Impacts of ethylene diurea (EDU) soil drench and foliar spray in *Salix sachalinensis* protection against O_3-induced injury

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ABSTRACT

It is widely accepted that elevated levels of surface ozone (O_3) negatively affect plants. Ethylenediurea (EDU) is a synthetic substance which effectively protects plants against O_3-caused phytotoxicity. Among other questions, the one still open is: which EDU application method is more appropriate for treating fast-growing tree species. The main aims of this study were: (i) to test if chronic exposure of *Salix sachalinensis* plants to 200-400 mg EDU L^{-1}, the usually applied range for protection against O_3 phytotoxicity, is beneficial to plants; (ii) to evaluate effects of chronic exposure to elevated O_3 on *S. sachalinensis*; (iii) to assess the efficacy of two methods (*i.e.* soil drench and foliar spray) of EDU application to plants; (iv) to investigate the appropriate dose of EDU to protect against elevated O_3-induced damage in *S. sachalinensis*; and (v) to compare the two methods of EDU application in terms of effectiveness and EDU consumption. Current-year cuttings grown in infertile soil free from organic matter were exposed either to low ambient O_3 (AOZ, 10-h≈28.3 nmol mol^{-1}) or to elevated O_3 (EOZ, 10-h≈65.8 nmol mol^{-1}) levels during daylight hours. Over the growing season, plants were treated every nine days with 200 ml soil drench of 0, 200 or 400 mg EDU L^{-1} or with foliar spray of 0, 200 or 400 mg EDU L^{-1} (in two separate experiments). We found that EDU *per se* had no effects on plants exposed to AOZ. EOZ practically significantly injured *S. sachalinensis* plants, and the impact was indifferent between the experiments. EDU did not protect plants against EOZ impact when applied as soil drench but it did protect them when applied as 200-400 mg L^{-1} foliar spray. We conclude that EDU may be more effective against O_3 phytotoxicity to fast-growing species when applied as a spray than when applied as a drench.

Keywords: air pollution, antiozonant, effect size, ethylenediurea, tropospheric ozone

Key message: Soil-drenched EDU was not effective in protecting against O_3 injury to willow, while foliar-sprayed EDU was effective even at the concentration of 200 mg L^{-1}.

1. INTRODUCTION

Surface ozone (O_3) levels have risen globally, especially in the Northern hemisphere (Young et al. 2013; Akimoto et al. 2015; Saitanis et al. 2015a). This phenomenon is more severe in Asia, due to rapid population growth and industrialization (Ohara et al. 2007; Yamaji et al. 2008; Verstraeten et al. 2015). It is also shown that O_3 levels in European and USA cities and remote sites are still increasing, although peak values are decreasing (Sicard et al. 2013; Paoletti et al. 2014).

Ozone enters plant tissues via stomata (Hoshika et al. 2015; Watanabe et al. 2015). Uptake of elevated O_3 doses by plants stimulates production of reactive oxygen species (and thus lipid peroxidation), activation of antioxidant mechanisms and other repair processes (Alexou et al. 2007; Pellegrini et al. 2015; Vaultier and Jolivet 2015). These negative effects may range from plant cell level to ecosystem level (Agathokleous et al. 2015a, 2016; McGrath et al. 2015; Sicard et al. 2016; Wang et al. 2016).

Due to the severity of the problem, countermeasures are required in order to protect plants against O_3 impact, both in rural and urban areas. However, there are hitherto no available countermeasures to protect plants in practice. Several substances have been tested as potential protectants but none has been proved effective enough, except ethylene diurea ($C_4H_{10}N_4O_2$; abbreviated as EDU (Agathokleous et al. 2015b; Saitanis et al. 2015b). Some studies focused on methods for preventing O_3 uptake into the mesophyll but their efficacy is questioned due to high variability in effectiveness or potential negative feedbacks in the long term by CO_2 deficiency (Francini et al. 2011; Agathokleous et al. 2014; Agathokleous et al. 2016d).

