



Ethylenediurea (EDU) spray effects on willows (*Salix sachalinensis* F. Schmid) grown in ambient or ozone-enriched air: implications for renewable biomass production

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Abstract Ground-level ozone (O_3) is a widespread air pollutant causing extensive injuries in plants. However, its effects on perennial energy crops remain poorly understood due to technical difficulties in cultivating fast-growing shrubs for biomass production under O_3 treatment on the field. Here we present the results of a two-year evaluation in the framework of which willow (*Salix sachalinensis* F. Schmid) shrubs were exposed to ambient (AOZ) or elevated (EOZ) O_3 in two successive growing seasons (2014, 2015) and treated with 0 (EDU0) or 400 mg L⁻¹ (EDU400) ethylenediurea spray in the second growing season. In 2014, EOZ altered the chemical composition of both top young and fallen leaves, and a novel mechanism of decreasing

Mg in fallen leaves while highly enriching it in young top leaves was revealed in shrubs exposed to EOZ. In 2015, EDU400 alleviated EOZ-induced decreases in leaf fresh mass to dry mass ratio (FM/DM) and leaf mass per area (LMA). While EDU400 protected against EOZ-induced suppression of the maximum rate at which leaves can fix carbon (A_{max}) in O_3 -asymptomatic leaves, it did not alleviate EOZ-induced suppression of the maximum rates of carboxylation (V_{Cmax}) and electron transport (J_{max}) and chlorophylls *a*, *b*, and *a + b* in the same type of leaves. In O_3 -symptomatic leaves, however, EDU400 alleviated EOZ-induced suppression of chlorophylls *a* and *a + b*, indicating different mode of action of EDU between O_3 -asymptomatic and O_3 -symptomatic leaves. Extensive herbivory occurred only in AOZ-exposed plants, leading to suppressed biomass production, while EOZ also led to a similar suppression of biomass production (EDU0 × EOZ vs. EDU400 × EOZ). In 2016, carry-over effects were also evaluated following cropping and transplantation into new ambient plots. Effects of EOZ in the preceding growing seasons extended to the third growing season in the form of suppressed ratoon biomass production, indicating carry-over effect of EOZ. Although EDU400 protected against EOZ-induced suppression of

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biomass production when applied in 2015, there was no carry-over effect of EDU in the absence of EDU treatment in 2016. The results of this study provide novel mechanistic understandings of O₃ and EDU modes of action and can enlighten cultivation of willow as energy crop.

Keywords Air pollution · Ethylenediurea (EDU) · Hormesis · Plant protection · Tropospheric ozone (O₃)

Introduction

Willows (Salicaceae) have been an integral part of the human societies since the ancient times due to their multiple uses, such as the use of their bark to alleviate pain (Vlachojannis et al. 2009) and the utilization of their wood to create willow coracles, which dates back to Herodotus in the fifth century BC (Karp et al. 2011). Nowadays, fast-growing willow (*Salix* spp.) clones are also cultivated in the framework of short-rotation forestry (Lumme and Tormala 1988), thus being important plants for cultivation as bioenergy crops throughout the world (Kopp et al. 2001; Adegbidi et al. 2001; Maruyama et al. 2002; Volk et al. 2006; Mola-Yudego and Pelkonen 2008; Mola-Yudego and González-Olabarria 2010; El Kasmioui and Ceulemans 2012; Guidi Nissim et al. 2013; Fabio et al. 2017; Nordborg et al. 2018; Kakuk et al. 2021). Willow short rotation coppice is either fertilized or unfertilized, although unfertilized cultivations may produce less biomass, and there is a trade-off between a high annual net energy yield and a high ratio of gross energy yield to total primary energy input (Dimitriou et al. 2011; Guidi Nissim et al. 2013; Nordborg et al. 2018). Willows are also important components of water-saturated ecosystems (e.g. rivers) and urban green spaces (e.g. parks with ponds) (Niiyama 1987; Agathokleous et al. 2016b). Hence, willows play versatile roles of both ecological/environmental and anthropocentric importance, and their cultivation may further increase in the light of the recent carbon-neutral targets for the mid. part of the current century.

Ground-level ozone (O₃) is a secondary pollutant whose formation closely depends upon the levels of primary precursor pollutants, mainly NO_x and volatile organic compounds (VOCs), under sunlight (Derstroff et al. 2017; Fenech et al. 2021; Liu et al. 2021; Querol et al. 2021). Due to its complex chemistry, the control of O₃ levels is challenging, and O₃ can remain at elevated concentrations even if the emissions of some O₃ precursors are highly decreased (Sicard et al. 2020; Querol et al. 2021; Sicard 2021). Being also affected by transboundary long-range transport (Gao et al. 2020; Erickson et al. 2020), which downplays national efforts to decrease O₃ concentrations, O₃ levels may remain elevated for several decades to come (Sicard et al. 2017). Such elevated O₃ levels can lead to inhibited photosynthesis

and suppressed woody biomass production and yields (Paoletti 2006; Karnosky et al. 2007a; Koike et al. 2013; Jolivet et al. 2016; Li et al. 2017; Cotrozzi 2021), suggesting increased risks to tree productivity (Sicard et al. 2017; Feng et al. 2019a; Proietti et al. 2021; Sacchelli et al. 2021). Therefore, it is important to develop methodologies to protect plants against elevated O₃-induced toxicity. However, it remains unknown whether elevated O₃ suppresses the biomass production of shrub/tree energy crops cultivated on the field with no root limitation due to pot effect, yet such a root limitation would influence the response of plants to gaseous treatments (Oksanen 2003; Karnosky et al. 2007b; Wang et al. 2013).

Because of the suitability of *Salix* species, considerable genetic engineering targeted the development of clones with improved traits making them advantageous for biomass production, nutrient fitters, phytoremediation, and stabilization of stream banks (Kopp et al. 2001; Adegbidi et al. 2001). Genetic improvement of willows for biofuels and bioenergy has attracted much attention in the recent years (Maruyama et al. 2002; Volk et al. 2006; Karp et al. 2011; Fabio et al. 2017). For example, new triploid hybrids even displayed yields that are 19–27% higher than commercial cultivars; yield is a major trait for genetic selection and biomass feedstock development (Fabio et al. 2017). However, for optimum cultivation as energy crops, breeding programs should consider that willows should be pest- and disease-free and their yield improvement should be achieved without increasing the demands for fertilizers and water (Karp et al. 2011). Therefore, innovative methodologies are needed to improve the performance of willows grown in an O₃-polluted atmosphere without adding more fertilizers.

Ethylenediurea (C₄H₁₀N₄O₂; hereafter EDU) is a chemical compound that is known for its efficacy to act as both antiozonant, i.e. protecting materials against O₃-induced damage, and phytoprotectant, i.e. protecting plants against O₃-induced toxicity (Manning et al. 2011). It has been applied in research programs as protectant against O₃-induced phytotoxicity for over 40 years, thus, it is the most widely studied substance for its capacity to protect a wide variety of plants against O₃-induced toxicity (Paoletti et al. 2009; Feng et al. 2010; Oksanen et al. 2013; Agathokleous et al. 2015, 2021a). As a hormesis-inducing agent (Agathokleous et al. 2021a), EDU has been found to improve diverse traits of structure and function, including photosynthesis, chlorophyll fluorescence, growth, and biomass and productivity (Singh et al. 2015; Agathokleous 2017; Tiwari 2017). However, the mechanisms explaining its mode of action as a protectant against O₃-induced toxicity vary among and within species, while the specific underpinning molecular/genetic mechanisms remain unclear (Paoletti et al. 2014; Agathokleous et al. 2015; Singh et al. 2015; Pandey et al. 2015, 2019; Jiang et al. 2018; Ashrafuzzaman et al.

2018). It also remains unknown whether EDU can improve the biomass production of energy-crop willows cultivated with no pot-imposed root limitation under elevated O_3 .

Salicaceous species are widely distributed in Northeast Asia, including Hokkaido, the northernmost of the four main islands of Japan (Agathokleous et al. 2016b). *Salix sachalinensis* F. Schmid is a major, fast-growing willow species in willow-occurring environments, such as across the floodplain of Hokkaido's Ishikari River where it is a broad niche species (Niiyama 1987). That *S. sachalinensis* grows in highly humid environments suggests a high potential for increased O_3 uptake due to relatively more open stomata, and *S. sachalinensis* saplings grown in pots were found susceptible to elevated O_3 (Agathokleous et al. 2016b, 2018). To this end, we became interested in revealing whether *S. sachalinensis* trees grown in communities and with no root limitation due to pot effect (planted directly on the ground) would be adversely affected by elevated O_3 . We also hypothesized that EDU can decrease O_3 -induced negative effects, thus offering a perspective for applying EDU to enhance *S. sachalinensis* biomass production in an O_3 -polluted atmosphere. This research consisted of three parts (Table 1). Ozone effect on nutrients other than C and N remains poorly understood in open field-grown shrubs/trees, especially for different types of leaves (Shi et al. 2017; Agathokleous et al. 2018; Shang et al. 2018). Hence, in the first part, we carried out an assessment of elevated O_3 effect on *S. sachalinensis* in the first growing season to evaluate chemical status (Mg, K, Ca, Mn, Fe, Ni, Al, Cr, P) in top and fallen leaves and identify mechanisms of elemental regulation that can potentially explain O_3 effects on plant physiology. In the second part, we evaluated whether EDU can protect against O_3 -induced phytotoxicity in the second growing season, i.e. after cuttings re-grew following the harvest of the shoots formed in the first growing season. In this part we also aimed at revealing underlying physiological mechanisms which

could potentially explain the so far obscure mode of action of EDU in protecting willow against O_3 (Agathokleous et al. 2018). In the third part, we became interested in investigating whether there are O_3 and EDU carry-over effects in the third growing season, following the harvest of the EDU- and O_3 -treated shoots formed in the second growing season and cultivation of cuttings in ambient air conditions. Carry-over effects would indicate potential existence of 'memory' in the newly-developed shoots exposed to ambient air conditions in the third growing season, even after the removal of the EDU- and O_3 -treated shoots formed in the second growing season. The results of this study provide a perspective to enhance biomass production of this important energy crop, especially considering that the cost of willow biomass is much higher than the cost of coal (Volk et al. 2006).

Materials and methods

Experimental site

The experiment was performed within the Sapporo Experimental Forest (43°0' N, 141°2' E, 15 m a.s.l.) of the Field Science Center of Northern Biosphere (FSC), Hokkaido University, Sapporo, Japan, during the growing seasons of 2014, 2015, and 2016. The snow-free period commonly lasts from late April to mid-November. Meteorological information was recorded at a nearby station (43°3.6' N 141°19.7' E) run and supervised by the Japan Meteorological Agency (<http://www.jma.go.jp/jma/indexe.html>). The averages (\pm indicates standard deviation for meteorological data only) of the main parameters data were calculated for the months April-September of the three years. The values of the monthly average, daily minimum, and daily maximum air temperature were $(17.1 \pm 0.1)^\circ\text{C}$, $(13.4 \pm 0.2)^\circ\text{C}$, and $(21.7 \pm 0.3)^\circ\text{C}$, respectively. The average values of the

Table 1 A synopsis of the experiment

Part	Year	Treatments	Response indicators
1	2014	Ambient or elevated ozone	SPAD, LMA, and nutrients (Mg, K, Ca, Mn, Fe, Ni, Al, Cr, P) in top and fallen leaves
	2015	Soon after snowmelt, before treatments, all the main branches but one developed in the previous year on each cutting were harvested	Shoot growth and biomass production
2	2015	Ambient or elevated ozone and no-ethylenediurea or ethylenediurea	Plant physiology (FM/DM, LMA, gas exchange in O_3 -asymptomatic leaves, photosynthetic pigments in both O_3 -symptomatic and -asymptomatic leaves), growth, biomass production, and foliage damage by insects
3	2016	None (carry-over assay) Note: all shoots were harvested, and cuttings with their root system were transplanted into new ambient sites for ratoon growth	Physiology (SPAD, N_{PPW} , LMA, and F_v/F_m), foliage damage caused by insects and number of insects grazing foliage, plant growth, and plant biomass

LMA stands for leaf mass per area and FM/DM for leaf fresh mass (FM) to dry mass (DM) ratio. NPPW indicates the estimated N content per leaf area, F_v/F_m the maximum efficiency of PSII photochemistry, and SPAD the leaf greenness

monthly total sunshine duration, relative humidity, and monthly precipitation were 194.7 ± 9.4 h, $67.6\% \pm 0.6\%$, and 107.0 ± 11.1 mm, respectively. Finally, the wind speed was 3.69 ± 0.19 m s⁻¹.

Plant material and cultivation within O₃ FACE system

Current-year cuttings of *S. sachalinensis*, originated from the river basin of Ebetsu city, were obtained from Hokkaido Horti-Tree Planting Center, Co. Ltd., and kept in a laboratory incubator (0–4 °C) for one month to break the dormancy. On 13 May, 2014, cuttings were planted in containers filled with commercial soil, and left on the field for rooting and establishment (under partial shading for the first two weeks). The soil substrate was a mixture (1:1) of Kanuma (well-weathered pumice) and Akadama (well-weathered volcanic ash), originated from Kanuma town, Tochigi prefecture (DCM Homac CO., LTD., Sapporo, JP). The characteristics of the soil substrate used in this study have been previously described in detail (Agathokleous et al. 2016b, 2021a). While the plants were rain-fed over the course of the experiment, on 1, 2 and 3 June, 2014, the willow cuttings were well irrigated due to abnormally high ambient air temperatures (reaching 34–37 °C). On 12 June, 2014, 72 uniform rooted cuttings were transplanted directly on the ground of the FACE plots (12 per FACE plot, 6 per soil type). At this stage, the 72 saplings had on average 4.4 ± 0.2 branches/shoots (\pm refers to standard error hereafter), 40.8 ± 1.8 leaves on all shoots, and 9.8 ± 0.3 leaves per shoot. The average shoot height was 12.6 ± 0.2 cm, while the average diameter at the base, middle, and upper part of the cutting was 19.4 ± 0.2 , 18.5 ± 0.2 and 18.5 ± 0.2 mm, respectively (measured in mm with an electronic caliber; 2-decimal accuracy). The plant-to-plant distance was 50 cm. In this mixed-species community grown in each plot, there were clusters of each species, i.e., plants of one species were not planted within clusters of other species. Species other than willow were grown in the same FACE plots for studies not related to this work (e.g., Agathokleous et al. 2016b, 2021a). In each plot, willow cuttings were planted in two soil types (3 cuttings per soil type), viz. brown forest soil (BF: Dystric Cambisols), which is native to the experimental field, and BF mixed (1:5 v/v) with immature volcanic ash plus pumice soil (VA-BF; Vitric Andosols). Further details of the experimental design, including the composition of the tree communities growing within the plots, and the soils can be found in supplementary materials of an earlier article (Agathokleous et al. 2021b).

