The response of marigold (*Tagetes erecta* Linn.) to ozone: impacts on plant growth and leaf physiology

Ning Yang^{1,2} · Xiaoke Wang¹ · Feixiang Zheng^{1,3} · Yuanyuan Chen^{1,2}

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Abstract Progressively increasing ozone (O₃) concentrations pose a potential threat to the value of marigold (*Tagetes erecta* Linn.), a plant widely used in urban landscaping. The response of marigold to elevated O₃ has been reported earlier, but the mechanisms underlying the O₃ effect have not been clearly elucidated. In the present study, we exposed marigold “Moonsong Deep Orange” plants to elevated O₃, including ambient non-filtered air (NF) plus 60 ppb (NF+60) and 120 ppb (NF+120) O₃, to assess visible injury and the possible physiological consequences of this pollutant. Yellow lesions appeared after 4 days under NF+120 treatment and 12 days under NF+60 treatment, with 85.6% and 36.8% of the leaves being injured at harvest time, respectively. Compared with NF, NF+60 inhibited leaf photosynthesis, stem-diameter growth, and biomass production significantly, while the parameters were decreased more by NF+120. Although the stomatal conductance decreased under elevated O₃ exposure, the O₃ flux into leaves increased by 28.0–104.8% under NF+60 treatment and 57.5–145.6% under NF+120 treatment. The total ascorbic acid (ASA) content increased due to elevated O₃ exposure, while the reduced ASA content did not, resulting in a decreased ratio of reduced to total ASA. A lower level

of jasmonic acid (JA) was observed under elevated O₃ exposure. In conclusion, the impacts of elevated O₃ on marigold plants may be ascribed to increased O₃ flux into leaves and reduced protective capacity of leaves to convert oxidized to reduced ASA and synthesize endogenous JA.

Keywords Ascorbic acid · Biomass · Jasmonic acid · Marigold · Ozone · Stomatal aperture

Introduction

Owing to rapid industrialization and urbanization, tropospheric ozone (O₃) concentration is thought to be increasing by 0.5–2% per year in the northern hemisphere based on predictive models (ICPP 2013). The average ambient O₃ concentration in the northern hemisphere is expected to rise to approximately 42–84 ppb (depending on space and time) by 2100 (Vingarzan 2004). Values exceeding 40 ppb will result in visible and invisible injuries to plants (Ashmore 2005) and will have detrimental effects on forests (Broadmeadow 1998), crops (Bao et al. 2014), and grasslands (Evans and Ashmore 1992). O₃ attenuates photosynthesis, resulting in reduced carbon accumulation (Ainsworth et al. 2012; Sun et al. 2014). Furthermore, O₃ accelerates leaf senescence and decreases leaf longevity by inducing injury (Zhang et al. 2011), which not only depresses plant productivity, but also affects their ornamental performance in human settlements.

As O₃ enters plant leaves through stomata, these small organs represent the first line of defense and the principal interface responsible for O₃ exchange between leaves and the atmosphere (Fares et al. 2014). Stomatal conductance controls O₃ uptake by leaves. Studies have demonstrated that O₃ stress reduces stomatal conductance (Sun et al. 2014) or stomatal aperture (Frey et al. 1996), resulting in

✉ Feixiang Zheng
zhengfeixiang@caas.cn

¹ State Key Laboratory of Urban and Region Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

² University of Chinese Academy of Sciences, Beijing 100049, China

³ Institute of Environment and Sustainable Development in Agriculture, Chinese Academy of Agricultural Sciences, Beijing 100081, China

reduced O₃ flux into leaves and less O₃-induced injury (Pääkkönen et al. 1996). However, opposite results have also been reported, i.e., plants exposed to O₃ had a slower stomatal response to this pollutant and a weaker ability to close their stomata than the control (Hoshika et al. 2014). These inconsistent results require further investigation into the responses of various plant species to different O₃ levels.

Ascorbic acid (ASA), the primary antioxidant scavenger of O₃, functions in the detoxification of O₃ in the leaf apoplast. Burkey et al. (2006) indicated that more reduced ASA is present in O₃-insensitive plants than in O₃-sensitive ones, with a lower percentage of O₃ injury detected in the former than the latter. However, other studies have suggested that ASA cannot be a primary O₃ scavenger because the level of reduced ASA in the leaf apoplast is substantially low (Booker et al. 2012). Hence, it is questionable whether ASA levels represent an effective index for assessing O₃ injury in plants. In fact, only a portion of the O₃ entering leaves participates in the reaction with ASA; another portion of O₃ remains to attack the plasma membrane and cause membrane lipid peroxidation, as well as influence phytohormone biosynthesis (Zhao et al. 2013).

Phytohormones function as second messengers in oxidative signal transduction; they regulate signal strength either alone or together (Baier et al. 2005). Jasmonic acid (JA) is a key signaling compound that functions in the plant response to stress (Wasternack 2014), and its roles in containing lesion propagation (Pellegrini et al. 2013) and protein storage (Creelman and Mullet 1997) are widely known. JA biosynthesis begins in the chloroplast, where linolenic acid is converted into hydroperoxylinolenic acid by lipoxygenases (Bartoli et al. 2013). JA-insensitive mutants and mutants blocked in JA biosynthesis could develop lesions under elevated O₃ exposure (Rao et al. 2000), demonstrating that JA can reduce O₃-induced injury to plants. However, less is known about whether O₃ injury to plants is driven by O₃-induced changes in endogenous JA content.