EDU is a substance which has been found to protect plants against O_3 impact (Carnahan et al. 1978) when appropriately applied in the usual range of doses, e.g. 200-400 mg L^{-1} (Paoletti et al. 2009, Feng et al. 2010). EDU has been studied as a protectant of plants against O_3, as an O_3 biomonitoring tool or as a comparative tool for screening other chemicals as to their efficacy to protect plants against O_3 impact (Paoletti et al. 2009; Feng et al. 2010; Manning et al. 2011; Agathokleous et al. 2015b; Singh et al. 2015). EDU has been applied to plenty of agricultural crops. However, it has been applied only to few tree species: *Fagus sylvatica* L., *Fraxinus americana* L., *F. excelsior* L. and *F. pennsylvanica* Marshall., *Liriodendron tulipifera* L., *Pinus taeda* L., *Prunus serotina* Ehrh, and different poplars (Paoletti et al. 2009; Agathokleous et al. 2015d; Xin et al. 2016). This is because such experimentations with trees are more difficult to be conducted (Manning et al. 2011). Notably, only a recent study (Agathokleous et al. 2016b) with the willow *Salix sachalinensis* Fr. Schmidt (syn. *Salix udensis* Trautv. & C.A.Mey.) investigated EDU effects on plants grown in an infertile soil substrate. However, soil infertility, and particularly phosphorus (P) scarcity, is one of the most critical issues nowadays as a large proportion of global soils are P deficient and acidic, phosphate rock reserves are decreasing, and P demands are increasing (von Uexkull and Mutert 1995; Van Vuuren et al. 2010; Cordell and Neset 2014; Ulrich and Frossard 2014). Thus, the effectiveness of EDU against O_3 injury is unknown under such a scenario of soil infertility and when plant demands of nutrients are high. Agathokleous et al. (2016b) investigated the potential toxicity of very high EDU doses, and rather found beneficial effects in willow plants grown in infertile and organic-matter-free soil and exposed to low background O_3 levels. It remains, however, unanswered whether EDU applied at the usual low concentrations (200-400 mg L^{-1}, Feng et al. 2010) has stimulatory effects on plants growing in nutrient-poor and organic-matter-free soil.

Willows are the major species for the production of salicin, the predominant pain reliever (Vlachojannis et al. 2009; Mahdi 2010), and are cultivated as short-rotation coppices for biofuel production as well (Karp et al. 2011). *Salix sachalinensis* is a hygrophilous and heliophilous willow, native to Japan, north-east China, North Korea and Russian Far East, which plays an important role in river ecosystem functioning (Tamura and Kudo 2000; Isebrands and Richardson 2014). Its tolerance to shade, drought and waterlogging scores 1, 1.5 and 4, respectively, with 5 being maximal tolerance (Niinemets and Valladares 2006). It can also be grown as ornamental plant, as in the case of the cultivar 'Sekka' (Japanese fantail willow). *Salix sachalinensis* is classified as pioneer species which grows fast and continuously (Ueno et al. 2006). Since this species is fast growing and grows in wet habitats, a high O_3 uptake through the stomata is expected. However, its response to elevated O_3 levels is unknown, as only one investigation had been previously carried out under low O_3 levels (Agathokleous et al. 2015c, 2016a).

The two main methods for applying EDU are soil drench and foliar spray (Paoletti et al. 2009; Agathokleous et al. 2015b), although stem injections were tested too (Ainsworth and Ashmore 1992; Paoletti et al. 2007). It was suggested that soil influences EDU effectiveness (Manning et al. 2011; Agathokleous et al. 2015b) while foliar applications of EDU are technically difficult in the case of big trees (Paoletti et al. 2010). In the present study, we aimed to assess the effectiveness of these two application methods of EDU, in the common range of 200-400 mg L^{-1} (Feng et al. 2010), to protect against O_3 damage in this fast-growing species.

We designed this study to address five principal research questions. The first question (Q1) was "Does EDU applied at low doses affect *S. sachalinensis* plants grown in infertile and organic-matter-free soil under ambient conditions?" Based on estimations of Agathokleous et al. (2016b), we hypothesized that EDU in the usual range of doses would not affect *S. sachalinensis* plants grown in infertile and organic-matter-free soil. The second question (Q2) was "Does elevated O_3 alone affect *S. sachalinensis* plants?" In order to investigate EDU soil drench, the third question (Q3) was "Do EDU soil-drench applications at the dosage of 200 ml with the common concentrations of 200-400 mg L^{-1} every nine days protect against O_3 impact on *S. sachalinensis* plants grown in infertile and organic-matter-free soil?", where dosage means the rate of application of a dose. Similarly, to investigate EDU foliar spray, the next question (Q4) was "Do EDU spray applications at the common concentration range of 200-400 mg L^{-1} every nine days protect against O_3 impact on *S. sachalinensis* plants grown in infertile and organic-matter-free soil?" Finally, we aimed to answer the question (Q5) "Which application method is more appropriate for protecting this fast growing species against O_3 phytotoxicity?". For this purpose, we also recorded the amount of EDU needed for foliar spray applications in order to estimate the consumption of EDU in relation to plant leaf area. This information would be important for designing future experiments. For our questions, we were further interested in estimating the magnitude of the effect in case the alternative hypothesis (H_1) is accepted.

