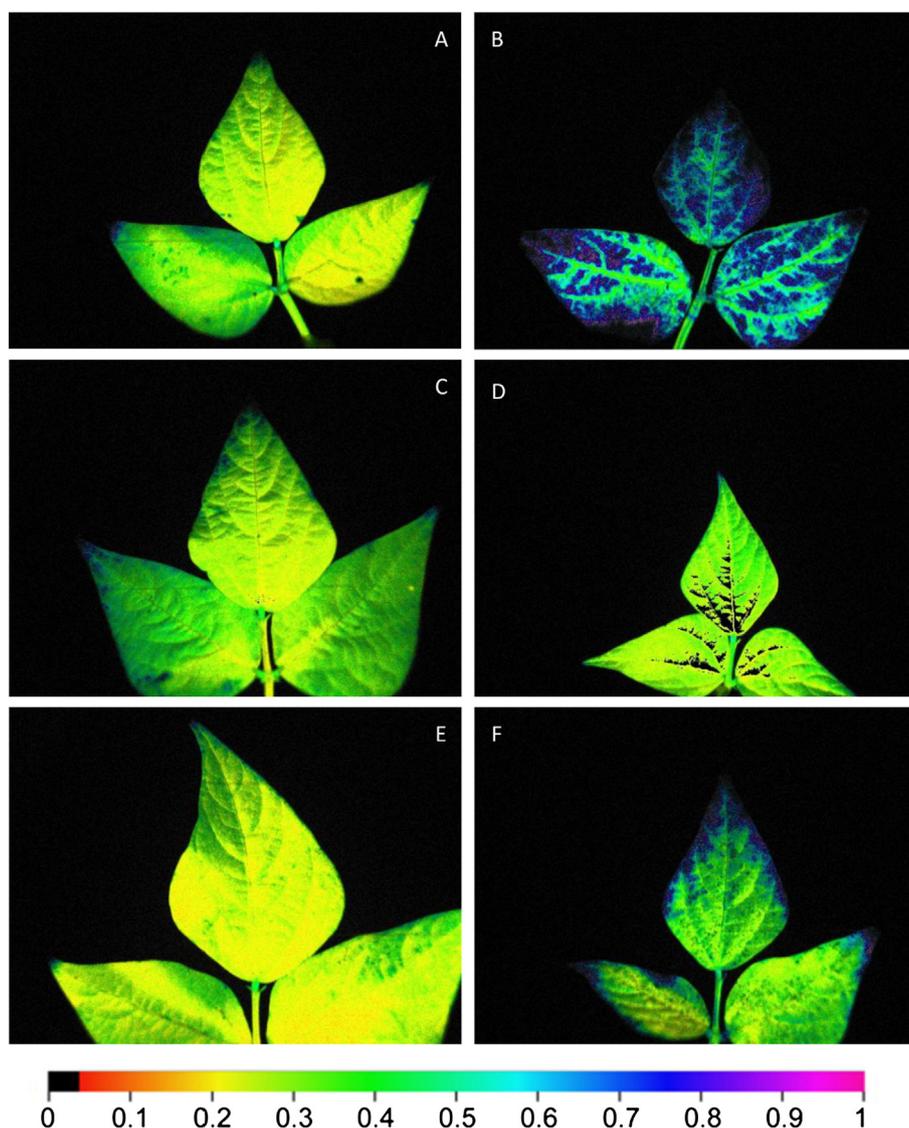


Fig. 5 – Chlorophyll fluorescence imaging of the quantum yield of nonregulated heat dissipation and fluorescence emission. Chlorophyll fluorescence imaging of the quantum yield of nonregulated heat dissipation and fluorescence emission (Y_{NO}) in snap bean plants exposed to ambient air for eight days ($AOT_{40} = 0.61$ ppm·hr) and sprayed with distilled water (A), 300 ppm EDU solution (C) or 1 mmol/L KIN solution (E) before the exposure or exposed to $2\times$ ambient ozone for eight days ($AOT_{40} = 4.17$ ppm·hr) and sprayed with distilled water (B), 300 ppm EDU solution (D) or 1 mmol/L KIN solution (F) before the exposure. Images were taken from a single leaf per treatment and normalized to the false color bar provided. The false color code depicted at the bottom ranges from black (0.0) to purple (ending at 1.0) via red, yellow, green, and blue.

able to mitigate the inhibition of CO_2 assimilation. This result is consistent with other reports (Feng et al., 2010; Zhang et al., 2017). On the other hand, our results showed that both g_s and E were decreased by elevated O_3 . The responses of g_s to elevated O_3 in plants sprayed with different agrochemicals were not significantly different, which probably meant that the mitigating effects of EDU and KIN were due to their role in detoxification rather than in avoidance. This result is consistent with other reports in which the protective role of EDU was attributed to biochemical detoxification processes such as maintenance of antioxidant enzymes (Paoletti et al., 2009a; Feng et al., 2010). The trend of E was similar to that of g_s suggesting that the water transpiration was also affected by

O_3 , which has already been confirmed by many studies (Biswas et al., 2008; Zhang et al., 2010).