Marigold (*Tagetes erecta* Linn.), a member of the Asteraceae family (Dasgupta et al. 2012), is widely cultivated in China. Marigold is native to Mexico, and its natural range extends from the Southeastern United States to Argentina. Marigold is well-known for a wide range of pharmacological, agricultural and industrial uses (Hussain et al. 2011; Maity et al. 2011; Nikkon et al. 2011). This species is also a common ornamental plant (bearing bright yellow and orange flowers) that is often used for decorative purposes (Kiranmai et al. 2011). As early as in the 1980s, marigold was found to be moderately sensitive to O₃ when exposed to 300 ppb O₃ (Sanders and Reinert 1982; Reinert and Sanders 1982). However, less is known about the physiological mechanisms of the ozone effect on marigold growth.

In this study, we chose marigold, a typical urban greening plant with a short life cycle, to investigate its growth response to elevated O₃ exposure and the underlying physiological mechanisms. The objectives of the study were to investigate (1) the stomatal response of marigold to elevated O₃ by measuring stomatal conductance (gas exchange) and stomatal movement (aperture and density); (2) whether O₃-induced changes in stomatal conductance affect O₃ uptake by calculating O₃ flux into leaves of marigold; and (3) the defensive mechanisms of marigold plants by analyzing the capacity of leaves to synthesize ASA and JA and convert oxidized to reduced ASA.

Materials and methods

Experimental site

The experimental site was located in Zhangtou village (40° 12' N, 116° 8' E), Changping district, Beijing, China. Beijing has a typical temperate, monsoonal climate, with four clearly distinct seasons. During the experimental period (4 August–28 September 2014), the average temperature, maximum temperature, and total precipitation were 22.6°C, 28.1 °C, and 36.1 mm, respectively. The monthly averages of background O₃ concentrations were 51.6 and 51.0 ppb in August and September 2014, respectively. According to data from the nearby air monitoring station (Beijing Urban Ecosystem Research Station), the average of all hourly averages (24 hr/day) and peak one hourly average for SO₂ concentrations were 3.1 and 17.4 ppb in August and 5.9 and 21.5 ppb in September 2014, respectively; the average of all hourly averages and peak one hourly average for NO₂ concentrations were 3.8 and 21.6 ppb in August and 9.5 and 47.1 ppb in September 2014, respectively.

Open top chambers

Open top chambers (OTCs) were designed as previously described (Zheng et al. 2011a) with slight modifications. Regular dodecagon OTCs (2.8 m in height and 4.0 m in diameter) were constructed from an aluminum alloy frame covered with stalinite (toughened glass). Ambient air or O₃ was delivered by a centrifugal blower (2800 r min⁻¹) through PVC pipes with an internal diameter of 110 mm, followed by release from driven-rotating acrylic tubes with downward-facing holes. The OTCs were placed 3 m apart. O₃ was generated from pure oxygen with an O₃ generator (HY003, Chuangcheng Co., Jinan, China) using high voltage discharge. The O₃ concentration within the OTCs was monitored via an O₃ analyzer (Model 49i, Thermo Scientific, Waltham, MA, USA) equipped with a flash memory to store the measurement data online. The Thermo iPort

Fig. 1 Nine-hour mean ozone (O_3 , vertical bar chart) and daily peak O_3 (line plot) concentrations in open top chambers during the experimental period (4 August–28 September 2014). NF, NF+60, and NF+120 indicate the ambient control (non-filtered air) and two elevated O_3 treatments (non-filtered air plus 60 ppb O_3 and 120 ppb O_3), respectively

software was installed on a computer connected with O_3 analyzer to download data weekly. The O_3 analyzer was calibrated using a 49i-PS calibrator (Thermo Scientific), before the experiment and once a month during the experimental period. To attenuate chamber effects, OTCs were arranged in a line from south to north to ensure uniform environmental conditions, including solar radiation.

Ozone treatment

Three treatments were applied: non-filtered air (NF, control), non-filtered air plus 60 ppb O_3 (NF+60), and non-filtered air plus 120 ppb O_3 (NF+120), with a single OTC per treatment. The plants were exposed to elevated levels of O_3 for 9 h (8:00 am–17:00 pm) every day from 4 August to 28 September 2014, except when it was raining. The door of chambers always opened at other time of the day for air exchange with the outside. The daily average of 9 h-averages and daily peak one hourly average for O_3 , and accumulated exposure above a threshold of 40 ppb (AOT40) based on 9-h O_3 exposure period per day from fumigation beginning to the key periods for each treatment are shown in Fig. 1 and Table 1.

Plant management

A hybrid, male-sterile marigold cultivar (*Tagetes erecta* Linn. “Moon song Deep Orange”) seedlings of approximately 7.0 cm in plant height and 4.0 mm in stem diameter were purchased from Jindetai Landscape Engineering Co. (Beijing, China). The plants were subsequently moved into larger pots (17.0 cm in height and 22.0 cm in diameter). One pot was allocated per plant, and 20 pots per treatment were used in each treatment. The substrate, which was purchased from the same company as the marigold seedlings, comprised a mixture of grass charcoal, vermiculite and perlite at a ratio of 100:1:2. Given the higher temperatures in the summer, the plants were watered with 900–1000 mL of tap

Table 1 Accumulated ozone exposure above a threshold of 40 ppb (AOT40, ppm h) during key periods for different treatments (including the time of injury recording and physiological index determination)

Date	NF	NF+60	NF+120
8 August	1.3	3.9	6.4
15 August	2.6	8.6	14.2
22 August	5.8	15.4	24.6
27 August	7.4	19.6	31.3
10 September	11.3	30.5	49.2
12 September	11.7	32.1	51.7
17 September	23.7	67.1	108.5
28 September	25.1	73.0	119.9

Note: NF, NF+60 and NF+120 represent non-filtered air and non-filtered air plus 60 ppb O_3 and 120 ppb O_3 , respectively

water after 17:00 pm every day when O_3 treatment was stopped. Additionally, the replicates were not true independent units, i.e., chambers, but were individual pots within a chamber, so we rotated pots to minimize any pseudoreplication effects: the plant position within individual OTCs was changed every 3–5 days, the plants and O_3 treatments were rotated among three OTCs every 10–15 days in order to minimize any individual chamber effects on growth. At each time of change among OTCs, the plants were first removed from the OTC; after the O_3 concentration was reassigned, the plants were rearranged into a random OTC where they continued to receive the same O_3 treatment as before (Feng et al. 2011a).