In order to answer the above questions, we selected production-related response variables rather than other ones, such as biochemical and physiological variables, because the O_3 impact on biomass production reflects the actual accumulated O_3 damage (Larch 2003; Agathokleous et al. 2015b, 2016a) and is used in O_3 risk assessment (U.S. EPA 2014).

2. MATERIALS AND METHODS

2.1. Study area

A two-year experiment was conducted at Sapporo Experimental Forest of Hokkaido University, Japan (43^o.04’ N, 141^o.20’ E, 15 m a.s.l.). The snow-free period lasted from early May to mid-November. Over the experimental period (August-October), data of temperature, wind speed, relative humidity, sunshine and precipitation were recorded by a nearby station at Sapporo (WMO, ID: 47412, 43^{o}03.6'N 141^{o}19.7'E), which is monitored by the Japan Meteorological Agency (2016). In addition, the photosynthetic photon flux density (PPFD) was recorded by a HOBO Pendant data logger (UA-002-64, Onset Computer, Co., MA, USA) located in the center of each experimental plot at a height of two meters.

2.2. Plant material & experimental design

Willows can be propagated clonally from branch fragments (Newsholme 1992) by rooting cuttings (Hayashi et al. 2005). One hundred fifty current-year cuttings of *S. sachalinensis* with height and basal diameter of 12.09±0.25 (mean ±s.e.) and 1.90±0.05 cm, respectively, were obtained from the Hokkaido Horti-Tree Planting Center, Co. Ltd; their origin was from the river basin of the Ebetsu city. The cuttings were stored at 0-4 ^{o}C, in an incubator, for a month, in order to break the dormancy. Plant growth containers were filled with a mixture (1:1) of Akadama (well-weathered volcanic ash) and Kanuma (well-weathered pumice) soil – free from organic matter. Volcanic ash soils are phosphorus deficient and poor in N, and are commonly found in Hokkaido (Schmincke 2004; Kam et al. 2015). Soils, originated from Kanuma town of Tochigi prefecture, were obtained (DCM Homac CO., LTD., Sapporo, JP) and opened just before the filling of the containers. Cuttings were planted for rooting on May 13th, in both 2014 and 2015, irrigated, and kept under field conditions. Irrigation was repeated two weeks later. On June 9th, when the cuttings were well rooted, 72 of them were selected for uniformity based on total number of leaves per plant (39 ±2) and transplanted into 15 L pots filled with the same soil mixture, irrigated, and left in the field until establishment and full adaptation. The pH of this pot soil mixture was 5.9 ±0.01; details on sampling and composition of Akadama and Kanuma soils are in Agathokleous et al. (2015e). Irrigation was repeated two times, every seven days. On August 14th, the potted plants were randomly assigned and transferred to six different plots (12 pots per plot), of which three served as elevated O_3 and three as ambient O_3 treatment, and, further, four plants were randomly assigned to each of the three EDU treatments in each plot. All the pots within each plot were subjected to a fortnight rotation and the three plots of each O_3 treatment were interchanged three times over each growing season, during late evening hours. Irrigation was done using tap water (pH=6.57 ±0.04). The plants were not fertilized. Plants were visually checked daily, and when insects were present they were manually removed. Visible injury by pests or pathogens was rarely observed, and thus plants were not treated by agrochemicals during the experiment.

In 2014, EDU was applied as soil drench whereas in 2015 it was applied as a foliar spray to different plants of the same age as those used in 2014. In order to achieve comparability, all the plant materials were handled and the treatments were conducted in the same manner and on the same dates each year following exactly the same protocol. The morphological characteristics of this species, when grown from cuttings, can be found in Koike et al. (1995).