On June 21, July 8, July 21, and August 25, 2014, the weeds in the plots were removed mechanically (by hand). Furthermore, on June 19, 2014, and May 7, 2015, plants were sprayed with insecticide due to high populations of insects grazing the foliage. The insecticide composition was

etofenprox 0.020% and water and surfactant etc. 99.98% (Earth Garden T, Earth Corporation, Chiyoda-ku, Tokyo, Japan) in 2014 and clothianidin 0.0080%, fenprothrin 0.010%, and water and surfactant etc. 99.982% (Benika-J spray, Sumitomo Chemical Garden Products Inc., Chuo-ku, Tokyo, Japan) in 2015. The insecticides were applied according to the manufacturer instructions. No other chemical or physical treatments were applied over the course of the experiments, except those described herein.

EDU treatments

EDU treatments were carried out in the second growing season (2015). After allocating 3 randomly-selected plants per soil type per plot to each EDU treatment (i.e. 6 plants per FACE plot per EDU treatment), plants were sprayed with water solution containing either 0 or 400 mg EDU L⁻¹ (no surfactant was included). An electric sprayer, which had two independent nozzles spraying at the same time, was used to spray both sides of leaf with fine mist based on Venturi effect. The EDU concentration tested was 400 mg L⁻¹ because it can be considered the upper limit of EDU concentrations applied in research programs with sufficient protection against O₃ phytotoxicity (Feng et al. 2010; Agathokleous 2017). Moreover, this concentration was found to offer sufficient protection against elevated O₃ to willows grown from the same cutting lot (Agathokleous et al. 2016b). EDU was applied as a foliar spray in the present study because it protected only when it was sprayed to plants (not when applied as soil drench) in the earlier experiment with plants grown in pots for one growing season (Agathokleous et al. 2016b). No more EDU was needed when applied as foliar spray to current-year willow plants grown in pots for one growing season, compared to soil drench (commonly given as 200 mL per plant), while the amount was even 2.3 times smaller for foliar spray when the plants had a small leaf area (Agathokleous et al. 2016b). EDU (100% a.i.) was prepared 30 min prior to each application by gently warming and continuous stirring on electric hotplates (Carnahan et al. 1978; Manning et al. 2011). The first EDU application took place on April 14, 2015, and repeated every 9 d on average, till early September, 2015; on rainy days EDU application was postponed to the next non-rainy day. Hence, EDU treatments were applied 17 times in total. EDU was applied every 9 d according to an abundant literature with successful results across various species, and because of its known persistence in the leaf apoplast (Paoletti et al. 2009; Agathokleous 2017).

O₃ treatments

The FSC FACE system with tree communities was used. This system consisted of 6 independent plots located at

different positions within the experimental forest; for details, see open-access information presented previously (Agathokleous et al. 2021b). Three plots were enriched with O₃ and served as elevated O₃ (EOZ) treatment plots, while the rest of the plots were not enriched with O₃, thus representing the ambient O₃ (AOZ) condition. The enrichment of the EOZ plots was active during the daytime (07:00–17:00, Japan Standard Time, JST). EOZ treatment was active from August 15 to October 26, 2014, and from April 24 to October 26, 2015; however, harvest took place on September 15, 2015, marking the end of the exposure to EOZ as only the main stem (cutting) was left. O₃ mixing ratios were logged at an 1-min interval using a TUV-1100 device (Tokyo Industries Inc. Tokyo, Japan). The average 10-h (07:00–17:00 Japan Standard Time, JST) O₃ levels of AOZ were 20.7 ± 5.5 and 34.7 ± 7.6 nmol mol⁻¹ in the growing seasons of 2014 and 2015, respectively. The average 10-h O₃ levels in the three EOZ plots were 65.7 ± 9.8, 61.7 ± 11.9, and 62.0 ± 11.5 nmol mol⁻¹ in 2014 and 71.5 ± 9.4, 76.0 ± 7.6, and 69.3 ± 10.5 nmol mol⁻¹ in 2015. Such elevated O₃ concentrations occur in wide areas of Japan and other regions of northeast Asia (Kume et al. 2009; Akimoto et al. 2015; Li et al. 2021), while the annual mean surface O₃ over Japan exhibits an increase of ≈2.7 nmol mol⁻¹ per decade (Nagashima et al. 2017). Further details about the O₃ exposures in the FACE system during 2014 and 2015 growing seasons have been described elsewhere (see supplementary materials of Agathokleous et al. (2021b)). The average 10-h ambient O₃ level in the growing season of 2016 (carry-over experiment) was 32.3 ± 5.7 nmol mol⁻¹ (Agathokleous et al. 2021a, b).

Treatments effect assessment

Ozone effects on leaf traits, as a function of soil, at the end of the first growing season (2014)

Mature and shed leaves were collected from 4 individuals of *S. sachalinensis* (1 per leaf type per plant) per soil type per FACE plot on 25 September, 2014. Mature (not senescing) leaves were collected from the base of the shoot, whereas the shoot was gently shaken and fallen leaves were collected. Leaf greenness (soil plant analysis development; SPAD) value was recorded as the average of two measurements per leaf (SPAD-502 m, Konica-Minolta, Osaka, Japan), and their leaf area was calculated using image analysis software; SPAD value positively correlates with chlorophyll *a* content in willow saplings of the same cutting source (Agathokleous et al. 2016a). Then, leaves were air-dried and their absolute dry mass was measured with an electronic, digital scale. Leaves were analyzed for main nutrients (Mg, K, Ca, Mn, Fe, Ni, Al, Cr, P) after digestion; the effect of O₃ on these nutrients remains poorly understood compared to foliar N

and C which have been extensively studied and well understood. The 50-mg leaf tissues (one sample per leaf type per plant per soil type per FACE plot) were digested with hydrogen peroxide and nitric acid, and element concentration analyses were carried out with an Inductive Coupled Plasma Mass Spectrometer (ICP-MS, IRIS/IRIS Advantage ICAP, Thermo Fisher Scientific Inc., MA, USA).

Ozone effects on plant growth and biomass production, as a function of soil, in early spring of the second growing season (2015)

Soon after snowmelt (4 May, 2015), all the main branches developed in the previous year on each stem/cutting, but the most vigorous, were harvested by cutting them at their base where they were attached to the main stem/cutting. There were on average 2–3 branches per stem/cutting, and each main branch represents a shoot with its own complex network of organs (e.g. branches of various orders/levels, buds, leaves). The most vigorous of the main branches was maintained for new growth to be able to manage EDU treatments and perform the experimentation in the second growing season. The length of each harvested branch was measured with a measuring tape with an 1-mm accuracy, while the lateral branches on each main branch were counted by visual observation. The basal diameter of each main branch was measured in mm (2-decimal accuracy) using an electronic caliber. Two cross-wise measurements were recorded, and the average value was calculated. The leaves were separated from the main branches (all wood, i.e. any-order branches), and the two types of organs (leaves and stems) were air-dried to constant mass in ovens. The average length of main shoots, number of lateral branches per main branch, dry mass of main branches, and diameter of main branches was calculated for each plant. Furthermore, the sum of dry masses of all main branches per plant was calculated as the total aboveground biomass.

EDU impact against O₃ effects on plant physiology, growth, and biomass production, as a function of soil, in the second growing season (2015)

To calculate LMA and leaf fresh mass (FM) to dry mass (DM) ratio (hereafter FM/DM), two randomly-selected mature leaves (from the base of the branch) were collected from each tree on 12 September, 2015, and their area and fresh and dry masses were measured (after air-drying at 60 °C to constant mass). The leaves were scanned (Canon LIDE 40, Tokyo, Japan), and the projected area was estimated (software LAI-32 v. 0.377e, Dr. K. Yamamoto, Graduate School of Bioagricultural Sciences, Nagoya University, Japan) after scanning the leaves.

Gas exchange measurements took place during morning hours (completed by noon) of days with ‘clean’ weather, from September 3–10, 2015, using LiCor instruments (LI-6400, Li-Cor Inc., Lincoln, NE, USA) measuring synchronously. Mature O_3 -asymptomatic leaves were randomly selected and subjected to 20-min acclimation to the chamber prior to analyzing A/Ci curve (the curve of net CO_2 assimilation rate, A, to intercellular CO_2 concentration, Ci). The measured leaves were collected and stored immediately in deep freeze to be used for photosynthetic pigments analysis. Gas exchange was measured in 1–3 plants per treatments combination (EDU and soil) per O_3 FACE plot, and LiCor instruments were rotated across plants of different treatment combinations and over time. Stomatal conductance (g_{s380}), net photosynthetic rate (A_{380}), and transpiration rate (E_{380}) were assessed at ambient CO_2 ($380 \mu\text{mol mol}^{-1}$). The leaf temperature was 25.5°C , the relative air humidity was 55–65%, the vapor pressure deficit (vpd) was 1.0, and the light saturated photosynthetic active photon flux density (PPFD) was $1500 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$. The A/Ci curve was established by changing the CO_2 level (12 levels) in the leaf chamber fluorometer, while maintaining constant temperature, PPFD, and relative humidity using the LI-6400 AutoProgram. Then, by means of a C3 plant biochemical model (Farquhar et al. 1980; Long and Bernacchi 2003), maximum rate of electron transport rate (J_{max}), maximum rate of carboxylation (V_{Cmax}), and net photosynthetic rate at saturated CO_2 of $1500 \mu\text{mol mol}^{-1}$ (A_{max}) were estimated.

Photosynthetic pigments were analyzed in both O_3 -asymptomatic and O_3 -symptomatic leaves. Ozone-asymptomatic mature leaves, which were collected after measured for gas exchange and stored in deep freeze, were used for pigment analyses. Regarding O_3 -symptomatic leaves, two randomly-collected mature leaves, with typical O_3 visible foliar injury in EOZ, were also collected from each tree per treatments combination per FACE plot in early September, and stored in deep freeze until further analyses. Two circular leaf disks (one per leaf; diameter = 1 cm) of standard area per plant were inserted in tubes containing 4 mL of DMSO and kept in an incubator (65°C) for 4 h, while shaking periodically (Shinano et al. 1996). Leaf extracts were assessed (GeneSpec III; Hitachi Genetic Systems; MiraiBio, Alameda, CA) instantaneously following incubation, and the absorbance was measured at 470, 543, 648, and 665 nm. Carotenoids were estimated according to the method of (Lichtenthaler 1987), using the recordings obtained at 543 and 470 nm. Chlorophyll *a*, chlorophyll *b*, and chlorophyll *a + b* were analyzed according to the method of Barnes et al. (1992) using the recordings obtained at the optical densities of 648 and 665 nm. The ratios of chlorophyll *a* to chlorophyll *b* and chlorophyll *a + b* to carotenoids were also calculated as indices of physiological stress.

On September 11, 2015, the height (in cm) of all the plants was measured with a measuring tape. The diameter of the shoot of each plant was also measured with a digital caliber (2-mm accuracy) as the average of two cross-wise measurements at the base of the shoot (old, lignified part).

The aboveground part of all plants was harvested on September 15, 2015; the initially planted cutting was left for regrowth in 2016. Harvested plants were separated into old shoot (developed in 2014; lignified), new shoot (developed in 2015; fresh/green), lateral branches, and leaves, and air-dried at 60°C to constant mass. The dry mass was measured with a digital balance with an accuracy of 1 mg. The total aboveground biomass was calculated as the sum of old shoot, new shoot, lateral branches, and foliage.

At harvest (before drying), all the leaves of each plant were placed next to each other with no overlapping, with the adaxial surface facing up, on a large white surface for contrast. The percentage of foliage (0–100% of total foliage area) damaged by insect grazing (mainly *Agelastica coerulea*) was estimated for each plant through visual observation.

EDU and O_3 carry-over effects following cutting re-growth in the third growing season (2016)

To evaluate potential carry-over effects of O_3 , soil, and EDU, the cuttings were transplanted from the FACE plots into three newly-created ambient plots scattered around the experimental forest after the snowmelt in 2016 (27 April). To excavate the root system with the cutting (left after the September, 2016, harvest), a circular area of soil surface, with a diameter of 20 cm, was marked around the cutting and excavated at a depth of 40 cm; the soil was gently removed from the root system. Rooted cuttings of each treatment combination (O_3 , EDU, soil) were randomly allocated to the three ambient plots while having cuttings from all FACE plot replicates, for all treatment combinations, in each new plot. Willow cuttings naturally re-grow by developing new shoots (main branches) following the harvest of the previous shoots, thus producing new biomass without needing to cultivate new cuttings. The distance from cutting to cutting was 50 cm, while a completely randomized experimental design was employed. The cuttings were irrigated immediately after transplantation, on May 19 and 21, and on August 12 due to high temperatures. No chemical treatments were applied over the course of the growing season.

Leaves morphological traits, chlorophyll (SPAD), and estimated N content per leaf area (N_{PPW}) were evaluated on September 18, 2016. Five mature sun-lit leaves were randomly sampled from each plant (all plants were evaluated). The fresh weight (g) was measured immediately. The length (cm) and width (cm, the widest point) of each leaf was measured with a ruler. The individual leaf area (g m^{-1} ; one-side projection) was estimated using the product of leaf width

and leaf length as predictor in the predictive model reported by Agathokleous et al. (2016a). From each leaf (all 5 leaves, all plants), the average value of 2 measurements was taken for SPAD (SPAD-502 m, Konica-Minolta, Osaka, Japan) and N_{ppw} (Agriexpert PPW-3000, Satake Corp., Hiroshima, Japan), which significantly correlates with measured N content in willows grown from the same cutting source (Agathokleous et al. 2016a). Leaves were air-dried to constant mass in an oven (60 °C). Fresh and dry mass of leaves was measured with an electronic balance with a 3-decimal accuracy.

Chlorophyll fluorescence was measured by a pulse amplitude modulation fluorometer (PAM, EM-FluorCam-800MF, Photon system inst., Drasov, Czech Republic) on September 26, 2016, from 08:30 to 12:00 (local time). One randomly-selected, sun-lit, mature leaf per plant was measured (all plants were evaluated). Each leaf was adapted in dark for 15 min by wrapping with aluminum foil before measurement. The minimum fluorescence (F_o) was measured using a low measuring modulated light, which is sufficient to induce significant variable fluorescence. Maximum fluorescence (F_m) was measured by applying a saturated light pulse. The light reaction centers were open during F_o measurement and closed during F_m measurements. The maximum efficiency of PSII photochemistry (F_v/F_m) was estimated (Sperdoui et al. 2021).

The percentage of insect damage of the foliage was ranked for each shoot (all plants) on October 8, 2016, as described earlier in the text, and the number of individual adult beetles (*A. coerulea*) grazing each shoot was counted for all plants.

All plants were harvested on October 8, 2016, and their growth and biomass were measured. The length and basal diameter of each shoot were measured with a measuring tape (cm; 1-decimal accuracy) and a digital caliper (mm; 2-decimal accuracy). The aboveground parts of all plants, which were developed in 2016, were air-dried (60 °C) to constant mass in ovens. The aboveground dry mass of each plant was measured with an electronic balance (g; 1-decimal accuracy).

For some of the data obtained in 2016, some of the plant treatments could not be identified due to technical errors (e.g., missing or unreadable plant ID number). Hence, these data were considered missing values and excluded from the calculations and data analyses. However, there were sufficient statistical units for all the analyses.