Leaf injury assays

Whole-plant and foliar injury were assessed every 7 days from 15 August to 28 September for 20 plants per treatment. All plants were observed macroscopically. For each plant, the percentage of injured leaves was quantified. Additionally, the percentage of injured area for each leaf was estimated according to six non-proportional classes,

and the leaf injury index (LII) was then calculated as described previously (Furlan et al. 2007):

$$\text{LII}(\%) = \frac{(N1 \times 1) + (N2 \times 2) + (N3 \times 3) + (N4 \times 4) + (N5 \times 5)}{(N0 + N1 + N2 + N3 + N4 + N5) \times 5} \times 100 \quad (1)$$

where N0, N1, N2, N3, N4, and N5 are the number of leaves with zero, first, second, third, fourth, and fifth degree symptoms of O₃ injury, respectively. The degree of symptoms was represented as follows: 0: no symptoms; 1: 0–5; 2: 6–25; 3: 26–50; 4: 51–75; and 5: >75% of foliar area with symptoms.

Growth, biomass and photosynthesis measurements

Sixty marigold seedlings with similar plant height and stem diameter were selected and equally distributed to the three treatments. At the beginning of the experiment, we marked the main stem of all plants at the substrate level. The initial plant height was measured from the marked position to the apex of the main stem and the initial stem diameter was measured at the marked position. During the experimental period, plant height and stem diameter were measured twice a month. To exclude the individual differences in marigold seedlings, the relative growth was calculated as the percentage increase in plant height and stem diameter compared with the last measurement. On 28 September, five plants per chamber were randomly harvested and separated into four components: leaves, flowers, stems, and roots. All parts were oven-dried at 80 °C to achieve a constant weight for biomass measurements (Zhang et al. 2011).

Net photosynthetic rate and stomatal conductance of fully expanded sun leaves were measured twice in situ, respectively, at pre-anthesis (22 August) and anthesis (12 September), with an Li-6400 portable system (Li-Cor Inc., Lincoln, NE, USA). Five plants per chamber were randomly selected and one leaf per plant was measured. The Li-6400 system controlled saturating photosynthetic photon flux density at 1,500 μmol m⁻² s⁻¹ and CO₂ at 400 ppm using pure CO₂ supplied by a cylinder. Measurements of net photosynthetic rate and stomatal conductance were conducted simultaneously, from 10:00 am to 12:00 pm, when maximum parameters were generally reached (Zhang and Gao 1999; Grantz et al. 2013). Data were stored in the Li-6400 system. O₃ flux into leaves (F_{st}) was calculated based on the following formula (Hu et al. 2015):

$$F_{st} = [O_3] \times g_{O_3} \quad (2)$$

where [O₃] is the O₃ concentration and g_{O₃} is the stomatal conductance of water vapor multiplied by 0.663.

Stomatal aperture and density analysis

Stomatal characteristics were measured as described previously (Wen et al. 2014) with minor modifications. Four fully expanded sun leaves per plant and five plants per treatment were harvested at about 16:00 pm when daily peak ambient O₃ concentrations occurred (Chen et al. 2015) on two occasions (pre-anthesis [22 August] and anthesis [12 September]). The fresh leaves were subsequently wiped with a wet towel and immediately placed into a 50-mL white plastic bottle containing a fixative solution (70% ethanol: glacial acetic acid: formalin [v:v:v = 90:5:5]) to protect leaf cell structure from deformation. One day later, each leaf sample was cut into 1.0 × 0.5 cm fragments and dehydrated in an ethanol gradient of 75, 85, 95, 100, and 100% ethanol at a time interval of 20 min. After dehydration, each sample was incubated overnight in isoamyl acetate. The samples were then critical point dried, mounted on stubs, and coated with gold in a high-vacuum evaporation unit, followed by examination under a scanning electron microscope (FE-SEM-EDS, SU-8020, Hitachi, Tokyo, Japan) at 3.0 kV acceleration voltage.

Stomatal aperture in the abaxial epidermis was determined microscopically by measuring the width of the inner lips of a minimum of 60 stomata per treatment from photographs captured using Image-Pro Plus 6.0 software (Media Cybernetics Inc., Rockville, MD, USA). Stomatal density was determined by manual counting using the convention of excluding stomata overlapping the margins of the image and was converted to the number of stomata per mm² of abaxial leaf surface (Wen et al. 2014).

Malondialdehyde, soluble protein and ascorbic acid measurements

Fully expanded sun leaves were sampled for plant physiological measurements on three occasions: pre-anthesis (22 August), anthesis (12 September) and harvest (28 September). Five plants per chamber and one leaf per plant were sampled randomly. The leaves were wrapped in aluminum foil, frozen in liquid nitrogen, and stored in a –80 °C freezer before analysis.