2.3. Ozone treatment

For the O_3 treatments, a novel free-air O_3-enrichment system was established in the Sapporo Experimental Forest of Hokkaido University, Japan (Agathokleous et al. 2016e). The O_3 treatments were ambient O_3 (AOZ) and elevated O_3 (EOZ). Exposure of plants to EOZ lasted from August 15th to October 26th, in 2014 and 2015, during daytime, when the PPFD exceeded 70 μmol m^{-2} s^{-1} (*i.e.* light compensation point of photosynthesis of targeted plants as determined by Koike, 1988). The PPFD in the experimental plots exceeded 70 μmol m^{-2} s^{-1} during the hours 07:00 up to 17:00, for both experiments, and was not different between AOZ and EOZ plots (not shown). The AOZ and EOZ 10-h means were 22.3±3.3 and 60.1±2.2 nmol mol^{-1}, respectively, in 2014 and 34.3±5.5 and 71.5±1.3 nmol mol^{-1}, respectively, in 2015. Details on the O_3 metrics can be found in Agathokleous et al. (2016e).

2.4. EDU treatment

EDU (100% a.i., N-[-2-(2-oxo-1-imidazolidinyl) ethyl]-N'-phenylurea]; Wat (1975)) was freshly prepared (30 min before application) using an electric hotplate, by dissolving the required EDU amount in 500 mL, so as the target concentration was achieved in the final desired volume, gently-warmed water (Manning et al. 2011) with continuous stirring. For the soil drench treatment (applied in 2014), 200 mL of the prepared volume were given to each plant at each application. For the foliar spray treatment (conducted in 2015), EDU was applied as fine mist with low fluid velocity (*Venturi effect*), until run-off, using an electric sprayer with two nozzles spraying simultaneously. Both abaxial and adaxial leaf surfaces were sprayed. Surfactant was not used for EDU treatments.

The first EDU application was carried out on July 29th, 50 days after transplanting, when the plants had 63±2 leaves (measured a day before). Taking into account that EDU may persist in the leaf apoplast for more than eight days (Paoletti et al. 2009), EDU application was repeated every nine days. The last (10th) EDU treatment was applied on October 18th. All the applications were conducted during morning hours (between 10:00 and 11:00).

In order to assess the amount of EDU needed for the two application methods, the amount of spray liquid spent for the EDU treatments of 200 mg L^{-1} and 400 mg L^{-1} was recorded; for the soil drench, 200 ml with either 200 or 400 mg EDU L^{-1} were given to each plant at each application. For the applications of EDU as foliar spray from September to the semi-final in October (pooled over time), 197±3 ml of spray liquid were needed for each plant. The variation among time points was very low as it is evidenced from the low s.e. However, for the semi-final and final applications in October, 206±4 and 88±6 ml, respectively, of spray liquid were needed for each plant. The 88 ml corresponded to 18±1 leaves or a total plant leaf area of 120.5±11.7 cm^2.

2.5. Data collection

Data were collected from all the 144 plants. On October 25th crown length (from the point to which the first shoot is attached on the stem to the highest point of the crown) and crown width (distance between the two farthest shoots, as observed from above) were measured using a measuring tape with 1-mm graduation.

Each shoot of each plant was photographed and the angle between the shoot and the stem was taken by using the software ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA; Schneider et al. 2012). Then, the average shoot-stem angle per plant was calculated.

On October 26th, the length and width of each leaf, for all the shoots and plants, were measured (cm) non-destructively using a ruler. Later, the area of each leaf y (hereafter leaf size) was calculated using the predicting model $y=0.5786x+1.6913$, where x is the product of leaf length × leaf width, as described by Agathokleous et al. (2016b). Then the total leaf area for each plant was calculated.

On October 27th, the entire root system of each plant was excavated, with no damage or loss due to absence of soil organic matter (SOM), and gently washed with tap water.

The basal diameter of each shoot was measured by a caliper (mm), and the average shoot diameter (shoot diameter) was calculated per plant. The length of each shoot was also measured and the average shoot length per plant was calculated.

The number of buds of each shoot was counted and the buds of all the shoots were summed up to give the total number of buds per plant.

At the end of each experiment, each shoot and each leaf were harvested and put in a separate paper bag with an ID so as to know the position for the leaves on the shoots and the position of the shoots on the stem and thus to group them into lower-level and upper-level compartments. Roots were also put into separate bags with an ID informing about the plant to which they belonged.

All plant compartments were air-dried until constant dry mass in an oven at constant air temperature of 65 ^{o}C. The dry mass (DM) of each leaf, shoot, root and stem was measured by an electronic balance (g), and the average leaf DM (leaf DM), average shoot DM (shoot DM), total foliage DM (foliage DM), mean shoot DM and total shoot DM (shoots DM) and the Root DM/Foliage DM ratio were calculated per plant. The sum of Foliage DM and Shoots DM constituted the aboveground plant dry mass (Aboveground DM) and the sum of Foliage DM, Shoots DM and Root DM constituted the total plant dry mass (Plant DM).