Data analyses

The level of statistical significance was defined at $\alpha = 0.05$. For datasets with more than one measurement per leaf and individual plant, data were averaged per leaf and plant. For statistical analyses, the average value of all measured

individual plants in each soil type and FACE plot was used, thus giving three real replicates to serve as statistical units. The data of all response variables were subjected to Box-Cox power transformation, as described previously (Agathokleous et al. 2016c). SPAD, LMA, and leaf nutrient data obtained in the first growing season (2014) and growth and biomass data obtained in early spring of the second growing season (2015) were analyzed with a General Linear Model (GLM) with Type III Sums of Squares (SS) attributable to an effect, which is calculated as the SS for the effect controlling for any effects of lower or equal degree and orthogonal to any effects (if any) of higher order interaction that contain it. The method for recoding categorical predictors used was the ‘overparameterized model’ for representing categorical predictor variables. Ozone and Soil were fixed factors and Plot was random factor. Physiological, growth, and biomass production data obtained later in the second growing season (September, 2015) were subjected to the same GLM model, but with EDU as an additional factor. Ozone, EDU, and Soil were fixed factors and Plot was random factor. Regarding EDU and O_3 carry-over effects following cutting re-growth in the third growing season (2016), Soil was excluded from the fixed factors to simplify the analysis because the previous statistical analyses of 2014 and 2015 data revealed no any important influence of soil on O_3 and EDU effects (see section Results). Hence, the data of 2015 were subjected to the same GLM analysis; however, with O_3 and EDU as fixed effect factors and FACE plot, soil, and ambient plot (created in 2016) as random effect factors. Since soil had no any important influence on O_3 and EDU effects (see section Results), the data specific to each soil are also not presented for the data of the second growing season ($O_3 \times EDU \times Soil$) to facilitate interpretation and presentation of results. For all GLM tests, multiple comparisons among means for significant main effects with more than two levels (groups) were evaluated with Bonferroni test. For elements content in top and fallen leaves exhibiting significant main effect of O_3 , associations between elements were evaluated with multiple linear regression using all raw (untransformed) data. To evaluate the effect magnitude of significant EOZ and EDU400 effects, the Cohen’s delta (δ) was computed as described by Agathokleous et al. (2016c), using raw (untransformed) data. The effect magnitude was categorized into neutral ($\delta = [0.00, 0.50)$), small ($\delta = [0.50, 1.50)$), moderate ($\delta = [1.50-3.00)$) or large ($\delta = 3.00 +$) (Cohen 1988; Agathokleous et al. 2016a). Calculations and data processing as well as statistical analyses were executed within EXCEL 2010 (Microsoft, Redmond, CA, USA) and STATISTICA (v.10, StatSoft, Tulsa, OK, USA). The effect size was computed using an available Excel application (Agathokleous and Saitanis 2020).

Results

Ozone effects on plant physiology, as a function of soil, in the first growing season (2014)

EOZ led to typical O₃ foliar visible injury of varied intensity in selected leaves of various ages (Fig. 1).

Regarding randomly-selected young, top leaves, neither O₃ nor soil was significant for SPAD (Fig. 2A) and LMA (Fig. 2B). Ozone was a significant main factor for Mg (Fig. 3A), Mn (Fig. 3B), Fe (Fig. 3C), Al (Fig. 3D), and Cr (Fig. 3E), but not for K (Fig. 4A), Ni (Fig. 4B), Ca (Fig. 4C), and P (Fig. 4D). In particular, EOZ led to 5.0, 1.4 and 1.8 times more Mg, Fe and Cr, respectively, and 1.5 and 1.5 times less Mn and Al, compared to AOZ (Fig. 3). The effect



Fig. 1 Ozone-induced visible injury on selected leaves of willow (*S. sachalinensis*) shrubs. The picture presents various degrees of injury on leaves of different levels of maturity. The shrubs were grown from cuttings in plots enriched with elevated ozone levels, in a free-air ozone-concentration enrichment (FACE) system. This observation took place at the end of the first growing season (2014). No such injuries were observed in shrubs grown in the ambient ozone plots. Photo credit: Evgenios Agathokleous

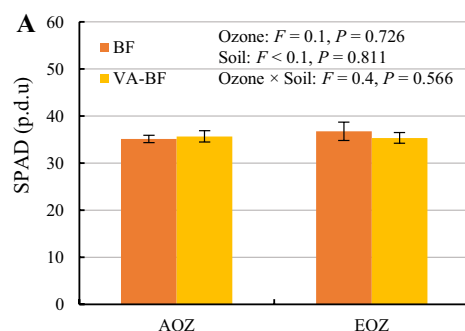
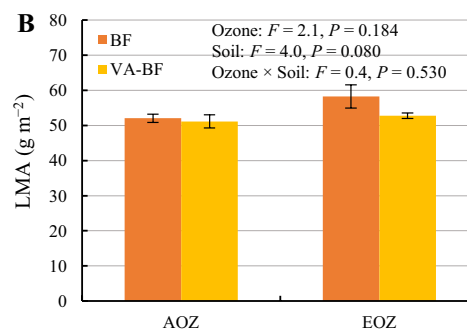


Fig. 2 Means (\pm se, $n=3$) of leaf greenness (SPAD) and leaf mass per area (LMA) in leaves of willow (*S. sachalinensis*) shrubs grown in brown forest (BF) or volcanic ash added to BF (VA-BF) soil and

of EOZ was moderate for Mn ($\delta=2.43$, CI [2.39, 2.45]), Fe ($\delta=1.64$, CI [1.59, 1.68]), Al ($\delta=1.97$, CI [1.95, 1.98]), and Cr ($\delta=2.26$, CI [2.24, 2.27]), and large for Mg ($\delta=4.49$, CI [2.78, 4.74]). Soil and O₃ × Soil interaction was non-significant for all the elements (Figs. 3, 4). Regarding the nutrients with a significant O₃ effect, Mg was significantly anticorrelated with Fe ($t=3.8$, $P<0.001$) and Al ($t=4.0$, $P<0.001$) and correlated with Cr ($t=5.2$, $P<0.001$). Mn was correlated with only Al ($t=4.5$, $P<0.001$). Fe was anticorrelated with Mg ($t=3.8$, $P<0.001$) and correlated with Cr ($t=3.3$, $P<0.01$). Al was anticorrelated with Mg ($t=4.0$, $P<0.001$) and correlated with Mn ($t=4.5$, $P<0.001$). Cr was correlated with Mg ($t=5.2$, $P<0.001$) and Fe ($t=3.3$, $P<0.01$).

Regarding randomly-selected fallen leaves, O₃ was a significant main factor for Mg (Fig. 5A), K (Fig. 5B), Mn (Fig. 5C), P (Fig. 5D), and Al (Fig. 5E) but not for Fe (Fig. 6A), Ca (Fig. 6B), Cr (Fig. 6C), and Ni (Fig. 6D). EOZ resulted in 1.3, 1.8, 2.4 and 1.5 times less Mg, Mn, Al, and P, respectively, and 1.2 times more K, compared with AOZ (Fig. 5). The EOZ effect was small for Mg ($\delta=1.41$, CI [1.19, 1.68]) and K ($\delta=1.12$, CI [-0.83, 3.82]), moderate for Mn ($\delta=1.97$, CI [1.87, 2.03]) and P ($\delta=1.86$, CI [1.26, 2.24]), and large for Al ($\delta=4.29$, CI [4.25, 4.31]). Soil and Ozone × Soil main effects were significant only for Fe and Cr (Fig. 6). Plants in VA-BF had 33.0% and 37.9% more Fe and Cr in fallen leaves relative to plants in BF. For both Fe and Cr, the only difference among means was between the means of BF and VA-BF in EOZ, i.e. plants grown in VA-BF had 78.5% and 65.7% more Fe and Cr in shed leaves, compared with plants grown in BF, in EOZ. According to correlation analysis of nutrients with a significant O₃ effect, Mg was anticorrelated with K ($t=4.0$, $P<0.001$) and Cr ($t=2.9$, $P<0.01$) and correlated with Fe ($t=2.9$, $P<0.01$) and Ni ($t=3.5$, $P<0.01$). Mn was significantly correlated only with Al ($t=4.0$, $P<0.01$) and Ca ($t=2.5$, $P<0.05$). Al was anticorrelated with Cr ($t=3.3$, $P<0.01$) and correlated with Mn ($t=4.0$, $P<0.001$), Fe ($t=3.1$, $P<0.01$), and Ni



exposed to either ambient (AOZ) or elevated (EOZ) ozone. Sampling was performed at the end of the first growing season (2014). Data were analyzed with a General Linear Model (GLM) at $\alpha=0.05$

Fig. 3 Means (\pm se, $n=3$) of elements content (with significant effects ozone) in top leaves of willow (*S. sachalinensis*) shrubs grown in brown forest (BF) or volcanic ash added to BF (VA-BF) soil and exposed to either ambient (AOZ) or elevated (EOZ) ozone. Sampling was performed at the end of the first growing season (2014). Data were analyzed with a General Linear Model (GLM) at $\alpha=0.05$

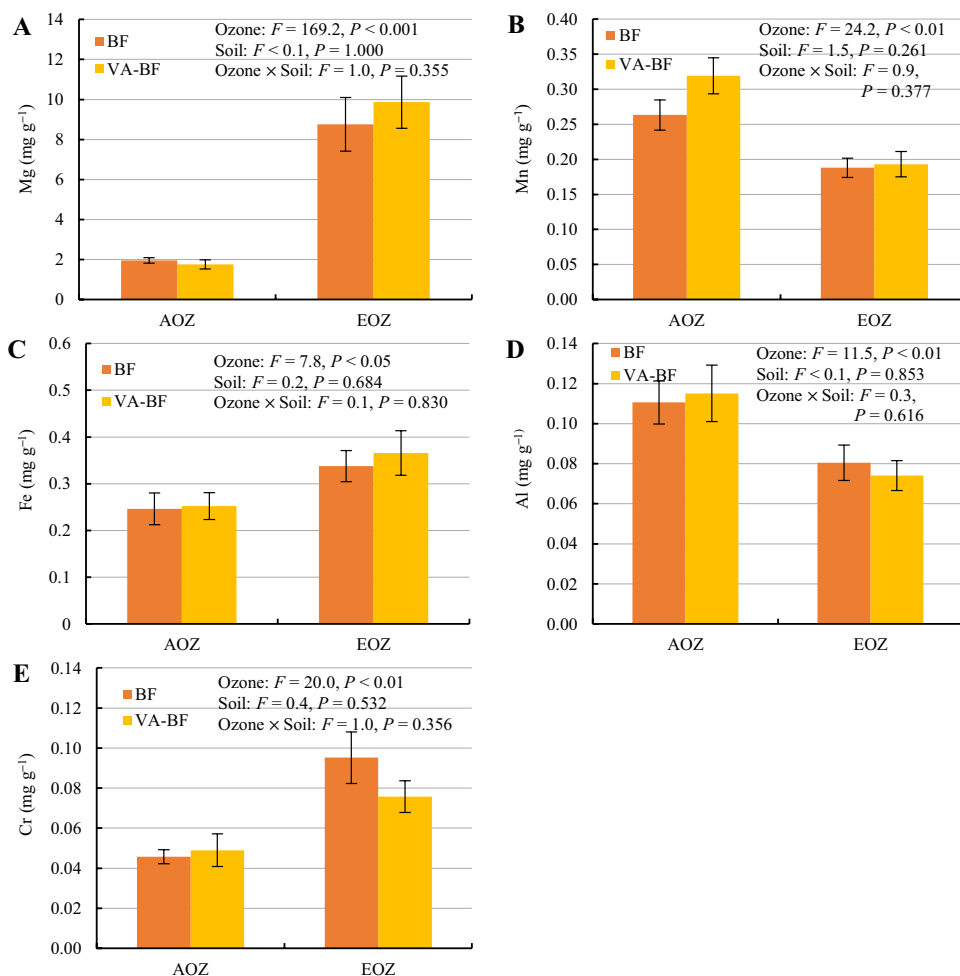


Fig. 4 Means (\pm se, $n=3$) of elements content (with non-significant effects of ozone) in top leaves of willow (*S. sachalinensis*) shrubs grown in brown forest (BF) or volcanic ash added to BF (VA-BF) soil and exposed to either ambient (AOZ) or elevated (EOZ) ozone. Sampling was performed at the end of the first growing season (2014). Data were analyzed with a General Linear Model (GLM) at $\alpha=0.05$

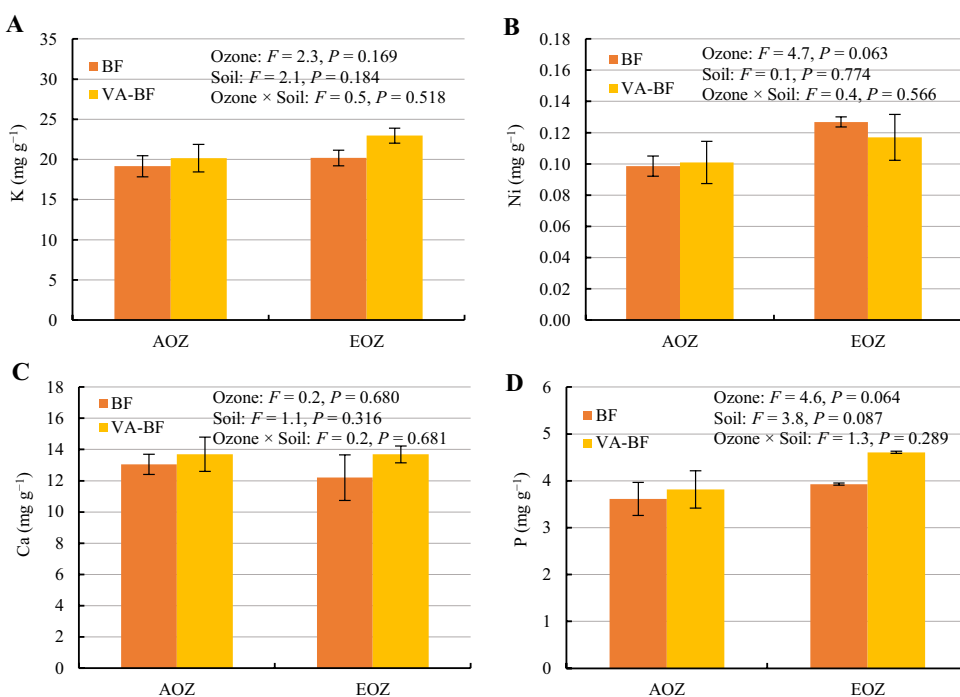


Fig. 5 Means (\pm se, $n=3$) of elements content (with significant effects of ozone) in fallen leaves of willow (*S. sachalinensis*) shrubs grown in brown forest (BF) or volcanic ash added to BF (VA-BF) soil and exposed to either ambient (AOZ) or elevated (EOZ) ozone. Sampling was performed at the end of the first growing season (2014). Data were analyzed with a General Linear Model (GLM) at $\alpha=0.05$