Malondialdehyde (MDA) was determined by microplate spectrophotometer (Molecular Devices, USA) using 2-thiobarbituric acid-reactive metabolite (Feng et al. 2011b). MDA concentrations were calculated using the following equation: MDA concentration (μmol L⁻¹) = 6.45 (A₅₃₂ – A₆₀₀) – 0.56A₄₅₀, where A₄₅₀, A₅₃₂, and A₆₀₀ represent the absorbance of the reaction solution at 450, 532, and 600 nm, respectively. MDA levels (nmol g⁻¹) were then normalized to fresh leaf mass, as determined by weighing before extraction.

Soluble proteins were extracted by homogenizing 50 mg of leaf tissue in 5 mL of phosphate buffer (50 mM, pH 7.8) with a prechilled mortar and pestle. After centrifugation at 3000 *g* for 10 min at 4 °C, the absorbance of a mixture containing 1 mL of supernatant and 5 mL of Brilliant Blue G-250 at 595 nm was measured. Quantification of soluble proteins was carried out according to the Bradford (1976) method, with albumin bovine used as a standard.

Ascorbic acid was measured as described by Gillespie and Ainsworth (2007) but with a sample weight of 100 mg fresh tissue. Total and reduced ASA levels were calculated based on a standard curve prepared using L-ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA). The ASA redox state was indicated by the ratio of reduced to total ASA content.

Jasmonic acid determination

Jasmonic acid was extracted as reported previously (Pan et al. 2010), with minor modifications. Each 50 μ L extract sample was injected into a reverse-phase C₁₈ high-performance liquid chromatography (HPLC) column (4.6 mm $\times$ 5 cm, 1.8 μ m) for HPLC-ESI-MS/MS (AB Qtrap 5500) analysis. The HPLC-ESI-MS/MS settings were as follows: source, turbo spray; ion polarity, negative; needle voltage, -4500 V; source temperature, 600 °C; gas, nitrogen; curtain gas, 30 psi; nebulizing gas (GS1), 40 psi; focusing gas (GS2), 50 psi; interface heater, on; collision activated dissociation gas pressure, medium; and scan type, multiple reaction monitoring. The mobile phases included acetonitrile (A) and distilled water containing 0.1% acetic acid (B). Separations were performed by altering the percentage of B as follows: 90% B at 0–0.5 min, 95% B at 0.5–5 min and 90% B at 5–8 min to stabilize the instrument. JA concentration (ng mL^{-1}) = (concentration of internal standard $\times$ peak area of JA)/peak area of internal standard in that sample. JA levels (ng g^{-1}) were then normalized to fresh leaf mass, as determined by weighing before extraction.

Statistical analysis

Prior to analysis, the original data were checked for normality (one-sample Kolmogorov-Smirnov test) and homogeneity of variance. Plant growth and physiological parameters were measured for all or five plants as replicates within each chamber. That if there was a significant interaction between ozone and time, we used One-Way analysis of variance (ANOVA) to analyze the data for ozone effects within a time, followed by Fisher LSD post-hoc test. We also examined the relationship between LII and AOT40 using linear regression analysis. The statistical significance

was accepted at $P \leq 0.05$. Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Leaf injury

O₃-induced yellowing lesions first occurred between the veins on the upper surfaces of marigold leaves (Fig. 2a). After 4 days of O₃ exposure, leaf yellowing was detected on the bottom leaves under NF+120 treatment (Fig. 2b), with AOT40 of 6.4 ppm h (Table 1). After 12 days O₃ exposure, leaf yellowing was detected under NF+60 treatment with AOT40 of 8.6 ppm h (Table 1). Almost all foliar surfaces were covered with yellow lesions after 55 days of O₃ treatment (Figs. 3a, b). The percentage of injured leaves and the LII significantly increased under O₃ treatment (Figs. 3a, b) from the beginning of observation on 15 August, when the AOT40 was 2.6, 8.6 and 14.2 ppm h for NF, NF+60 and NF+120, respectively (Table 1). After 55 days of O₃ exposure, only 19.5% of the leaf area was affected under NF treatment, whereas NF+60 and NF+120 treatments produced injured leaf areas of 36.8 and 85.6%, respectively. A significant linear relationship was detected between LII and AOT40 based on observations made on the six sampling dates (Fig. 3c), suggesting that O₃ injury to marigold leaves increased with increasing O₃ exposure.

Photosynthesis, growth and biomass

Elevated O₃ exposure suppressed leaf net photosynthetic rate in marigold plants at the pre-anthesis and anthesis stages, with reductions of 34.1 and 16.5% under NF+60 treatment and 67.5 and 42.2% under NF+120 treatment, respectively (Fig. 4a). The relative increase in stem diameter was reduced by 16.7–30.7% under NF+60 treatment and 37.0–46.3% under NF+120 treatment (Fig. 4b); however, no significant changes were detected in the relative increase in plant height after elevated O₃ exposure (Fig. 4c). NF+60 treatment was sufficient to inhibit the increase in marigold biomass, with a 28.9% reduction detected by the end of the experiment. A greater reduction (40.4%) in biomass was detected under NF+120 treatment (Fig. 4d).

Stomatal traits and O₃ flux

Stomatal conductance and O₃ flux, as well as stomatal aperture and density, were analyzed at both pre-anthesis and anthesis (Fig. 5). Stomatal conductance and stomatal aperture showed different responses to O₃ stress, with a 27.2–67.4% decline in the former (Fig. 5a) and a 29.1–121.7% increase in the latter (Fig. 5b).