2.6. Data handling & Statistics

Each comparison of interest derived from a particular hypothesis, requiring thus straightforward interpretation. Yet, the total number of possible pair-wise comparisons was quite huge (high number of independent variables with at least two levels each), the majority of which was meaningless, increasing thus the experimental error and further making the *a posteriori* comparisons inappropriate. Thus, based on prior theoretical knowledge and in order to answer only the most biologically meaningful questions (Ruxton and Beauchamp 2008) the approach of contrasts was chosen and applied to *a priory* planned comparisons which offer a better trade-off between type I and type II errors than unplanned comparisons.

For more conservative conclusions, regarding the experimentwise type I error rate (EER) (Ruxton and Beauchamp 2008), all the statistical comparisons were conducted at level of significance lower than 0.05, calculated according to the Dunn–Šidák correction equation:

$$a_{[PC]}= 1-(1-a_{[PF]})^{1/C}=0.0085,$$

where $\alpha_{[PC]}$ is the Type I error for the group of contrasts, $\alpha_{[PF]}$ the Type I error per contrast and *C* the sum of contrasts. Such a correction is particularly important with respect to orthogonality regarding the independence of the contrasts (Ruxton and Beauchamp 2008).

To answer the research questions (Q1-Q4b), 6 of the 11 degrees of freedom were partitioned to the following straightforward comparisons where Q_x = component A *vs*. component B (* indicates interaction). Each predefined question was tested by the contrasts shown in the below corresponding simple contrast (Q3b, Q4b) or complex contrast (Q1, Q2, Q3a, Q4a) null hypothesis (H_0). The standard form of each population contrast is indicated by the equation gamma (γ), where μ indicates each mean. It should be noted that preliminary analysis of the data (Q1) confirmed that EDU by itself had no effects on AOZ plants, as expected based on prior suggestions (Manning et al. 2011; Agathokleous et al. 2015b). Thus, to make more robust estimates of Q2, the EDU200*AOZ and EDU400*AOZ treatments were considered EDU0*AOZ. Questions 3 and 4 were partitioned into two questions each.

Q1: Is the mean of plants treated with 200 or 400 mg EDU L^{-1} different from those treated with 0 mg EDU L^{-1} in AOZ?

H_0: Mean ($EDU0_{DRENCH*}AOZ$ + $EDU0_{SPRAY*}AOZ$) = Mean ($EDU200_{DRENCH}$*AOZ + $EDU400_{DRENCH}$*AOZ + $EDU200_{SPRAY}$*AOZ + $EDU400_{SPRAY}$*AOZ), that is

$\gamma_1=(1/2)\mu_1+(1/2)\mu_2+(-1/4)\mu_3+(-1/4)\mu_4+(-1/4)\mu_5+(-1/4)\mu_6$

Q2: Is the mean of EOZ plants different from the mean of AOZ plants?

H_0: Mean ($EDU0_{DRENCH*}EOZ$ + $EDU0_{SPRAY*}EOZ$) = Mean ($EDU0_{DRENCH}$*AOZ + $EDU200_{DRENCH}$*AOZ + $EDU400_{DRENCH}$*AOZ + $EDU0_{SPRAY}$*AOZ + $EDU200_{SPRAY}$*AOZ + $EDU400_{SPRAY}$*AOZ), that is

$\gamma_2=(1/2)\mu_1+(1/2)\mu_2+(-1/6)\mu_3+(-1/6)\mu_4+(-1/6)\mu_5+(-1/6)\mu_6+(-1/6)\mu_7+(-1/6)\mu_8$

Q3a: Is the mean of plants treated with 200 ml soil drench of 200 or 400 mg EDU L^{-1} comparable to those treated with 0 mg EDU L^{-1} in EOZ?

H_0: Mean ($EDU200_{DRENCH}$*EOZ + $EDU400_{DRENCH}$*EOZ) = Mean ($EDU0_{DRENCH*}$EOZ), that is

$\gamma_{3\alpha}=(1/2)\mu_1+(1/2)\mu_2+(-1)\mu_3$

Q3b: Is the mean of plants treated with 200 ml soil drench of 400 mg EDU L^{-1} comparable to those treated with 200 mg EDU L^{-1} in EOZ?