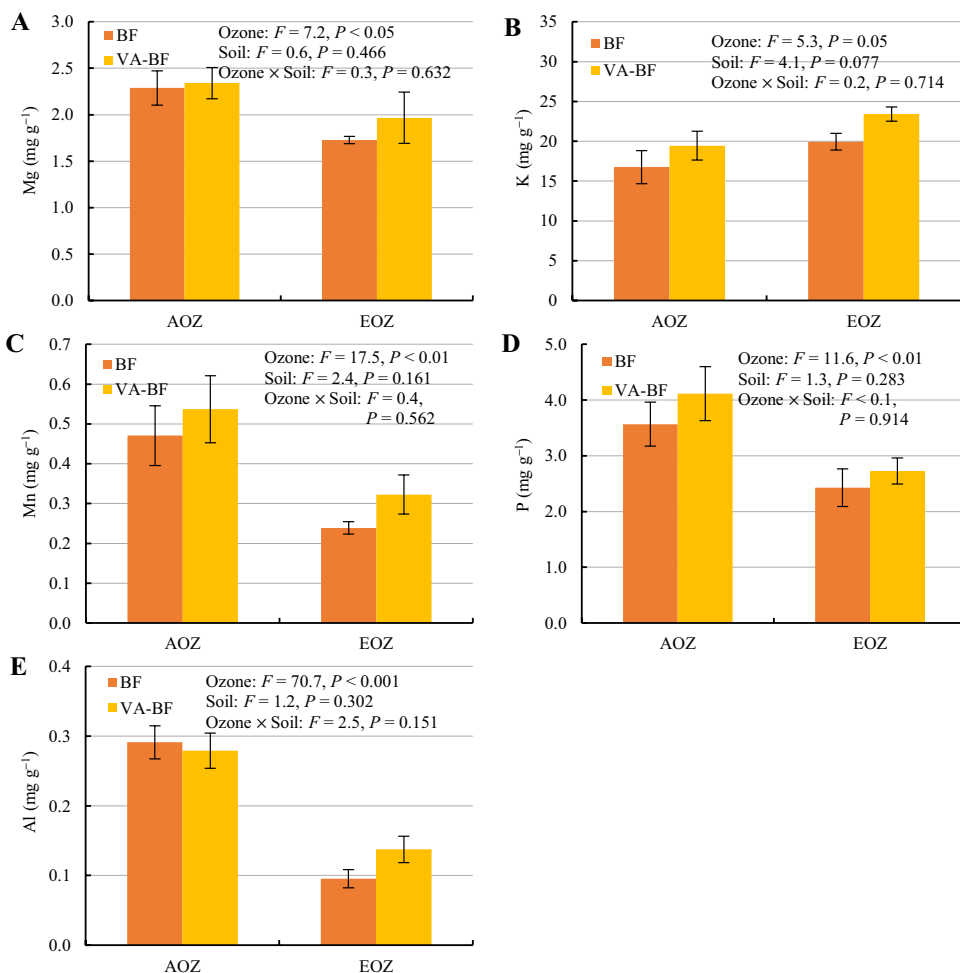
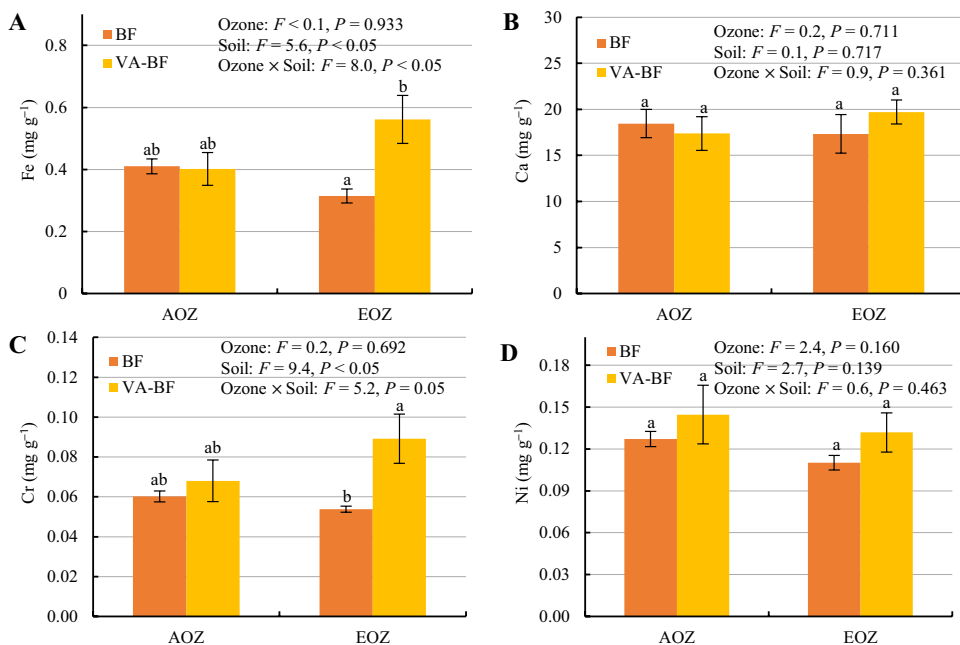


Fig. 6 Means (\pm se, $n=3$) of elements content (with non-significant effects of ozone) in fallen leaves of willow (*S. sachalinensis*) shrubs grown in brown forest (BF) or volcanic ash added to BF (VA-BF) soil and exposed to either ambient (AOZ) or elevated (EOZ) ozone. Sampling was performed at the end of the first growing season (2014). Data were analyzed with a General Linear Model (GLM) at $\alpha=0.05$. For elements exhibiting a significant Ozone \times Soil interaction, different letters above means indicate statistically significant differences between means based on Bonferroni post-hoc test ($\alpha=0.05$)



($t=2.9$, $P<0.01$). The only significant association of K was the anticorrelation with Mg ($t=4.0$, $P<0.001$). P was correlated with Ni ($t=3.4$, $P<0.01$) and anticorrelated with Ca ($t=2.2$, $P<0.05$) and Cr ($t=2.1$, $P<0.05$).

Ozone effects on plant growth and biomass production, as a function of soil, in early spring of the second growing season (2015)

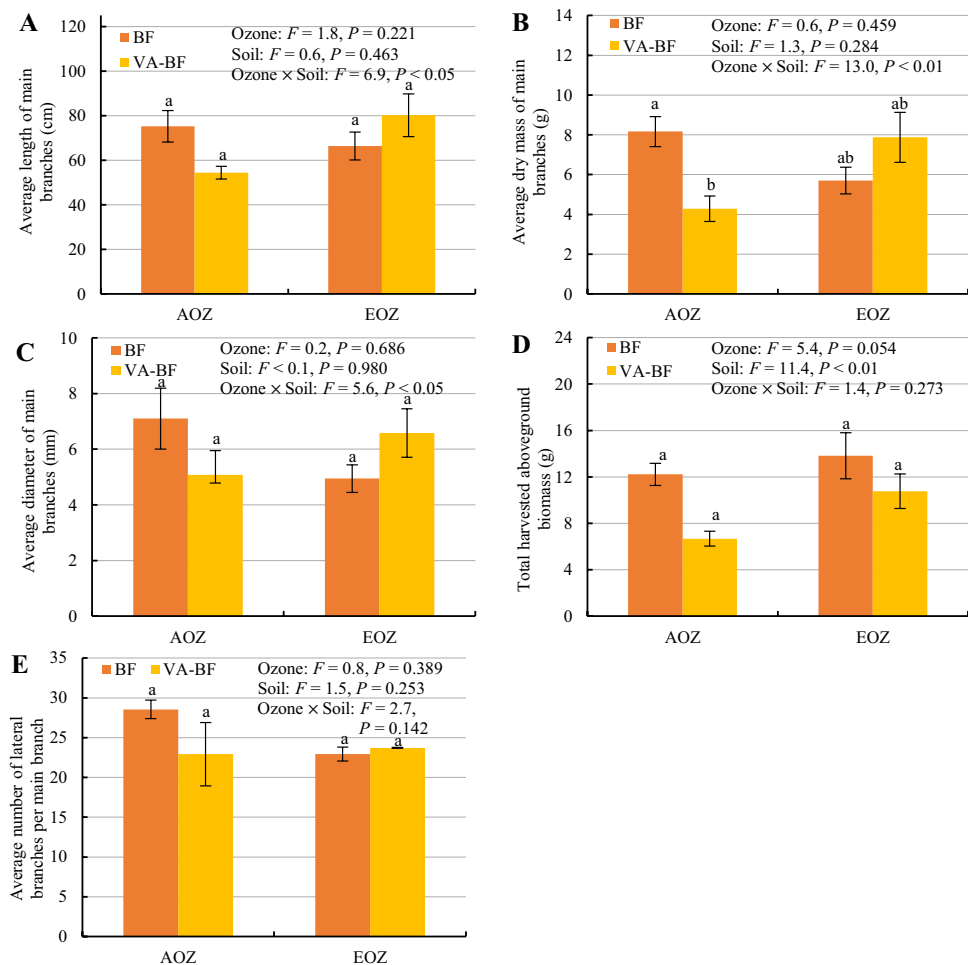
Ozone was a significant main factor for only total harvested aboveground biomass (Fig. 7D), where plants exposed to EOZ had 30.1% greater mean value than plants exposed to AOZ, an effect that was small in magnitude ($\delta=0.81$, CI [-1.75, 3.44]). Ozone did not have a significant effect on traits at the level of average main branch, viz. length (Fig. 7A), dry mass (Fig. 7B), basal diameter (Fig. 7C), and number of lateral branches (Fig. 7E). Soil was a significant main factor for only total harvested aboveground biomass (Fig. 7D). In particular, plants grown in BF soil had 49.4% higher total harvested aboveground biomass than plants grown in VA-BF soil. Ozone \times Soil interaction was significant for the average length, diameter, and dry mass of main

branches. However, according to the 'strict' Bonferroni comparisons, there were no significant differences among means for average length and diameter of main branches. Regarding the average dry mass of main branches, the only significant difference among means was between plants grown in BF soil (83.1% higher) and plants grown in VA-BF soil in AOZ.

EDU impact against O₃ effects on plant physiology, growth, and biomass production, as a function of soil, in the second growing season (2015)

Regarding FM/DM (Fig. 8A) and LMA (Fig. 8B), the only significant factors were EDU and O₃ \times EDU interaction. Plants treated with EDU400 had 7.7% higher mean value of FM/DM (small effect; $\delta=1.11$, CI [1.02, 1.19]) and 10.0% lower mean value of LMA (small effect; $\delta=0.99$, CI [-2.34, 5.48]) compared with plants treated with EDU0 (O₃ and soil treatments pooled). An examination of the O₃ \times EDU interaction revealed that the only significant difference among means was between EDU0 and EDU400 within EOZ, for both FM/DM and LMA. In particular, leaves treated with EDU400 had 16.9% greater FM/DM

Fig. 7 Means (\pm se, $n=3$) of plant growth and biomass production of willow (*S. sachalinensis*) shrubs grown in brown forest (BF) or volcanic ash added to BF (VA-BF) soil and exposed to either ambient (AOZ) or elevated (EOZ) ozone. Sampling was performed after snowmelt, in early spring of the second growing season (2015). Data were analyzed with a General Linear Model (GLM) at $\alpha=0.05$. For traits exhibiting a significant Ozone \times Soil interaction, different letters above means indicate statistically significant differences between means based on Bonferroni post-hoc test ($\alpha=0.05$)



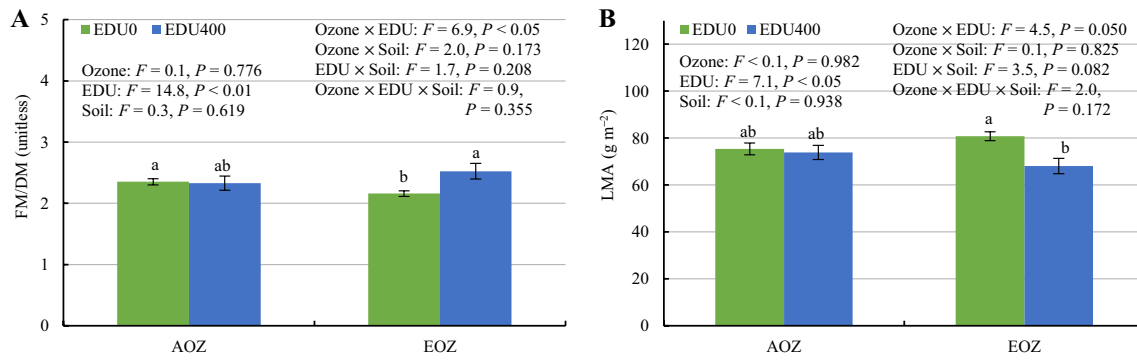


Fig. 8 Means (\pm se, $n=6$) of fresh mass (FM) to dry mass (DM) ratio (FM/DM) and leaf mass per area (LMA) of mature leaves of willow (*S. sachalinensis*) shrubs grown in brown forest (BF) or volcanic ash added to BF (VA-BF) soil, treated with 0 (EDU0) or 400 (EDU400) mg EDU L⁻¹ every 9 d, and exposed to either ambient (AOZ) or elevated (EOZ) ozone. Sampling was performed in early September of the second growing season (2015). Data were analyzed

value (moderate effect; $\delta=2.53$, CI [2.41, 2.63]) and 18.6% smaller LMA value (moderate effect; $\delta=1.81$, CI [-1.87, 8.13]), compared with leaves treated with EDU0, in EOZ (Fig. 8).

Treatments effects on gas exchange parameters were minimal in O₃-asymptomatic leaves (Fig. 9). Ozone was a significant factor for only A_{max} , for which a 16.1% lower mean was observed in EOZ-exposed plants, relatively to AOZ-exposed plants (Fig. 9F). The effect of EOZ on A_{max} was small ($\delta=0.81$, CI [-1.49, 4.46]). EDU had a significant main effect on only A_{380} (Fig. 9A) and E_{380} (Fig. 9E), which were increased (18.4%) and decreased (20.8%) by EDU400, compared to EDU0 (Fig. 9), effects that were small (A_{380} : $\delta=0.82$, CI [-1.11, 3.09]; E_{380} : $\delta=0.68$, CI [0.07, 1.40]). Soil had a significant main effect on g_{s380} (Fig. 9C) and E_{380} (Fig. 9E). Plants grown in BF (g_{s380} : mean = 0.53 ± 0.025 mol m⁻² s⁻¹; E_{380} : mean = 0.46 ± 0.029 mmol m⁻² s⁻¹) had 16.4% and 41.8% higher g_{s380} and E_{380} than plants grown in VA-BF (g_{s380} : mean = 5.2 ± 0.23 mol m⁻² s⁻¹; E_{380} : mean = 3.7 ± 0.33 mmol m⁻² s⁻¹). The only significant interaction was that of O₃ × EDU for A_{max} (Fig. 9F), which occurred due to 33.2%, 36.4%, and 34.3% lower A_{max} in EDU0-EOZ plants than in plants of EDU400-EOZ ($\delta=1.45$, CI [-1.47, 6.47]; small effect), EDU0-AOZ ($\delta=1.40$, CI [-2.81, 6.43]; small effect), and EDU400-AOZ ($\delta=1.57$, CI [-0.78, 6.59]; moderate effect), respectively.