Fig. 2 Foliar (a) and whole-plant (b) injury in marigold under different ozone levels. Treatment abbreviations are as in Fig. 1

Fig. 3 The percentage of injured leaves (a), leaf injury index (LII, b), and relationship between LII and accumulated ozone exposure above a threshold of 40 ppb (AOT40, c) in marigold under different ozone levels. Treatment abbreviations are as in Fig. 1. Data in a and b are shown as mean $\pm$ standard error ($n = 20$). For each parameter, results

of one-way repeated measures ANOVA are reported, with asterisks showing the significance of factors (ozone and time) and their interaction for: *** $P \leq 0.001$. Different lowercase letters indicate significant differences between the ambient control (NF) and elevated O₃ treatments (NF+60 and NF+120) ($P \leq 0.5$)

Stomatal conductance exhibited stronger responses to higher O₃ exposure than to lower O₃ exposure, while aperture responses to both higher and lower O₃ exposure were not significantly different based on the statistical analysis. No significant difference was detected in stomatal density between NF and elevated O₃ treatments (Fig. 5c). The O₃ flux into leaves showed an increase of 28.0–104.8% under NF+60 treatment and 57.5–145.6% under NF+120 treatment, compared with the control (Fig. 5d).

Malondialdehyde and soluble protein contents

Elevated O₃ exposure accelerated membrane lipid peroxidation in marigold leaves, as indicated by an increase in MDA contents (Fig. 6a). After elevated O₃ exposure, the MDA contents at pre-anthesis, anthesis, and harvest significantly increased, with values of 83.7, 50.2, and 17.3% higher, respectively, under NF+60 treatment and 73.1, 50.1, and 21.4% higher, respectively, under NF+120 treatment. Elevated O₃ treatment

Fig. 4 Leaf photosynthetic rate (a, 10:00–12:00 am on 22 August and 12 September), plant growth expressed as percentage increase in stem diameter and plant height (b and c, 22 August, 5 September, 28 September, and 10 October), and biomass (d, 28 September) in marigold under different ozone levels. The reference for each measurement date of relative stem height and diameter was the value from the previous date. Treatment abbreviations are as in Fig. 1. Data are shown as mean $\pm$ standard error ($n_a = n_d = 5$, $n_b = n_c = 20$). For growth and photosynthetic parameters, results of one-way repeated measures ANOVA are reported, with asterisks showing the significance of factors (ozone and time) and their interaction for: *** $P \leq 0.001$, * $P \leq 0.05$, ns = $P > 0.05$. Different lowercase letters indicate significant differences between the ambient control (NF) and elevated O_3 treatments (NF+60 and NF+120) ($P \leq 0.5$)

also induced oxidative damage to soluble proteins in marigold leaves at both the pre-anthesis and anthesis stages, with a decrease of 11.2–12.6% in protein content under NF+60 treatment and 12.9–21.7% under NF+120 treatment (Fig. 6b).

Ascorbic acid system and jasmonic acid content

Elevated O_3 exposure promoted ASA synthesis and disturbed the ASA redox state (Fig. 7). The total ASA contents significantly increased throughout growth, with increases of 20.7–45.5% under NF+60 treatment and 41.2–66.7% under NF+120 treatment. However, the ASA redox state decreased, with 12.2–27.0% and 23.0–27.2% reductions under NF+60 and NF+120 treatment, respectively. Additionally, elevated O_3 exposure reduced JA content, with declines of 51.9–80.5% under NF+60 treatment and of 75.9–78.8% under NF+120 treatment compared with the control (Fig. 8).

Discussion

Foliar injury and depression of plant growth by elevated O_3

The foliar injury symptoms in plant species are of major interest for realistically assessing the risks imposed by O_3

(Furlan et al. 2007). In the current study, we found that elevated O_3 levels accelerated the occurrence of leaf yellowing in marigold plants, which was first observed on the fifth day under NF+120 treatment (AOT40 = 6.4 ppm h) and thirteenth day under NF+60 treatment (AOT40 = 8.6 ppm h). The results suggest that, in addition to AOT40 (Table 1), peak O_3 has contributed to the development of foliar injury symptoms in marigold plants. As previously reported, plants respond more strongly to exposure to a higher peak O_3 concentration (Musselman et al. 1983).

As a well-known pollutant, O_3 has a negative impact on plant biomass due to the reduction in photosynthesis caused by the direct effect on light or dark reaction (Power and Ashmore 2002), or the indirect effect of stomatal conductance (Noormets et al. 2001). In the present study, O_3 suppressed stomatal conductance, whereas the corresponding internal CO_2 concentration tended to increase or remained constant at the control level (271, 301, and 312 mmol mol⁻¹ under NF, NF+60 and NF+120 treatments, respectively, at pre-anthesis; 300, 298, and 302 mmol mol⁻¹, respectively, at anthesis). A similar result was reported in Monarch birch by Watanabe et al. (2014), who indicated that decreased stomatal conductance is a passive response of plants to lower carboxylation efficiency, in order to maintain the ratio of intercellular to ambient CO_2 concentration. We therefore speculate that increased O_3

Fig. 5 Stomatal conductance (a, 10:00 am–12:00 pm), stomatal aperture (b, 16:00 pm), stomatal density (c, 16:00 pm), and ozone flux (d, 10:00–12:00 pm) in leaves of marigold under different ozone levels.