H_0: Mean ($EDU400_{DRENCH*}$EOZ) = Mean ($EDU200_{DRENCH}$*EOZ), that is

$\gamma_{3b}=(1)\mu_1+(-1)\mu_2$

Q4a: Is the mean of plants treated with foliar spray of 200 or 400 mg EDU L^{-1} comparable to those treated with 0 mg EDU L^{-1} in EOZ?

H_0: Mean ($EDU200_{SPRAY}$*EOZ + $EDU400_{SPRAY}$*EOZ) = Mean ($EDU0_{SPRAY*}$EOZ), that is

$\gamma_{4\alpha}=(1/2)\mu_1+(1/2)\mu_2+(-1)\mu_3$

Q4b: Is the mean of plants treated with 200 ml soil drench of 400 mg EDU L^{-1} comparable to those treated with 200 mg EDU L^{-1} in EOZ?

H_0: Mean ($EDU400_{SPRAY*}$EOZ) = Mean ($EDU200_{SPRAY}$*EOZ), that is

$\gamma_{3b}=(1)\mu_1+(-1)\mu_2$

According to homoscedasticity (Levene's test), in 7.4% of the cases the H_0 was rejected and therefore the *P* values were calculated with correction assuming unequal variance.

Since the prior results (Q3a-Q4b) showed no protection of EDU soil drench, it would be meaningless to further test statistically the difference between the two application methods. Hence, Q5 was excluded from further statistical hypothesis testing.

To quantify the effect magnitude for Q2 and Q4a (plant DM) and of EOZ for each of the 18 plant response variables for each experiment (EDU0*EOZ vs. (EDU0*AOZ + EDU200*AOZ + EDU400*AOZ)), the unbiased Cohen δ was estimated (Hedges and Olkin 1985; as described in

Agathokleous et al. 2016d). The effect magnitude was arbitrarily classified as neutral (δ=[0.00, 0.50)), small (δ= [0.50, 1.50)), moderate (δ= [1.50-3.00)) or large (δ=3.00+) (Cohen 1988; Agathokleous et al., 2016b). Absolute δ values in the interval [0.50-1.50] indicate educational significance while δ values >1.50 indicate practical significance (Wolf 1986; Agathokleous et al. 2016b).

Data management and statistical analyses were performed with MS EXCEL 2010 (© Microsoft) and PASW Statistics 18 (formerly SPSS Statistics, IBM ©) software.

3. RESULTS

With regard to the *a priori* comparisons set as Q1 to Q4b, the orthogonal contrast test returned the following results:

Q1 tested if EDU affected the plants in the absence of O_3 exposure (AOZ). H_0 was accepted (α=0.0085) for all response variables in this species (Table 1, Fig 1-3) suggesting that EDU by itself did not affect *S. sachalinensis* plants when grown in infertile and organic-matter-free soil under ambient conditions. There was only a trend (P<0.05) towards increased shoot DM and lower number of shoots (Table 1, Fig 2).

Q2 tested if EOZ alone affected the plants in the absence of EDU exposure (0 mg EDU L^{-1}). H_0 was rejected (α=0.0085) for all leaf traits variables (Table 1, Fig 1), crown width, shoots DM (total DM of shoots per plant), foliage DM, aboveground DM and plant DM (Table 1, Fig 3), suggesting a significant effect of EOZ on *S. sachalinensis* plants grown in infertile and organic-matter-free soil. EOZ did not affect the shoot traits (Table 1, Fig 2). EOZ led to decreased number of leaves, average leaf size, average leaf DM, plant leaf area, crown width and foliage DM (Table 1, Fig 1-3). It further led to reduced DM of shoot and aboveground DM. There was a trend for root DM reduction (P<0.05) by EOZ as well. As a result, there was a small effect of EOZ on plant DM (δ = -1.43, CI [-3.15, -0.28]); however, the biomasses of aboveground and belowground parts were equally suppressed by EOZ as indicated by the shoot:root ratio (S/R=1.18±0.16 for AOZ and 1.23±0.07 for EOZ). The effect magnitude of EOZ on plant DM was close to moderate and very close to the conservative margin for practical significance. Still, δ of the 18 plant response variables was -1.63±0.36 in 2014 and -1.39±0.35 in 2015, showing no difference in the effect magnitude of EOZ. The average δ of the two experiments across all the 18 plant response variables was -1.51, indicating an overall moderate effect of EOZ on plants which is of practical significance.