Apart from O₃, no other factor or factor interactions were significant for photosynthetic pigment traits in O₃-asymptomatic leaves (Fig. 10). EOZ significantly decreased chlorophyll *a* (23.0%; Fig. 10A), chlorophyll *b* (23.9%; Fig. 10C), chlorophyll *a* + *b* (23.3%; Fig. 10E), and chlorophyll to carotenoid ratio (12.1%; Fig. 10F), compared to AOZ, effects that were small ($\delta=0.89$, CI [-3.60, 4.00]), small ($\delta=0.97$, CI [-0.58, 1.94]), small ($\delta=0.89$,

with a General Linear Model (GLM) at $\alpha=0.05$. For traits exhibiting a significant Ozone × EDU interaction, different letters above means indicate statistically significant differences between means based on Bonferroni post-hoc test ($\alpha=0.05$). Since soil had no any important influence on O₃ and EDU effects, the data specific to each soil are not presented for the data of the second growing season to facilitate interpretation and presentation of results

CI [-5.02, 5.25]), and small ($\delta=0.84$, CI [-0.07, 2.04]), respectively.

Ozone had a significant main effect on all photosynthetic pigment traits, except chlorophyll *a* to *b* ratio (Fig. 11D), in O₃-symptomatic leaves. Specifically, EOZ inhibited chlorophyll *a* (Fig. 11A), chlorophyll *b* (Fig. 11C), chlorophyll *a* + *b* (Fig. 11E), carotenoid (Fig. 11B), and chlorophyll to carotenoid ratio (Fig. 11F) by 54.0%, 47.1%, 52.0%, 23.7%, and 26.4%, respectively, compared to AOZ. The effect of EOZ on chlorophyll *a*, chlorophyll *b*, chlorophyll *a* + *b*, carotenoid, and chlorophyll to carotenoid ratio was moderate ($\delta=2.03$, CI [0.51, 5.54]), moderate ($\delta=2.15$, CI [1.44, 3.21]), moderate ($\delta=2.13$, CI [-0.02, 6.57]), small close to moderate ($\delta=1.48$, CI [0.79, 2.17]), and small ($\delta=1.33$, CI [0.90, 2.12]), respectively. EDU had a significant main effect on chlorophyll *a* (Fig. 11A), chlorophyll *a* + *b* (Fig. 11E), and chlorophyll *a* to *b* ratio (Fig. 11D), which were 22.6%, 20.0%, and 12.4% higher in EDU400-treated plants, compared to EDU0-treated plants, respectively. The EDU400 effect on chlorophyll *a* ($\delta=0.70$, CI [-1.88, 5.34]), chlorophyll *a* + *b* ($\delta=0.65$, CI [-2.82, 6.83]), and chlorophyll *a* to *b* ratio ($\delta=0.98$, CI [0.84, 1.16]) was small. Ozone × EDU interaction was significant for all pigment traits except carotenoid and chlorophyll *a* to *b* ratio (Fig. 11D). For chlorophyll *a* (Fig. 11A) and chlorophyll *a* + *b* (Fig. 11E), the means of EDU0 × EOZ group were 1.6 and 1.6, 2.0 and 1.9, and 2.1 and 2.0 times smaller than the means of EDU400 × EOZ, EDU0 × AOZ, and EDU400 × AOZ, respectively, effects that were moderate, large, and large, respectively. The mean of EDU400 × EOZ did not significantly differ from the means of EDU0 × AOZ and EDU400 × AOZ for both chlorophyll *a* and chlorophyll *a* + *b*. Regarding chlorophyll *b* (Fig. 11C) and chlorophyll to carotenoid ratio (Fig. 11F),

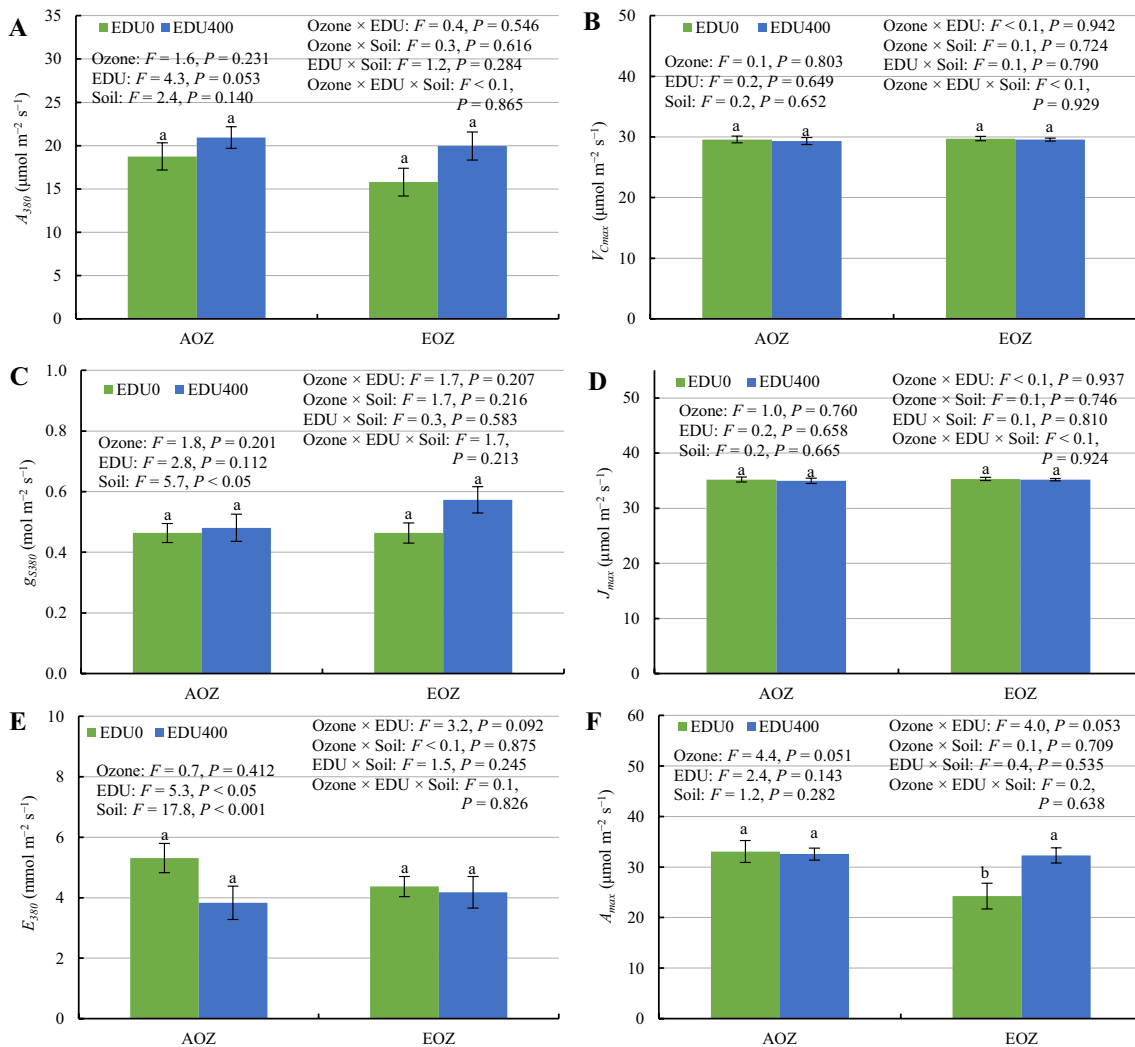


Fig. 9 Means (\pm se, $n=6$) of gas exchange parameters of mature O_3 -asymptomatic leaves of willow (*S. sachalinensis*) shrubs grown in brown forest (BF) or volcanic ash added to BF (VA-BF) soil, treated with 0 (EDU0) or 400 (EDU400) mg EDU L^{-1} every 9 d, and exposed to either ambient (AOZ) or elevated (EOZ) ozone. The parameters were net photosynthetic rate (A_{380}), stomatal conductance (g_{S380}), transpiration rate (E_{380}), maximum rate of electron transport rate (J_{max}), maximum rate of carboxylation ($V_{c_{max}}$), and net photo-

synthetic rate at saturated CO_2 of 1500 $\mu\text{mol mol}^{-1}$ (A_{max}). Measurements were performed in early September of the second growing season (2015). Data were analyzed with a General Linear Model (GLM) at $\alpha=0.05$. For traits exhibiting a significant Ozone \times EDU interaction (only A_{max}), different letters above means indicate statistically significant differences between means based on Bonferroni post-hoc test ($\alpha=0.05$)

the only significant differences in the multiple comparisons were between EDU0 \times EOZ group and EDU0 \times AOZ (1.8 and 1.5 times higher) and EDU400 \times AOZ (1.8 and 1.4 times higher). The two pairs of means displayed a difference of large magnitude for chlorophyll *b* and of moderate magnitude for chlorophyll to carotenoid ratio. Soil and interactions other than $\text{O}_3 \times \text{EDU}$ were non-significant for all the traits (Fig. 11).

Apart from a significant effect of O_3 on plant height, there was no other significant main effect of single factors or interactions on plant height (Fig. 12A) and shoot basal diameter (Fig. 12B) at the end of the second growing season.

EOZ-exposed plants were 16.6% taller than AOZ-exposed plants, an effect that was small ($\delta=0.99$, CI [-15.34, 28.25]).

Regarding biomass production, O_3 main effect was significant only for old shoot dry mass (Fig. 13A) and total aboveground dry mass (Fig. 13D). Specifically, EOZ-exposed plants had 25.0% greater old shoot dry mass ($\delta=1.03$, CI [-14.79, 14.03]) and 14.3% greater total aboveground dry mass ($\delta=0.73$, CI [-29.41, 22.76]), effects that were small in magnitude. EDU main effect was significant only for new shoot dry mass (Fig. 13C) and total aboveground dry mass (Fig. 13D), which were

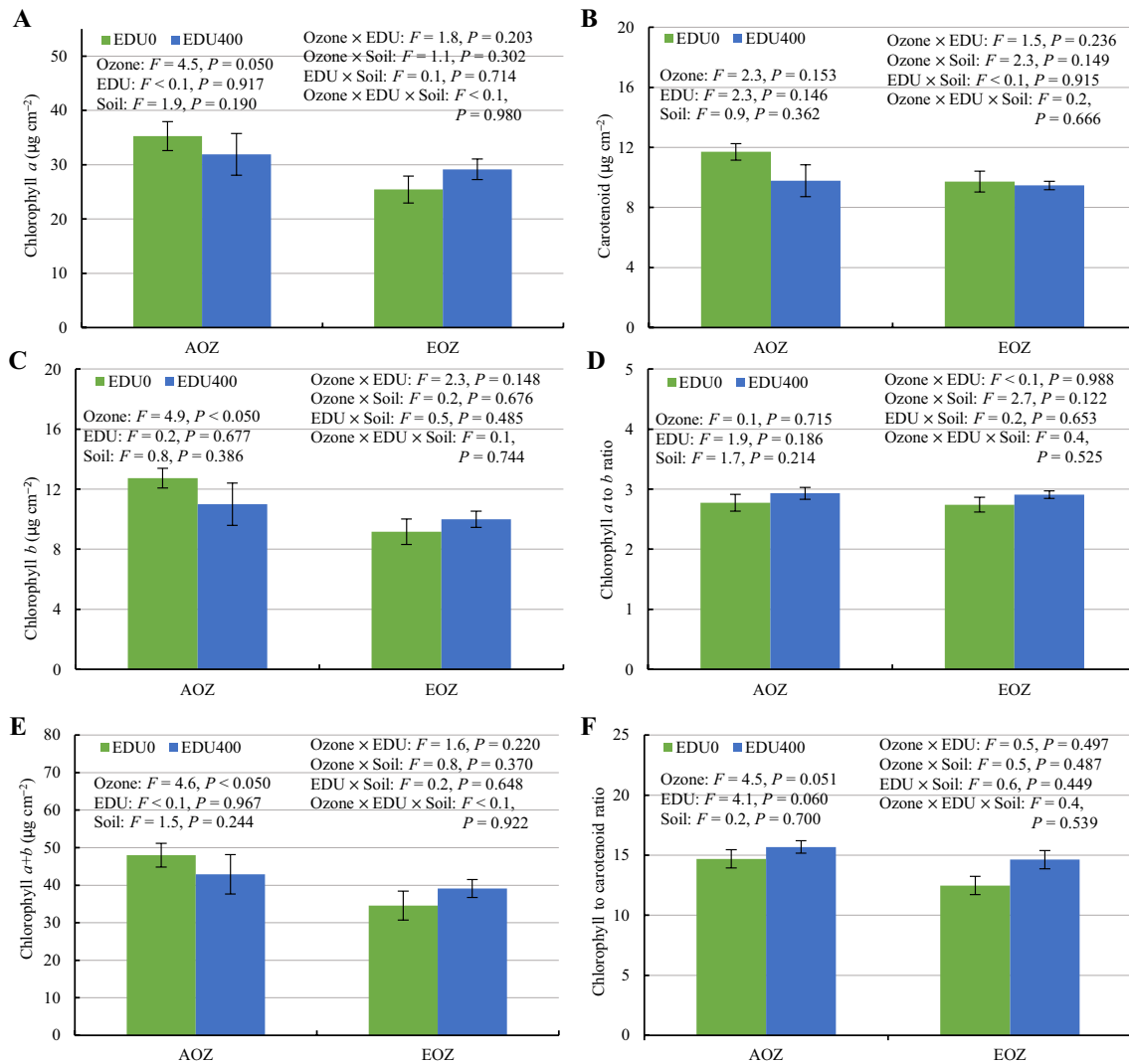


Fig. 10 Means (\pm se, $n=6$) of photosynthetic pigments of mature O_3 -asymptomatic leaves of willow (*S. sachalinensis*) shrubs grown in brown forest (BF) or volcanic ash added to BF (VA-BF) soil, treated with 0 (EDU0) or 400 (EDU400) mg EDU L^{-1} every 9 d, and

exposed to either ambient (AOZ) or elevated (EOZ) ozone. Sampling was performed after gas exchange measurements in early September of the second growing season (2015). Data were analyzed with a General Linear Model (GLM) at $\alpha=0.05$