Chl-a fluorescence parameters have been widely used as valuable indicators for early assessment of photosynthetic injury by O_3 (Guidi and Calatayud, 2014). In this study, plants sprayed with distilled water had higher F_0 and lower F_v/F_m under $2\times$ ambient O_3 compared with ambient air, which indicated that photoinhibition and photoinactivation of photosystem II complexes occurred (Bradbury and Baker, 1986; Leipner et al., 2001; Thwe et al., 2014). This result is in accordance with other studies of O_3 effects on snap bean (Flowers et al., 2007; Wang et al., 2015). The stable F_0 and F_v/F_m in plants sprayed with EDU or KIN between different O_3

concentrations suggested that the EDU or KIN spraying protected photosystem II complexes and avoided the occurrence of photoinhibition.

Chl fluorescence imaging provides detailed information about the spatial heterogeneity of fluorescence parameters in a whole leaf, and thus provides a more comprehensive view for the alterations to photosynthetic apparatus caused by abiotic stress such as O₃ (Guidi et al., 2007; Guidi and Degl'Innocenti, 2011; Chen et al., 2009). Ozone caused slight injury in localized regions on the leaf, and this response could be better detected using Chl fluorescence imaging rather than conventional fluorometers (Leipner et al., 2001). In this study, the F_v/F_m ratio was lower in cells in the interveinal area than those near the leaf veins, suggesting that the leaf area close to the veins is less prone to photoinhibition (Guidi et al., 2007). It is unknown whether these spatial differences reflect differences in O₃ uptake, detoxification, or some combination. It has been observed that there were no stomata in the interveinal area of the adaxial surface, and the stomatal density in the interveinal areas was higher than that across veins of the abaxial surface in this genotype (Agathokleous et al., 2017). We speculate that O₃ was absorbed mainly from stomata in the interveinal areas of abaxial surface and the generated ROS were transported to the adjacent cells in the interveinal areas of the adaxial surface. Although significant difference of F_v/F_m in plants sprayed with KIN was not observed between ambient air and 2× ambient O₃ using the PEA fluorometer, the Chl fluorescence images showed that decreases in F_v/F_m occurred in some parts of the leaves. This result is similar to other studies (Leipner et al., 2001), suggesting that Chl fluorescence imaging may have detected more precisely the differences in O₃ injury among local sites on a leaf. From the Chl fluorescence imaging, distinct effects of EDU and KIN spraying on F_v/F_m and Y_{NO} were found. EDU was more efficient in mitigating the negative effects of O₃ on photoinhibition or photodamage than KIN, probably due to its higher detoxification ability. Compared with F_v/F_m , which requires dark adaptation of plant leaves for measurement, Y_{NO} have a potential for undestructive and *in vivo* monitoring plant health in the field due to its possibilities of being calculated under natural light (Ivanov and Bernards, 2016). Our results also indicated that Chl fluorescence imaging of Y_{NO} could be a sensitive method for monitoring O₃ injury of plants in the field.

4. Conclusions

This present comparative study of O₃-protecting ability of EDU and KIN showed that elevated O₃ decreased P_N , g_s , F_v/F_m , increased F_o and Y_{NO} , and induced visible injury. Both EDU and KIN spraying alleviated the O₃-induced changes of these parameters. It is interesting to notice that EDU and KIN had similar mitigating effects according to both gas exchange and conventional Chl fluorescence parameters, however, other indices (visible injury and images of F_v/F_m and Y_{NO}) showed that EDU had the best ameliorating impact on the O₃ caused injury. These results suggest that we should pay more attention to visible injury, and images of F_v/F_m and Y_{NO} when selecting the best agrochemicals to cope with increasing O₃. It

has also been reported that visible foliar injury is more consistent than yield for detecting O₃ effects across different environments (Agathokleous et al., 2017). As a main conclusion, EDU showed a better mitigating ability than KIN for protecting snap bean from O₃ impacts.

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