Q3a tested if EOZ plants treated with soil drench of 200 and 400 mg EDU L^{-1} had similar performance with those treated with 0 mg EDU L^{-1}. H_0 was rejected (α=0.0085) only for number of leaves (Table 1, Fig 1), evidencing that, for all the other response variables, the means of plants treated with 200 ml soil drench of 200 and 400 mg EDU L^{-1} were comparable to those treated with 0 mg EDU L^{-1} in EOZ. Thus, there was a trend for lower foliage DM (P<0.05) and plant leaf area (P=0.058) in plants treated with 0 mg EDU L^{-1} than those treated with 200 or 400 mg EDU L^{-1} (Table 1, Fig 1).

Q3b tested if the performance of EOZ plants treated with soil drench of 400 mg EDU L^{-1} differed from that of EOZ plants treated with 200 mg EDU L^{-1}. H_0 was accepted (α=0.0085) for all plant response variables (Table 1, Fig 1-3), evidencing that the means of plants treated with 200 ml soil drench of 400 mg EDU L^{-1} were comparable to those treated with 200 mg EDU L^{-1} in EOZ. However, there was a trend for increased (P<0.05, Table 1) number of shoots (Fig 2) and crown width (Fig 3) in plants treated with 400 mg EDU L^{-1} than those treated with 200 mg

EDU L^{-1}. In addition, there was an insignificant decrease (P=0.066) in shoot diameter (Table 1, Fig 2) in plants treated with 400 mg EDU L^{-1} than those treated with 200 mg EDU L^{-1}.

Q4a tested if EOZ plants treated with foliar spray of 200 and 400 mg EDU L^{-1} had similar performance with those treated with 0 mg EDU L^{-1}. H_0 was rejected (α=0.0085, Table 1) for number of leaves, plant leaf area, average leaf DM (Fig 1) and root DM (Fig 3). Furthermore, average leaf size (Fig 1) and DM of foliage and plant (Fig 3) showed a trend for higher (P<0.05, Table 1) means of plants treated with foliar spray of 200 or 400 mg EDU L^{-1} than those treated with 0 mg EDU L^{-1} in EOZ. Yet, there was an insignificantly higher crown width (16%, Fig 3), shoots DM (16%, Fig 3) and aboveground DM (18%, Fig 3) of EOZ plants treated with 200 or 400 mg EDU L^{-1} than those treated with 0 mg EDU L^{-1} (Table 1). H_0 was accepted (α=0.0085) for all the response variables of shoot traits (Table 1, Fig 2). The effect magnitude of EDU on plant DM was close to moderate (δ = 1.41, CI [0.45, 2.59]) and very close to the conservative margin for practical significance.

Q4b tested if the performance of EOZ plants treated with foliar spray of 400 mg EDU L^{-1} differed from that of EOZ plants treated with 200 mg EDU L^{-1}. H_0 was accepted (α=0.0085) for all the plant response variables (Table 1, Fig 1-3), with the means being similar between the components, proving that the means of plants treated with foliar spray of 400 mg EDU L^{-1} were indifferent from those treated with 200 mg EDU L^{-1} in EOZ. Only a trend was observed towards lower shoot-stem angle (Table 1, Fig 2) of EOZ plants treated with 400 mg EDU L^{-1} than those treated with 200 mg EDU L^{-1}, which, however, was insignificant (P>0.05). Except the shoot-stem angle, there was no difference between plants treated with 200 mg EDU L^{-1} and those treated with 400 mg EDU L^{-1} in EOZ.

As to the meteorological conditions, average air temperature and maximum air temperature were 0.1 and 0.3 ^oC higher in 2014 than in 2015 while minimum air temperature was 0.3 ^oC lower in 2014 than in 2015 (Table 2). Wind speed was 0.1 m s^{-1} lower in 2014 compared to 2015 and relative humidity was indifferent between years. Sunshine duration was 17.2 h longer and precipitation 20 mm higher in 2014 than in 2015. Moreover, the average daily PPFD, as measured within the experimental plots, was 161.7 ±6.8 μmol m^{-2} s^{-1} (n=6) in 2014 and 141.6 ±13.9 μmol m^{-2} s^{-1} (n=6) in 2015. These variations in meteorological conditions were not biologically significant (both for O_3 and EDU effects) as the effect magnitude of EOZ was indifferent between 2014 and 2015. In addition, these variations were insignificant for comparison between the two EDU application methods due to the binomial effect of the methods ("failure" of soil drench and "success" for foliar spray).