Treatment abbreviations are as in Fig. 1. Data are shown as mean $\pm$ standard error ($n_a = n_d = 5$, $n_b = 60$, $n_c = 20$). For each parameter, results of one-way repeated measures ANOVA are reported, with asterisks showing the significance of factors (ozone and time) and their interaction for: *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, ns = $P > 0.05$. Different lowercase letters indicate significant differences between the ambient control (NF) and elevated O_3 treatments (NF+60 and NF+120) ($P \leq 0.5$)

Fig. 6 Leaf malondialdehyde (MDA; a) and soluble protein (b) contents in leaves of marigold under different ozone levels. Treatment abbreviations are as in Fig. 1. Data are shown as mean $\pm$ standard error ($n = 5$). For each parameter, results of one-way repeated measures ANOVA are reported, with asterisks showing the significance of factors (ozone and time) and their interaction for: *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, ns = $P > 0.05$. Different lowercase letters indicate significant differences between the ambient control (NF) and elevated O_3 treatments (NF+60 and NF+120) ($P \leq 0.5$)

concentration decreased photosynthesis in marigold leaves by inhibiting light or dark reaction (reduced capability to take advantage of CO_2), rather than CO_2 diffusion from the atmosphere to the leaves.

Both stem diameter and plant height are major components of shoot architecture and are associated with biomass yield (Dubouzet et al. 2013; Okello et al. 2001). However, in the present study, reductions in marigold biomass were

only accompanied by inhibited stem-diameter growth during the growing season. This result indicates that the reduction in stem diameter growth plays a more significant role in suppressing marigold biomass than changes in plant height. Previous work reported a similar response in poplar, Norway spruce, and European beech (single-stemmed plants) under high O_3 environment, where stem diameter was inhibited more easily because O_3 fumigation induced a

Fig. 7 Total ascorbic acid (ASA; **a**), reduced ASA (**b**) and the ratio of reduced to total ASA (**c**) in leaves of marigold under different ozone levels. Treatment abbreviations are as in Fig. 1. Data are shown as mean $\pm$ standard error ($n = 5$). For each parameter, results of one-way repeated measures ANOVA are reported, with asterisks showing the

significance of factors (ozone and time) and their interaction for: *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, ns = $P > 0.05$. Different lowercase letters indicate significant differences between ambient control (NF) and elevated O_3 treatments (NF+60 and NF+120) ($P \leq 0.5$)

Fig. 8 Leaf jasmonic acid (JA) content in leaves of marigold under different ozone levels. Treatment abbreviations are as in Fig. 1. Data are shown as mean $\pm$ standard error ($n = 5$). Results of one-way repeated measures ANOVA are reported, with asterisks showing the significance of factors (ozone and time) and their interaction for: *** $P \leq 0.001$. Different lowercase letters indicate significant differences between the ambient control (NF) and elevated O_3 treatments (NF+60 and NF+120) ($P \leq 0.5$)

shift in the resource allocation into height growth at the expense of diameter growth (Pretzsch et al. 2010; Carriero et al. 2015). O_3 -induced reductions in stem diameter could result in decreased stem stability and thus negatively affect the greening and ornamental values of marigold. Another important reason for the decline in marigold biomass is the increased percentage and severity of injured leaves (Fig. 3), which reduced the availability of healthy leaves for photosynthesis. Therefore, the increase in ground-level O_3 concentration not only affected the ornamental value of marigold, but it also led to a loss of potential ecological benefits by inhibiting the formation of biomass.

While the NF+120 treatment was a high O_3 exposure, and represented the 'worst case' O_3 exposure; in the present

study, plant growth responses to NF+120 treatment were consistent with that to NF+60 treatment, and more severe in the former than the latter.

Stomatal response to elevated O_3 exposure

In our experiment design, the responses of gas exchange and stomatal openness to O_3 stress were measured separately: stomatal conductance was measured at about 11:00 a.m. in the morning when peak photosynthetic rate occurred using Li-6400 photosynthetic analyst; stomatal aperture was destructively measured at about 16:00 p.m. in the afternoon when the stomatal responses to O_3 peak occurred.

Extensive studies have assessed the effects of O_3 on stomatal movement due to its irreplaceable role in determining the rate of O_3 uptake into leaves (Wilkinson and Davies 2010; Yamaguchi et al. 2014). Most reports indicate that O_3 induces stomatal closure to protect plants from further injury through reduced O_3 flux (Sun et al. 2014). Therefore, when guard cells are fully functional, the stomata can be regulated effectively to avoid O_3 injury (Musselman and Minnick 2000). In the present study, elevated O_3 treatment decreased the stomatal conductance of marigold leaves at both pre-anthesis and anthesis; this result is consistent with previous findings in deciduous trees, such as *Fagus crenata*, *Fraxinus*, *Populus*, and *Prunus species* (Hoshika et al. 2015; Wittig et al. 2007). However, the decline in stomatal conductance did not prevent injury to marigold by elevated O_3 , as shown by the significant decreases in biomass production and increased percentages of injured leaves.

The effects of O_3 on plants depend on the amount of O_3 entering the leaves and on the capacity of leaves for detoxification of O_3 (Hu et al. 2015; Musselman et al. 2006). POD_Y (phytotoxic O_3 dose over a threshold of Y),

an index based on F_{st} (stomatal uptake rate of O_3), has been widely used to indicate the critical levels of O_3 needed to affect crops (Gonzalez-Fernandez et al. 2010), trees (Hu et al. 2015) and herbs (Danielsson et al. 2013). In the present study, the O_3 flux into leaves increased under elevated O_3 treatments compared with the control (Fig. 5d). By analyzing the correlation of O_3 flux, O_3 concentration, and stomatal conductance in smoke tree, rose, and marigold (data not shown), we concluded that O_3 concentration, rather than stomatal conductance, is the predominant factor influencing O_3 flux into leaves. Therefore, O_3 -induced reduction in stomatal conductance is likely the result of plant physiological response to elevated O_3 exposure, but it is not an effective measure for reducing O_3 injury due to high O_3 concentrations (Sun et al. 2014).