28.6 and 13.9% greater in EDU400-treated plants than in EDU0-treated plants. The effect of EDU400 on new shoot dry mass ($\delta=1.22$, CI $[-4.60, 5.51]$) and total aboveground dry mass ($\delta=0.71$, CI $[-30.03, 22.16]$) was small. Soil was a significant main factor for new shoot dry mass only (Fig. 13C), with an 18.3% higher mean for plants grown in VA-BF (49.4 ± 2.4 g), relative to plants grown in BF (41.8 ± 3.3 g). Ozone \times EDU interaction was significant for lateral branches dry mass (Fig. 13E), foliage dry mass (Fig. 13B), and total aboveground dry mass (Fig. 13D). However, there was no significant difference among means of lateral branches dry mass with the strict Bonferroni test. Regarding foliage dry mass, the only significant difference observed was a 36.1% higher mean of EDU400-treated plants compared to EDU0-treated

plants in EOZ, an effect that was moderate (Fig. 13B). Regarding total aboveground dry mass (Fig. 13D), the mean of EDU400 \times EOZ group was 29.9%, 27.9%, and 30.4% greater than the means of EDU0 \times EOZ (moderate effect), EDU0 \times AOZ (small near moderate effect), and EDU400 \times AOZ (moderate effect) groups, respectively. Ozone \times Soil interaction was significant for lateral branches dry mass (Fig. 13E); however, there was no significant difference among means with the strict Bonferroni. EDU \times Soil interaction was a non-significant factor for all the traits. Ozone \times EDU \times Soil interaction was significant for new shoot dry mass (Fig. 13C), lateral branches dry mass (Fig. 13E), and total aboveground dry mass (Fig. 13D). However, there was no significant difference among means for lateral branches dry mass with the strict

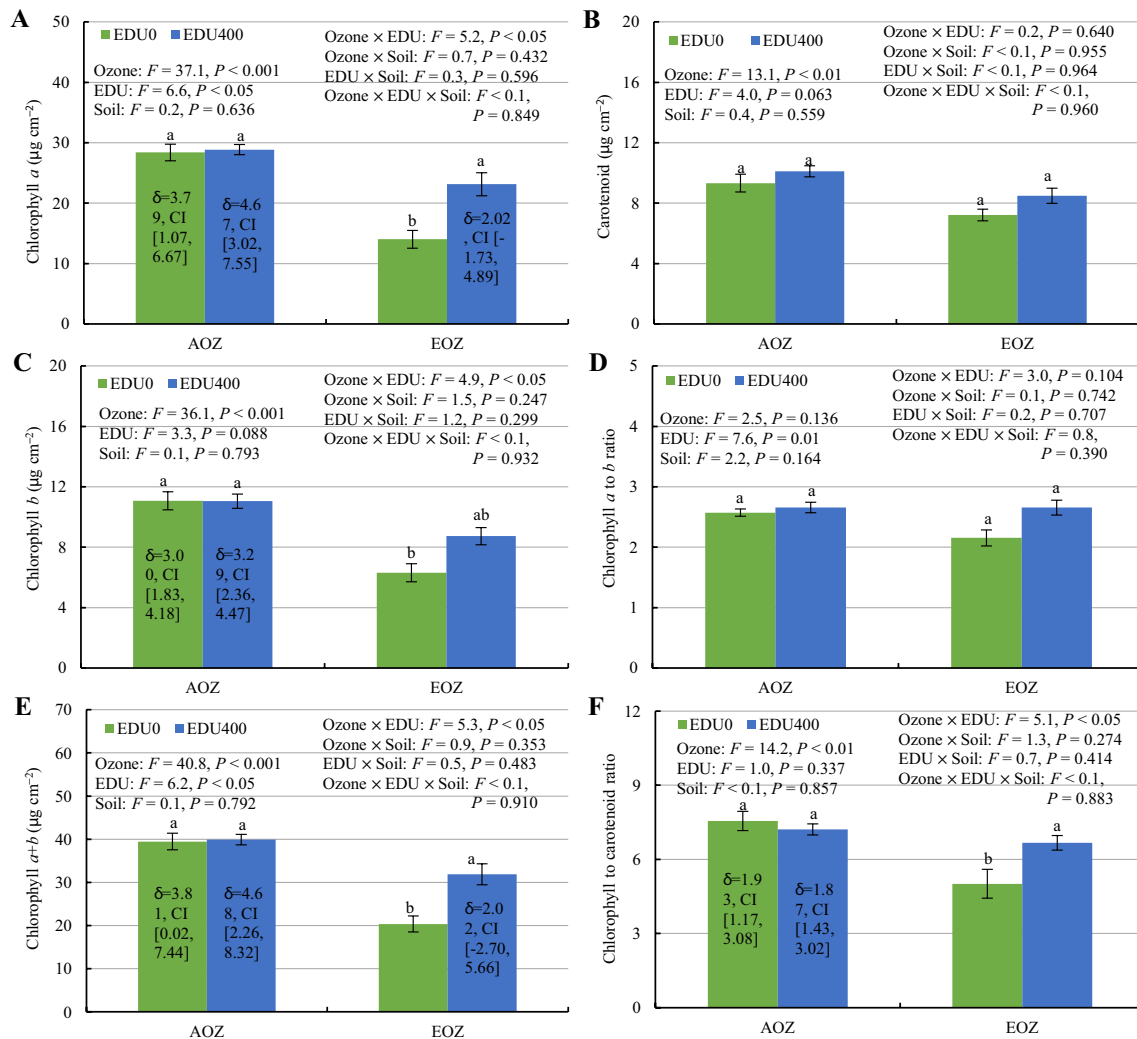


Fig. 11 Means (\pm se, $n=6$) of photosynthetic pigments of mature O_3 -symptomatic leaves of willow (*S. sachalinensis*) shrubs grown in brown forest (BF) or volcanic ash added to BF (VA-BF) soil, treated with 0 (EDU0) or 400 (EDU400) mg EDU L^{-1} every 9 d, and exposed to either ambient (AOZ) or elevated (EOZ) ozone. Sampling was performed after gas exchange measurements in early September of the second growing season (2015). Data were analyzed with

a General Linear Model (GLM) at $\alpha=0.05$. For traits exhibiting a significant Ozone \times EDU interaction, different letters above means indicate statistically significant differences between means based on Bonferroni post-hoc test ($\alpha=0.05$). Cohen's delta (δ) and its confidence interval (CI) shown on a bar reflects the size of the difference between that group and the group (EDU0 \times EOZ) from which it differs significantly based on Bonferroni results

Bonferroni, and no significant difference of biological importance for new shoot dry mass and total aboveground dry mass (i.e. only EOZ \times EDU400 \times VA-BF differed from AOZ \times EDU400 \times VA-BF and AOZ \times EDU0 \times BF).

Only O_3 had a significant main effect on foliage damage by insects, which was $68.5 \pm 3.3\%$ ($n=12$) in AOZ and $0.6 \pm 0.3\%$ ($n=12$) in EOZ, indicating a large difference ($\delta=8.07$, CI [1.59, 8.57]). None of the other single factors or interactions were significant ($P > 0.900$; data not shown). The insect damage observed was commonly destruction of leaf area (by chewer adult beetles of *A. coerulea*) or parenchyma eating (skeletonizer larvae of *A. coerulea*), thus reducing the photosynthesizing leaf area of plants.

EDU and O_3 carry-over effects following shoots harvest and cutting re-growth in the third growing season (2016)

Ozone and EDU treatments in the previous growing seasons did not have any single or interactive effect on SPAD (Fig. 14A), LMA (Fig. 14B), N_{PPW} (Fig. 14C), and F_v/F_m (Fig. 14D) in mature leaves of newly developed shoots in September, 2016. This was also the case for the foliage damage caused by insect herbivores (average of all shoots per plant) (Fig. 15A). Shrubs re-grown from cuttings exposed to EOZ in the two previous growing seasons, however, had 2.3 times fewer adult beetles (*A. coerulea*) on their foliage

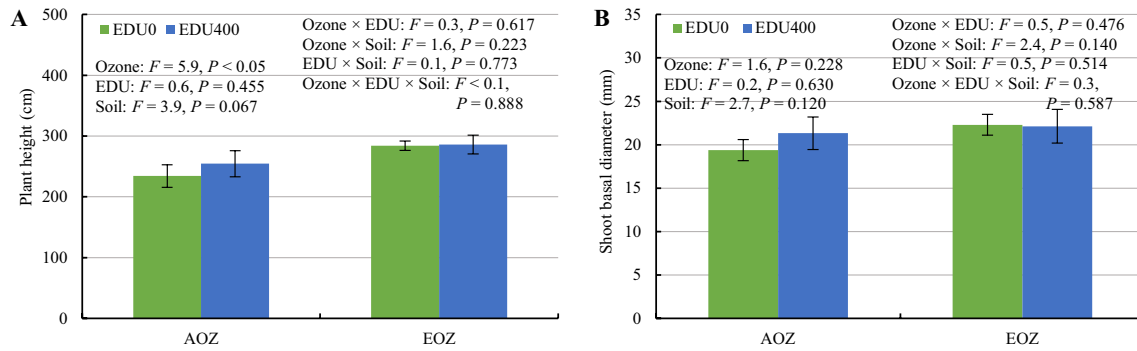


Fig. 12 Means (\pm se, $n=6$) of plant growth traits of willow (*S. sachalinensis*) shrubs grown in brown forest (BF) or volcanic ash added to BF (VA-BF) soil, treated with 0 (EDU0) or 400 (EDU400) mg EDU L⁻¹ every 9 d, and exposed to either ambient (AOZ) or

elevated (EOZ) ozone. Measurement was performed in mid. September of the second growing season (2015). Data were analyzed with a General Linear Model (GLM) at $\alpha=0.05$

(Fig. 15B), compared to AOZ group; this effect was marginally small ($\delta=0.50$, CI $[-4.92, 6.20]$). There was no significant single or interactive effect of O₃ and EDU treatments in the previous growing seasons on the average shoot height of re-grown shrubs (Fig. 16A). However, shrubs re-grown from cuttings exposed to EOZ in the two previous growing seasons had 1.2 and 1.3 times smaller average shoot basal diameter (Fig. 16B) and total aboveground dry mass (Fig. 16C) than shrubs re-grown from cuttings exposed to AOZ in the preceding growing seasons. This effect was small for both average shoot basal diameter ($\delta=0.83$, CI $[-0.17, 2.65]$) and total aboveground biomass ($\delta=0.51$, CI $[-11.10, 26.51]$).

Discussion

Negligible influence of soil condition

Through a multi-year, open-field experimentation, the effects of O₃, soil type, and EDU on willow shrubs were studied at multiple levels. However, the effects of soil were negligible, especially as a function of O₃ and EDU treatments. The absence of wide significant effects of soil may be explained by the fact that the addition of VA into the native soil was performed in the first 30 cm from the soil surface, while these very fast-growing shrubs were considerably big by the end of the first growing season, suggesting that their roots were deeper. Since there were negligible effects of soil, soil is excluded from the succeeding discussion.

Ozone effects on plant physiology in the first growing season (2014)

Visible injury appeared in leaves of various ages in EOZ, and the degree of severity was diverse. The injury was mainly

discoloration of leaves, which eventually became yellowish and then brownish. Necrosis started from the peripheral leaf blade toward the central vein, and covered almost the entire interveinal spaces with progressing stress. These injuries are typical O₃-induced foliar visible injuries, which have been observed in various plant species (Hoshika et al. 2013; Feng et al. 2014; Wan et al. 2014; Gottardini et al. 2014). The occurrence of foliar visible injuries, with decreased greenness, indicates that the shrubs grown in EOZ were under considerable O₃-induced stress from the first growing season. However, such toxicities were mainly observed in older leaves, which were exposed to EOZ for a longer time, and evaluation of young top leaves revealed no significant effect of EOZ on SPAD and LMA. Leaf greenness and LMA are major determinants of O₃ stress (Li et al. 2017).

In young top leaves, EOZ increased Mg, Fe, and Cr and decreased Mn and Al. Because the effect of EOZ on Mn, Fe, Al, and Cr was moderate and on Mg large, it can be postulated that, among the studied elements, Mg played a more pronounced and dominant role in the mechanisms of plant stress response to O₃. Across all treatments, Mg concentrations were negatively associated with Fe and Al and positively associated with Cr, suggesting that the regulation of Mg depends upon Fe, Al, and Cr. Mg is an important element in plant stress signaling, although its molecular and physiological roles have been neglected for a long time due to technical limitations (Guo et al. 2015). It also plays a vital role in the biosynthesis of chlorophylls and the fixation of carbon by acting as a cofactor of major enzymes participating in carbon metabolism (Hermans and Verbruggen 2005). Chlorophyll biosynthesis and carbon metabolism are major target sides of O₃ stress, both of which are disturbed and inhibited by elevated O₃ (Dizengremel et al. 1994; Dizengremel 2001; Feng et al. 2008, 2019b; Guidi et al. 2009; Pellegrini 2014; Kinose et al. 2020). The studies evaluating elemental composition in leaves of O₃-treated trees are

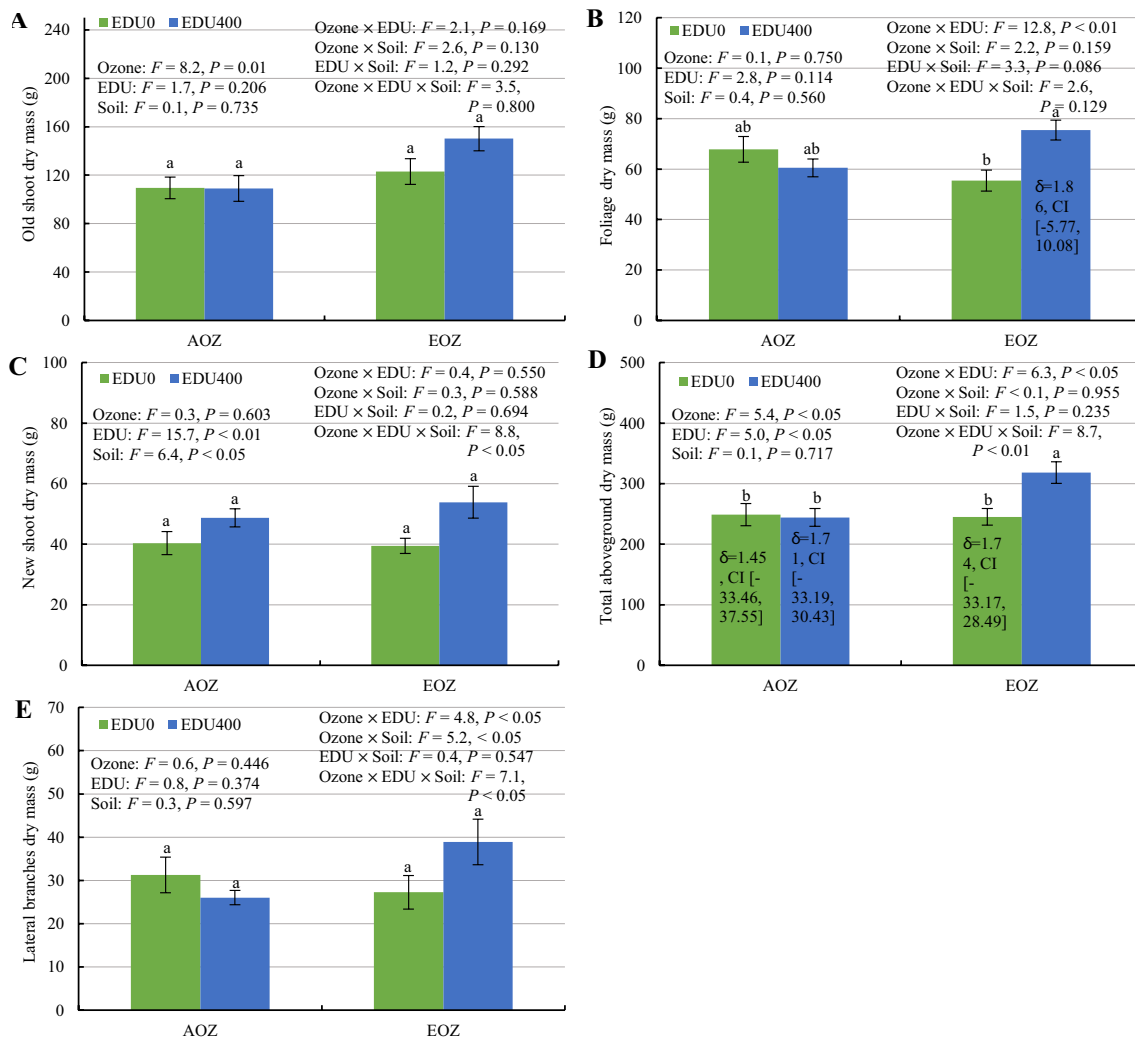


Fig. 13 Means (\pm se, $n=6$) of biomass production traits of willow (*S. sachalinensis*) shrubs grown in brown forest (BF) or volcanic ash added to BF (VA-BF) soil, treated with 0 (EDU0) or 400 (EDU400) mg EDU L⁻¹ every 9 d, and exposed to either ambient (AOZ) or elevated (EOZ) ozone. Old shoot is the part of the main shoot that was developed in 2014 (lignified in 2015), while new shoot is the part of the main shoot that was developed in 2015 (fresh/green). Harvest was performed in mid. September of the second growing season (2015). Data were analyzed with a General Linear Model (GLM) at $\alpha=0.05$.

limited, and the status of young, top leaves has rarely been studied because commonly the mature/older leaves are studied. Considering that chlorophylls have an Mg atom in the center of closed ring tetrapyrroles, our study newly suggests that Mg in young top leaves may be enriched in the framework of a strategy to precondition new leaves so to protect them against O₃-induced inhibition of chlorophylls; thus, keeping chlorophylls at ‘normal’ levels (no significant effect on SPAD was found) (Agathokleous 2021).

Mg contributes to plant health, and, in addition to its physiological benefits for active growth, is involved in plant resistance to diseases, such as protecting tissues

For traits exhibiting a significant Ozone×EDU interaction, different letters above means indicate statistically significant differences between means based on Bonferroni post-hoc test ($\alpha=0.05$). Cohen’s delta (δ) and its confidence interval (CI) shown on a bar reflects the size of the difference between that group and the group from which it differs significantly based on Bonferroni results. For the few significant Ozone×Soil and Ozone×EDU×Soil interaction, there was either no significant difference among means or there was no biologically meaningful significant difference among means

against degradation by pectolytic enzymes of some pathogens (Huber and Jones 2012). Our results extend these understandings by suggesting that Mg is also involved in the mechanisms of defense against O₃ stress in this plant species. They also suggest that management of Mg nutrition may protect plants against O₃ toxicities, a hypothesis that needs to be confirmed experimentally by exogenous application of various Mg concentrations. Although the interaction of soil Mg and O₃ has been examined in loblolly pine (*Pinus taeda* L.) seedlings, only two Mg doses were included (12 or 35 $\mu\text{g Mg g}^{-1}$) and no significant treatments interaction was found (Edwards et al.

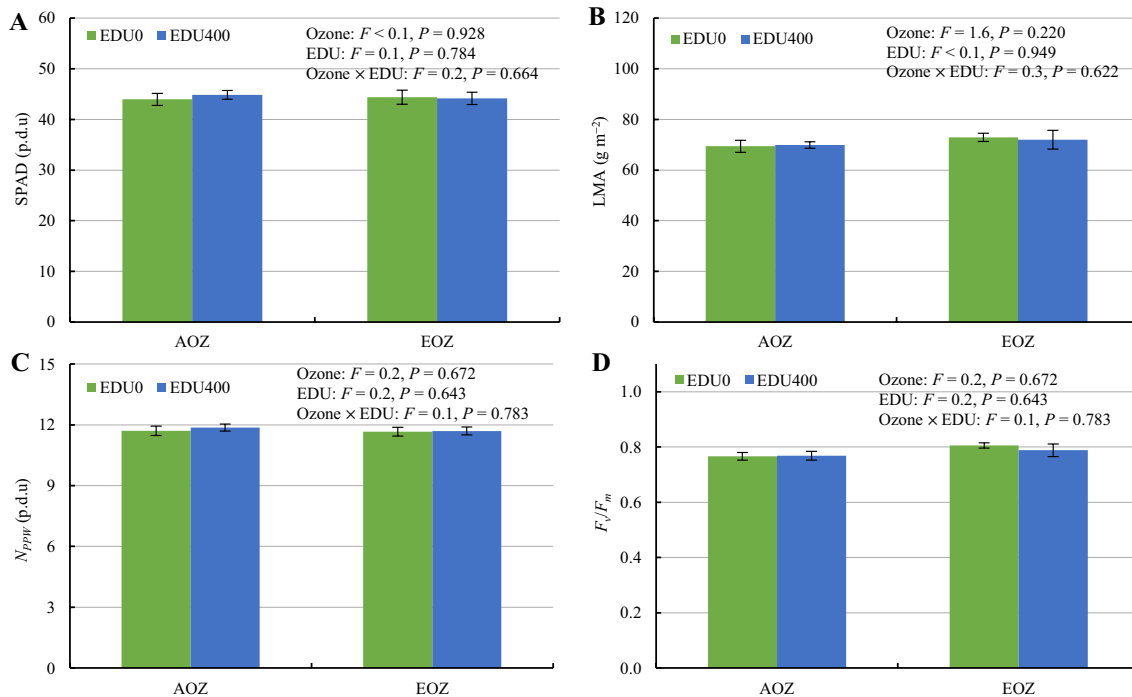


Fig. 14 Means (\pm se, $n=5-15$) of biomass production traits of willow (*S. sachalinensis*) shrubs grown in brown forest (BF) or volcanic ash added to BF (VA-BF) soil in 2014–2015, treated with 0 (EDU0) or 400 (EDU400) mg EDU L^{-1} every 9 d in 2015, and exposed to either ambient (AOZ) or elevated (EOZ) ozone in 2014–2015. Cuttings were transplanted into ambient plots newly created in 2016 and

cultivated with no any treatments. Sampling and measurements of re-grown cuttings were performed in mid-late September of the third growing season (2016). N_{ppw} indicates the estimated N content per leaf area, F_v/F_m the maximum efficiency of PSII photochemistry, and SPAD the leaf greenness. LMA stands for leaf mass per area. Data were analyzed with a General Linear Model (GLM) at $\alpha=0.05$

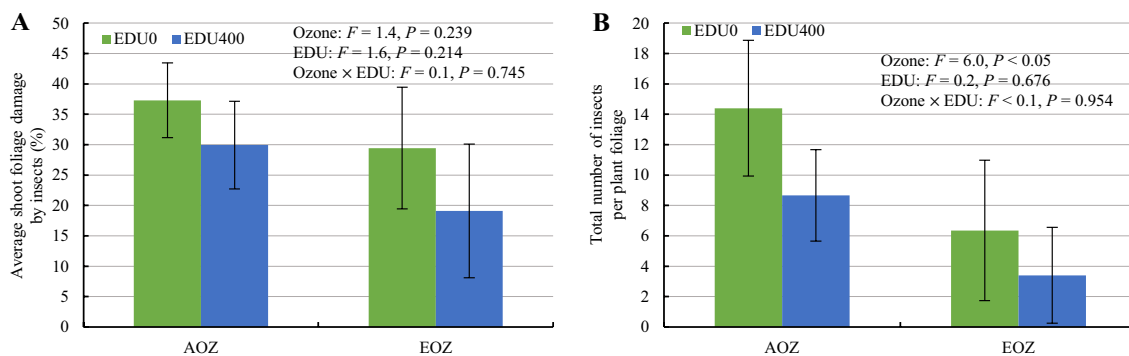


Fig. 15 Means (\pm se, $n=5-15$) of foliage damage by insect herbivores and number of adult beetles (*Agelastica coerulea*) in willow (*S. sachalinensis*) shrubs grown in brown forest (BF) or volcanic ash added to BF (VA-BF) soil in 2014–2015, treated with 0 (EDU0) or 400 (EDU400) mg EDU L^{-1} every 9 d in 2015, and exposed to

either ambient (AOZ) or elevated (EOZ) ozone in 2014–2015. Cuttings were transplanted into ambient plots newly created in 2016 and cultivated with no any treatments. Harvest of re-grown cuttings was performed in early October of the third growing season (2016). Data were analyzed with a General Linear Model (GLM) at $\alpha=0.05$

1990). Therefore, there is a need for more studies that will include several Mg doses to study the dose response under O_3 stress. Previous studies with trees suggested that O_3 alters the ecological stoichiometry (Agathokleous et al. 2018; Shang et al. 2018); however, the elements playing

major roles in the responses seem to vary across species (Shi et al. 2017).

In fallen leaves, EOZ had a small effect on Mg (decrease) and K (increase), a moderate effect on Mn (decrease) and P (decrease), and a large effect on Al (decrease). The direction of the effect was opposite between young top leaves

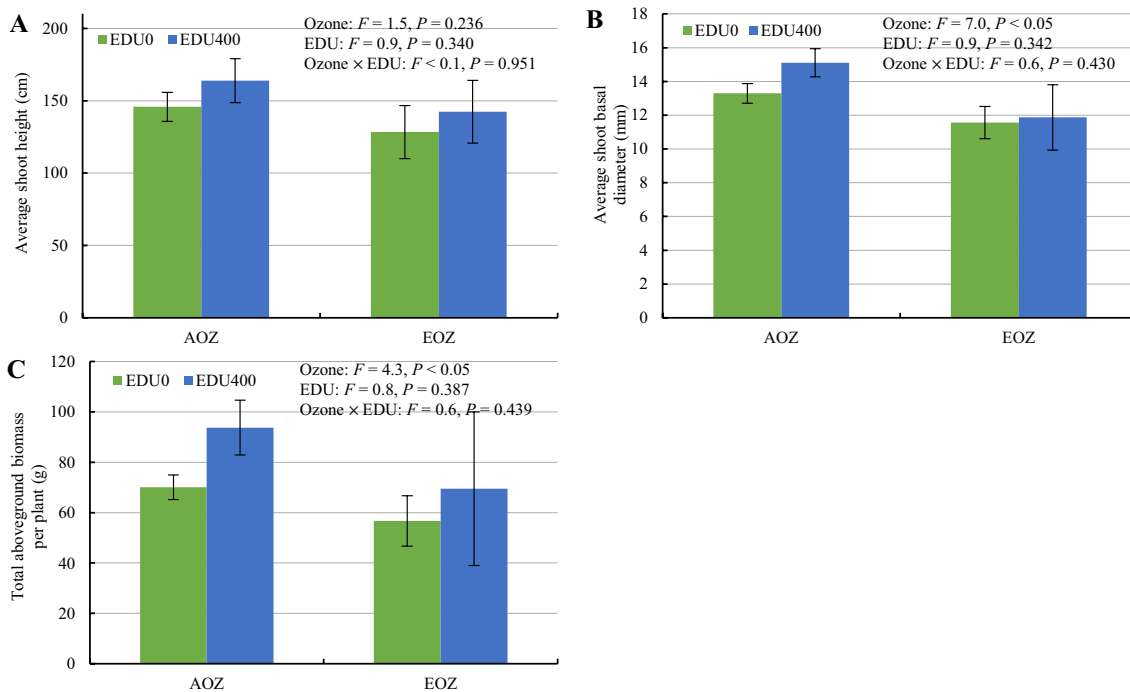


Fig. 16 Means (\pm se, $n=5-15$) of growth and biomass production traits of willow (*S. sachalinensis*) shrubs grown in brown forest (BF) or volcanic ash added to BF (VA-BF) soil in 2014–2015, treated with 0 (EDU0) or 400 (EDU400) mg EDU L⁻¹ every 9 d in 2015, and exposed to either ambient (AOZ) or elevated (EOZ) ozone in 2014–

2015. Cuttings were transplanted into ambient plots newly created in 2016 and cultivated with no any treatments. Harvest of re-grown cuttings was performed in early October of the third growing season (2016). Data were analyzed with a General Linear Model (GLM) at $\alpha=0.05$

and fallen leaves for Mg and same for Mn and Al. These results suggest that a greater amount of Mg might have been transferred to the young top leaves before shedding, thus contributing to the large increase in Mg in the young top leaves in EOZ. On the other hand, Mn and Al concentrations were decreased by EOZ in both fallen and top leaves, suggesting no significant contribution of elemental retranslocation from fallen to top leaves (e.g., Marschner 2012). However, because the decrease of Mg in fallen leaves was small whereas the increase in top leaves was large, some unidentified mechanisms might be involved in the enrichment of top leaves with Mg, even if the mature senescing leaves may be proportionally more compared to the top leaves. For example, young leaves in this willow may act as a sink of Mg and carbon after activation of *STAY-GREEN* gene encoding Mg-dechelataze enzyme that catalyzes the reaction of extracting Mg from chlorophyll to synthesize phaeophytin (Shimoda et al. 2016; Sato et al. 2018). Whether root- or leaves-associated microorganisms (e.g., endophytes; Ueno et al. 2020, 2021) are involved in these mechanisms remains unknown and warrants further studies. Across all treatments, Mg was positively associated with Fe and Ni, and negatively associated with K and Cr in fallen leaves, suggesting that the effect of EOZ on Mg can be driven by changes in Fe, Ni, K, and Cr. Nutrients P and K were significantly affected by

EOZ only in fallen leaves, while elements Fe and Cr were affected by EOZ only in young top leaves. These results indicate different effects of EOZ between the two leaf types. In a previous study with pot-grown cuttings of the same source with the ones used in this study, elevated O₃ affected the cycling of K, Cr, Fe, and Ni throughout crown, while it reduced the resorption efficiency of Fe and Cr and increased P/Fe ratio (Agathokleous et al. 2018). Although Ni and Fe are microelements, they can cause nutritional imbalances with potentially negative effects on plants at high concentrations (Kayama et al. 2005). Moreover, Cr is often accumulated in roots and becomes harmful for plants at high concentrations, although some plants require trace amounts of Cr regulated by Ca (Marschner 2012). Most of P and Fe in soil exists in unavailable form, thus, plants absorb them by releasing some kinds of organic acids (or by associating with mycorrhizae) (Marschner 2012). Therefore, elemental imbalance in top and fallen leaves of *S. sachalinensis* due to EOZ might affect the expenditure of energy in an effort to return to homeostasis.