Examination of stomatal ultrastructure showed that the stomatal aperture increased under elevated O_3 exposure. This increase may be attributed to the following factors: (1) higher soluble sugar contents under O_3 treatment (data not shown), as soluble sugar is a general osmolyte responsible for maintaining stomatal aperture (Lawson 2009); (2) inhibition of stomatal sensitivity to abscisic acid due to O_3 -induced ethylene production (Mills et al. 2009); (3) damage to the stomatal apparatus, as more O_3 entered the substomatal cavities, which could injure the parenchyma and adjacent epidermal cells, followed by loss of cytoplasm; further, these changes could cause cell collapse and the functional loss of stomata (Hassan et al. 1994). The increase in stomatal aperture is proportional to the increase in pore depth, resulting in a longer diffusion path and thus increasing stomatal resistance (decreasing stomatal conductance) (Franks and Beerling 2009; Monda et al. 2016). In addition to stomatal aperture, stomatal density is another key factor affecting stomatal conductance (Franks and Beerling 2009). However, in the current study, no significant changes were detected in stomatal density in marigold plants under elevated O_3 exposure. The above results suggest that, for “Moonsong Deep Orange”, stomatal conductance may have a more closer relationship with stomatal aperture than density.

Reduced ASA and its capacity to detoxify O_3 /ROS under elevated O_3 exposure

Stomata represent the first line of plant defense against O_3 damage. As stomatal regulation alone cannot prevent O_3 uptake, plants have developed an antioxidant system to alleviate O_3 injury, including enzymatic and non-enzymatic antioxidants (Castagna and Ranieri 2009). Among the non-enzymatic ones, ASA is considered to be a major soluble antioxidant in photosynthetic tissue and it is thought to minimize the initial formation of ROS by O_3 (ROS, e.g., hydrogen peroxide and superoxide) in the liquid phase of

apoplast (Chaparzadeh et al. 2004; Booker et al. 2012). Plöchl et al. (2000) found that leaf ASA concentration is inversely related to O_3 sensitivity. In the present study, although elevated O_3 treatment significantly increased the total ASA content and tended to increase the levels of reduced ASA, O_3 injury was not alleviated in marigold leaves (Fig. 3). One possible reason for this result is that additional O_3 was still present to attack the plasma membrane after reaction with ASA under elevated O_3 treatment.

According to the literatures, approximately 10% of the ASA pool is localized within the apoplastic space (Conklin and Barth 2004; Eltayeb et al. 2006; Horemans et al. 2000b; Vanacker et al. 1998). The ratio of reduced to total ASA in apoplast varies with plant species in a wide range of 5–87%, with an average of 45.1% (Booker et al. 2012; Luwe et al. 1993; Ranieri et al. 1996, 2000). If 0.2% of O_3 flux can be decomposed by 2 μ M reduced ASA as suggested by Booker et al. (2012), the amount of O_3 reacting with reduced ASA can be estimated by the equation: total ASA content $\times 10 \times 45\% \times (0.2\%/2 \times O_3 \text{ flux})$. Here, the O_3 flux represents the phytotoxic O_3 dose over a threshold of 1 (POD_1) on the two measurement dates (pre-anthesis and anthesis). Therefore, the residual O_3 level after reacting with ASA under NF, NF+60, and NF+120 treatment was approximately 3.1, 6.2, and 6.9 $mmol\ m^{-2}$ at pre-anthesis and 5.6, 9.1, and 8.5 $mmol\ m^{-2}$ at anthesis, respectively. Only 1.0–2.0% of the accumulated O_3 was reduced. Urbach et al. (1989) demonstrated that a major portion of the O_3 pool is broken down inside the plasmalemma, damaging the biomembrane. However, the residual O_3 amounts that may damage the plasmalemma were estimated based on the averaged percentage of the ratios of reduced ASA to total ASA in the apoplast from available reports (Booker et al. 2012; Luwe et al. 1993; Ranieri et al. 1996, 2000). In future studies, to accurately quantify the O_3 amount reacting with ASA, direct measurement of reduced ASA in apoplast of marigold leaves will be required. Other antioxidants, i.e. enzymes and phenolics (Booker et al. 2012), can also detoxify O_3 in protection against O_3 injury to the plasmalemma. Thus, additional research is needed to investigate the relationship between these antioxidants and O_3 injury.

The ASA redox state in O_3 -treated plants was disturbed, with 16.4–28.1 and 20.7–28.4% reductions detected under NF+60 and NF+120 treatments, respectively. This process disrupts the homeostasis of the ASA pool, indicating the presence of a sensitive mechanism for the response to O_3 stress (Horemans et al. 2000a). ASA flux across the plasmalemma is slower than O_3 flux across the stomata (Luwe et al. 1993), which may have led to an irreversible reaction between O_3 and other cellular components (e.g., biomembranes; Fig. 6) before ASA could detoxify O_3 (Luwe et al. 1993). The toxicity of O_3 is derived from the strong oxidizing capacity of both itself and its breakdown products,