It was suggested that Fe homeostasis and Cr concentration regulation play an important role early in O₃ stress; the assessment was conducted before leaves develop visible O₃ symptoms (Agathokleous et al. 2018). Hence, the results of this study with tall shrubs grown directly onto the soil

provide further support to the previous suggestions based on small pot-grown saplings.

Ozone effects on plant growth and biomass production in early spring of the second growing season (2015)

Plant growth and biomass production were evaluated in early spring of the second growing season, following snow-melt. Therefore, these growth and production characteristics reflect outcomes of the preceding growing season. All shoots, but one, produced by each plant in the first growing season were harvested to make possible the inclusion of EDU treatment in the study during the second growing season.

Despite the changes in elemental composition in leaves, which were observed in the first growing season, EOZ had no significant effect on the average length, basal diameter, number of lateral branches, and dry mass of branches per plant. However, EOZ caused a small increase in the total harvested aboveground biomass per plant. These results suggest physiological alterations at leaf level but no adverse effects of EOZ at individual plant level in the first growing season. Instead, that EOZ increased the total harvested aboveground biomass may indicate overcompensation responses of plant growth, similar to observations made in different species of trees exposed to elevated O_3 (Kitao et al. 2015; Tobita et al. 2019). Although photosynthetic pigments were not directly measured in the first growing season, the large enrichment of Mg in top leaves by EOZ may relate to enhanced chlorophylls potential, and, thus, increased photosynthates that can lead to more biomass.

EDU impact against O_3 effects on plant physiology, growth, and biomass production in the second growing season (2015)

The plasticity of LMA varies with plant functional groups and is an important determinant of plant fitness, affecting various ecosystem processes (Poorter et al. 2009). Deciduous shrubs and trees have smaller LMA than evergreen species (Poorter et al. 2009), and LMA is more affected by elevated O_3 in deciduous broadleaved species than in evergreen broadleaf tree species (Li et al. 2017). The range of LMA values found in mature leaves of the deciduous willow of this study is in line with the values reported for deciduous shrubs (on average 73 g m^{-2}) and trees (on average 75 g m^{-2}) in the literature (Poorter et al. 2009). However, that EDU400-treated leaves had moderately higher FM/DM value and lower LMA value than EDU0-treated leaves in EOZ suggests reduction of FM/DM and increase of LMA by EOZ, effects that were alleviated by EDU400. An increased LMA due to EOZ may indicate a mechanism to increase cell wall mass fraction and extend leaf lifespan

at the expense of the efficiency of photosynthesis (Onoda et al. 2017). Although meta-analyses of woody species to O_3 suggest a general decrease of LMA by considerably elevated O_3 concentrations ($83\text{--}128 \text{ nmol mol}^{-1}$; Li et al. (2017)), our study indicates that elevated O_3 -induced stress can have a positive effect on LMA. While a positive effect, the alleviation of this EOZ effect by EDU400 suggests the presence of oxidative stress induced by EOZ. The decreased FM/DM value by EOZ (or its increase by EDU400) may show that EOZ decreased the water content in mature leaves as a result of oxidative stress. Leaf water content is an important factor for insect herbivory (Bisigato et al. 2015), and one that had not been evaluated in previous studies of plant–insect interactions in this FACE system (Masui et al. 2021). However, since EDU400 led to a higher FM/DM value in EOZ while there was no difference in the herbivory between EDU400- and EDU0-treated shrubs within EOZ, it can be postulated that leaf water content was not a major factor explaining the herbivory patterns.

The vast majority of studies investigated O_3 effect on photosynthesis-related traits in symptomatic leaves. However, symptomatic leaves are expected to have considerably impacted photosynthetic apparatus due to the damaged chloroplast in leaves with O_3 -induced visible injury. Here, we found that EOZ had a small effect on gas exchange in O_3 -asymptomatic leaves too, in particular a decrease of A_{max} , which was counteracted by EDU400. The lack of significant effect of EOZ on V_{Cmax} and J_{max} as well as on gas exchange parameters other than A_{max} suggests that EOZ had a rather biochemical mode of action in asymptomatic leaves. EDU400 also had a small effect on A_{380} (increase) and E_{380} (decrease) regardless of O_3 treatment, suggesting that leaves in ambient O_3 might also be under small stress. The small effect of EOZ on A_{max} is in agreement with its small negative effect on chlorophylls *a*, *b*, and *a + b* and chlorophyll to carotenoid ratio in O_3 -asymptomatic leaves, effects against which EDU400 did not protect. The decrease in chlorophyll to carotenoid ratio by EOZ indicates that chlorophylls were relatively more affected by EOZ than carotenoids. Overall, despite no visible injuries, EOZ suppressed photosynthetic pigments, while EDU400 did not protect asymptomatic leaves against O_3 by improving photosynthetic pigments. Conversely, based on well over 100 observations from several studies, EDU generally protects against O_3 -induced decrease in chlorophylls (Feng et al. 2010; Jiang et al. 2018; Gupta et al. 2021; Jabeen and Ahmed 2021; Chaudhary and Rathore 2021). However, the protection was genotype/cultivar-specific (Jiang et al. 2018), and commonly chlorophylls were not evaluated in asymptomatic leaves. These observations suggest that EDU mode of action in protecting plants against O_3 -induced toxicity may differ between O_3 -symptomatic and O_3 -asymptomatic leaves. This assumption is further supported by the findings of photosynthetic

pigments assessment in O_3 -symptomatic leaves. While EOZ (EOZ \times EDU0) caused a moderate (vs. EOZ \times EDU400) to large (vs. AOZ \times EDU0) decrease in chlorophyll *a* and chlorophyll *a + b* in O_3 -symptomatic leaves, EDU400 significantly protected to a moderate extent. However, the protective action of EDU in O_3 -symptomatic leaves was pigment-specific as EDU400 did not protect against EOZ-induced reduction of carotenoids.

Despite the negative effects of EOZ on leaf-level physiology, EOZ-treated plants displayed a small increase in plant height, old shoot dry mass, and total aboveground dry mass, which may indicate overcompensation responses similar to the biomass production of the first growing season and observations in different studies with other species exposed to elevated O_3 (Kitao et al. 2015; Tobita et al. 2019). Contrariwise, EDU400 increased new shoot dry mass and total aboveground dry mass, compared EDU0-treated plants, irrespective of O_3 treatment, a finding suggesting that shrubs in both AOZ and EOZ were under stress. Moreover, the moderate increase in foliage dry mass noted in EDU400-treated shrubs, compared to EDU0-treated shrubs, within EOZ suggests that EDU400 protected against EOZ-induced premature senescence and/or directly increased total leaf area. As a result of the individual biomass components responses, EDU400 \times EOZ treatment led to a small to moderate increase of total aboveground dry mass compared to EDU0 \times EOZ, EDU0 \times AOZ, and EDU400 \times AOZ treatments, suggesting that plants of EDU400 \times EOZ treatment were more vigorous/productive than plants of the other treatment combinations.

Because it was observed during routine field work that insect herbivore activity and thus herbivory were considerably lower in EOZ plots than in AOZ plots, foliage damage by insects was recorded. The large destruction of leaf area by chewer adult beetles and parenchyma eating by larvae observed in AOZ plots, while it was negligible in EOZ plots, is in agreement with extensive observations in different plant species in the same plots from 2014 to 2016 (Masui et al. 2021). Since this difference in herbivory was irrespective of EDU treatment, this study further indicates that the difference in herbivory between AOZ and EOZ plots is primarily not due to plant qualitative status but rather due to atmospheric status, further hinting to a potentially more important role of volatile organic compounds in mediating insect-plant communication (Blande 2021; Masui et al. 2021). Within EOZ, there was negligible damage by herbivores, indicating that O_3 was the plant-inhibiting factor. The widespread herbivory in AOZ, by imposing stress to plants, may also explain the small increase in A_{380} and decrease in E_{380} by EDU400 irrespective of O_3 treatment. EDU is widely used as an O_3 biomonitoring tool and chemical to attribute differences between EDU-treated and -untreated plants to O_3 (Manning et al. 2011; Yuan et al. 2015; Singh et al. 2018;

Pandey et al. 2019; Fatima et al. 2019; Giovannelli et al. 2019). Hence, plants treated with EDU400 in EOZ can be considered as a control that theoretically approximates plant status under no O_3 stress. By comparison, response of plants treated with EDU0 in EOZ represents the effect of EOZ when compared with plants treated with EDU400 in EOZ. Moreover, the response of plants treated with either EDU0 or EDU400 in AOZ represents the effect of herbivory when compared with plants treated with EDU400 in EOZ. Hence, both EOZ and herbivory in AOZ suppressed biomass production. The increased biomass produced in the first growing season in EOZ might also be the result of largely more severe herbivory of AOZ plants compared to EOZ plants, instead of being positive effects of EOZ. This is highly plausible since similar herbivory patterns were observed in this exposure system in the first growing season, although not monitored in these willow shrubs (Masui et al. 2021). These results suggest that herbivory complicated O_3 effects estimation, highlighting the need to consider herbivory when evaluating O_3 effects on vegetation as well as the importance of FACE experimentation in providing more realistic evaluation of O_3 effects on vegetation by permitting consideration of biotic interactions. Many of the results could lead to incorrect conclusions if EDU were not included in the second growing season, indicating that EDU can be incorporated into such FACE studies as a tool to account for potential differences between O_3 treatments due to factors other than O_3 .

EDU and O_3 carry-over effects following shoots harvest and cutting re-growth in the third growing season (2016)

Ratoon cropping is a cultivation technique applied in the agronomical practice of various crops since a long time ago (Plucknett et al. 1970; Wang et al. 2020). New shoots (ratoon) emerge from the base of a plant following cropping, a practice that facilitates green and resource-efficient production (Wang et al. 2020). In this study, ratoon cropping was applied for the first time to investigate potential treatment effects on ratoon developed in the third growing season without the ratoon being directly exposed to elevated O_3 or EDU. To this end, harvested cuttings with their root system were transplanted into new ambient sites after snowmelt in the third growing season.

O_3 and EDU treatments in the previous years did not significantly affect leaf-level physiology (SPAD, N_{PPW} , LMA, and F_v/F_m) in the current year, suggesting no carry-over and/or memory effects. However, while the average foliage damage by insect herbivores was also not significantly affected by the O_3 and EDU treatments in the previous years, shrubs re-grown from cuttings exposed to EOZ in the two previous growing seasons had fewer *A. coerulea* adult individuals on their foliage, compared to AOZ group, irrespective

of EDU treatment. This suggests that (1) leaves on ratoon developed from cuttings/roots previously exhibiting to EOZ stress might be less attractive to insects, and (2) some unidentified biochemical alterations (e.g., in secondary metabolites concentrations) might be present in leaves of ratoon developed from cuttings/roots subjected to EOZ stress in the preceding two years. Furthermore, shrubs re-grown from cuttings exposed to EOZ in the two previous growing seasons exhibited a small inhibition of average shoot basal diameter and total aboveground dry mass, compared to AOZ group, irrespective of EDU treatment. Therefore, the negative effect of EOZ on biomass production of this energy crop (EDU0×EOZ vs. EDU400×EOZ) was carried over in the third growing season, which may be due to inhibition of root by EOZ in the previous years (Grantz et al. 2006). The results of these assessments suggest a carry-over effect. A potential memory effect through signals generated or processed in the cutting and/or root cannot be excluded either. It should be supplemented here that a recent study also provides evidence suggestive of the generation and/or process of signals in the root involved in transgenerational effects of oxidative stress, specifically by Cd (Nogueira et al. 2021). Therefore, further experimentation is needed to reveal whether carry-over and/or memory effects of O₃ are primarily driven by roots.

Conclusion

In the first growing season, the interactive effects of O₃ and soil were studied; however, soil had negligible impact on O₃ effects. EOZ led to various alterations in the leaf elemental composition. Specifically, among the evaluated elements, a predominant role of Mg was revealed. Although Mg exhibited a small decrease by EOZ in fallen leaves, it was largely enriched by EOZ in young top leaves. This novel finding suggests a mechanism to condition young top leaves, potentially to prevent O₃-induced suppression of chlorophylls, and warrants further studies to investigate whether exogenous application of Mg can protect plants against elevated O₃-induced phytotoxicity. EOZ exposure resulted in considerable changes in the elemental composition of young top leaves and fallen leaves, suggesting altered ecological stoichiometry. Such elemental changes can have implications to ecological processes such as litter decomposition.

In the second growing season, EDU was added in the experimental design. EDU400 protected against EOZ-induced reduction of FM/DM and LMA in randomly-selected mature leaves as well as of A_{max} in O₃-asymptomatic leaves. EDU400, however, did not protect against EOZ-induced suppression of V_{Cmax} , J_{max} , and chlorophylls *a*, *b*, and *a + b* in O₃-asymptomatic leaves. The mode of action of EDU in O₃-asymptomatic leaves

differed from O₃-symptomatic leaves in which EDU400 protected against EOZ-induced suppression of chlorophyll *a* and chlorophyll *a + b*, indicating that the mode of action of EDU may vary with the severity of O₃-induced stress in the leaf. Despite the negative effects of EOZ on leaf-level physiology, shrubs grown in EOZ produced more biomass than shrubs grown in AOZ. Based on consideration of shrubs performance under EDU treatment and herbivory, both EOZ and insect herbivore-induced damage in AOZ-exposed shrubs resulted in suppression of biomass production. These results signify the profound importance of considering biotic interactions when assessing O₃ effects on vegetation.

In the third growing season, carry-over effects of treatments were studied following ratoon cropping and transplantation of all plants into new ambient plots. EOZ treatment in the preceding growing seasons imposed a carry-over effect as indicated by suppressed ratoon biomass production. EDU400 treatment in the preceding growing season did not protect against EOZ carry-over effect, indicating that such an intervention based on hormesis priming should be applied in each growing season in ratoon cropping systems to protect plants against O₃-induced phytotoxicities.

We conclude that EOZ can adversely affect willow shrubs cultivated as energy crops, with long-lasting impacts, whereas EDU can enhance plant physiological performance and biomass production. Further work is needed to translate these scientific results into practical applications under real-world cropping systems.

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Declarations

Conflict of interest Evgenios Agathokleous (E.A.) is Associate Editor-in-Chief of this journal; however, he was not involved in the peer-review process of this manuscript. Parts of this research were presented in talks given by the first author (E.A.): (i) at the IUFRO's Regional Congress for Asia and Oceania 2016 "Air pollution and climate change impacts on forest ecosystems", 24–27 October, 2016, Beijing, People's Republic of China; (ii) at the 29th Task Force Meeting of the UNECE ICP Vegetation, 29 February–4 March, 2016, Dubna, Russian Federation; (iii) at the 3rd International Life-Science Symposium, 26 November, 2015, Sapporo, Japan; (iv) at the 126th Annual meeting of Japan Forest Society, 27–29 March, 2015, Sapporo, Japan. The at-

tendance of E.A. to present was financially supported by JSPS (event i) and the Frank Laboratory of Neutron Physics, Joint Institute for Nuclear Research, Dubna, Russian Federation (event ii). The authors declare that there are no conflicts of interest.

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