ROS. After reacting with reduced ASA, residual O_3 and its breakdown products can come in contact with water, plasmalemma, or other cellular components, initiating subsequent cellular damage (Agrawal et al. 2002). In this study, we observed the effects of O_3 levels that exceeded detoxification capacity in marigold leaves after elevated O_3 exposure, even though the ASA contents were higher under elevated O_3 treatment compared with the control. Higher ROS production could occur in the presence of more residual O_3 . It should be noted that ROS are produced as a normal product of plant cellular metabolism. In the normal environment, there is an equilibrium between ROS production and reduction. On this occasion, ROS act as secondary messengers regulating plants development, i.e., stomatal closure (Yan et al. 2007), root gravitropism (Joo et al. 2001), and conferment of tolerance to abiotic and biotic stresses (Miller et al. 2008). Elevated O_3 exposure in the present study could lead to excessive production of ROS, exceeding the ROS-scavenging capacity of specific components and thus leading to injury to the plasma membrane. This process was documented by Singh et al. (2014), who found that the increase in H_2O_2 levels in stress-treated maize plants matched the increase in lipid peroxidation. Compared with the control, higher MDA levels appeared much earlier under elevated O_3 exposure in this study, suggesting accelerated and aggravated leaf senescence in O_3 -treated marigold plants. Senescence is a time-dependent stress response that occurs in all living organisms. Senescence can trigger the expression of autophagy genes and thus promote the autophagy, a critical pathway of protein degradation (Wang et al. 2013). Total protein concentrations decline in parallel with the senescence process (Wang et al. 2013). Additionally, the increase in lipid peroxidation can result in enhanced proteolysis (Moran et al. 1994). Therefore, soluble proteins are expected to degrade and their contents are expected to decline in marigold leaves under elevated O_3 treatment. Ribulose 1,5-bisphosphate carboxylase (RuBisCO), an enzyme catalyzing photosynthetic CO_2 fixation, accounts for half or more of the total soluble proteins, e.g., 50% (Lin et al. 2014) to 70% (Zheng et al. 2011b). The decline in soluble proteins indicates a possible decrease in RuBisCO, which was expected to inhibit photosynthesis after elevated O_3 exposure.

Reduction in endogenous jasmonic acid levels under elevated O_3 exposure and its implications for O_3 -induced injury

As stated above, Elevated O_3 exposure promoted plasmalemma peroxidation in marigold leaves, as expressed by increased MDA content. This suggests that the levels of accumulated ROS derived from O_3 stress exceeded the ROS-scavenging capacity of specific cellular components,

which could induce active production of additional ROS, called “second oxidative burst” (Burkey et al. 2006; Sawada et al. 2012). Increasing evidences indicate that ROS can affect plant growth, development, and productivity (Karuppanapandian et al. 2011). To limit ROS injury and to maintain growth, plants tend to increase their defenses against peroxidative stress by triggering the JA signaling pathway in response to plasmalemma peroxidation (Ren et al. 2015). In the present study, however, the JA contents decreased significantly in O_3 -treated marigold leaves, which is inconsistent with previous findings for other plants such as tomato and hybrid poplar (Koch et al. 2000; Ren et al. 2015). The decline in JA contents detected in the present study may be attributed to O_3 -induced chloroplast degradation (data not shown), as the chloroplast is the first site of JA biosynthesis (Bartoli et al. 2013). Oksanen et al. (2004) also reported that O_3 significantly reduces chloroplast size in a sensitive aspen clone due to H_2O_2 accumulation. The breakdown of chloroplasts implies that the JA biosynthesis pathway is blocked, and a decrease in JA levels is therefore expected.

Jasmonic acid is considered to function antagonistically with ethylene by inhibiting the O_3 -induced biosynthesis of ethylene due to the conjugation of JA to the ethylene precursor 1-amino cyclopropane-1-carboxylic acid (Wasternack 2007), further containing the spread of ethylene-induced cell death (Cho et al. 2011). The decrease in JA levels may increase the release of 1-amino cyclopropane-1-carboxylic acid and thus the chance of cell death. Therefore, severe plant injury due to O_3 stress was expected, as a reduction in endogenous JA levels contributed to the effects of O_3 stress in marigold plants. JA also affects the normal growth of plants by regulating vegetative storage proteins (Creelman and Mullet 1997). Decreases in JA content indicate that fewer nutrients (especially nitrogen) are provided by vegetative storage proteins for the biosynthesis of novel proteins associated with plant defense responses (Beardmore et al. 2000; Staswick et al. 1991). Consequently, the levels of defensive enzymes that function downstream of the JA pathway are reduced (Ren et al. 2015). Therefore, the decrease in the protective ability of JA in the present study is thought to be a possible indirect reason for O_3 -induced injury to marigold leaves. This hypothesis can be supported by a study in Arabidopsis (Rao et al. 2000), which showed that JA-insensitive and JA-biosynthesis blocked mutants develop larger lesions than the one with the capacity to synthesize JA.

Conclusions

The results show that elevated O_3 causes severe injury to ‘Moonsong Deep Orange’, as indicated by accelerated,

pronounced leaf yellowing, as well as a significant decline in biomass production. The investigated cultivar of marigold therefore is a good bioindicator for O₃. The response of marigold to elevated O₃ is driven by complex physiological mechanisms: (1) Despite a reduction in stomatal conductance, O₃ flux into leaves increased remarkably under elevated O₃ exposure. The reduction in stomatal conductance is likely the result of plant physiological response to elevated O₃ exposure, but it is not an effective measure for reducing O₃ injury due to high O₃ concentrations. (2) With increased O₃ flux entering leaves, there were no significant changes in the content of reduced ASA after elevated O₃ exposure. Thus, the existing reduced ASA may be insufficient to eliminate additional O₃ and/or its breakdown products ROS under elevated O₃ exposure. (3) Disturbed ASA redox state (decreased ratio of reduced to total ASA) provides a sensitive mechanism for the response to O₃ stress. (4) The protective effects of JA on plants may be weakened after elevated O₃ exposure due to decreased capacity of leaves to synthesize JA.

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Compliance with ethical standards

Conflict of interest Ning Yang declares that she has no conflict of interest. Xiaoke Wang declares that he has no conflict of interest. Feixiang Zheng declares that he has no conflict of interest. Yuanyuan Chen declares that she has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animal performed by any of authors.

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