4. DISCUSSION

At low ambient O_3 levels which are not expected to impact plants (AOZ), the present findings confirm suggestions made by Manning et al. (2011) and Agathokleous et al. (2015b) for absence of EDU-induced side effects on plants when EDU is applied in the appropriate range of doses (Q1). Regarding the trend of EDU-treated plants in AOZ towards increased shoot DM (DM per shoot) and decreased number of shoots, *i.e.* more biomass to be allocated to fewer shoots, it should be taken into account that shoots were formed before the exposure to the treatments. Thus, these observations are likely due to pre-treatment differences since plants were allocated to the treatments based on number of leaves. Further, our findings support recent evidence on the absence of EDU side effects in the range of 150-300 mg L^{-1} when hydrophyte communities (*Lemna minor* L.) were treated with EDU in an O_3-free atmosphere (Agathokleous et al. 2016c).

EOZ impacted all leaf traits (Q2) that are common targets of O_3 phytotoxicity (Agathokleous et al. 2016a). *Salix sachalinensis* unfolds and sheds leaves over a long time during the growing season (Ueno et al. 2006). In our experiments, self-shedding of leaves started early in the growing season. At the final harvest, the AOZ-treated plants had approximately three times lower number of leaves than that at the beginning of EDU treatments because new leaves were no longer produced at the end of the season (i.e. preparation for over wintering). EOZ-treated plants, however, had a lower number of leaves than AOZ-treated plants. Ozone-induced accelerated leaf senescence is a phenomenon which has been often observed and is considered a characteristic symptom of O_3-caused phytotoxicity (Iriti and Faoro 2008; Paoletti et al. 2009; Agathokleous et al. 2015a). The lower average leaf size and DM suggests that each leaf of EOZ-exposed plants had less photosynthetic area than each leaf of AOZ-exposed plants. Unaffected S/R allometry is in agreement with 68% out of 104 reviewed cases of trees where there was no significant EOZ-induced change in S/R and in disagreement with 5% of cases where S/R was significantly reduced and 27% where S/R was significantly increased (Agathokleous et al. 2016a). No effect of EOZ on shoot traits was due to the fact that the shoots were well-developed before the treatments started.

EDU did not protect against EOZ-induced injury to this species when applied as soil drench, either at 200 or at 400 mg L^{-1} (Q3a and Q3b). EDU protected only against EOZ-induced accelerated senescence, as it is indicated by a higher number and DM of leaves and by an insignificant trend towards higher plant leaf area in plants treated with 200 or 400 mg EDU L^{-1} than those treated with 0 mg EDU L^{-1}. The impact of EOZ on leaf size and DM, root DM, shoots DM, aboveground DM and plant DM was similar in plants treated with 0 or 200 or 400 mg EDU L^{-1}. Less sink of photosynthetic products, indicated by lower average leaf size or DM, led to reduced biomass production. The only differences between plants treated with 400 mg EDU L^{-1} and those treated with 200 mg EDU L^{-1} were increased number of shoots (P<0.007) and crown width (P<0.050) in plants treated with 400 mg EDU L^{-1} than those treated with 200 mg EDU L^{-1}, which should be attributed to pretreatment differences as explained above.

In contrast to previous experiments where tree plants were treated with EDU soil drench (Paoletti et al. 2010, 2011; Hoshika et al. 2013; Carriero et al. 2015), this experiment was conducted with current-year cuttings grown in infertile soil. The plant leaf area of these fast-growing plants was higher early in the treatments than it was at harvest when the autumn senescence was at the final stages, as it is indicated by the 63 leaves at first EDU application and the higher amount of EDU needed for the spray treatments in the second experiment. We thus postulate that EDU as a soil drench was not enough for the high plant leaf area early in the treatments.

As observed for EDU applied as soil drench, EDU protected against EOZ-induced accelerated senescence in this species when applied as foliar spray at 200 and 400 mg L^{-1} (Q4a and Q4b), as indicated by number of leaves, plant leaf area and foliage DM. A loss of leaves was more obvious around the middle of October, when the air temperature dropped suddenly to very low levels. This observation is supported by the more than two times higher amount of EDU needed to spray the plants at the semi-final EDU treatment, compared to the final one. The harvest was done at the end of the growing season when plants stopped producing new leaves and, therefore, cannot be proved if plants treated with spray of 200 and 400 mg EDU L^{-1} compensated the accelerated leaf senescence by producing more leaves during the growing season (Kolb and Matyssek 2001). The reviews by Paoletti et al. (2009) and Singh et al. (2015) suggested that EDU delays the O_3-induced accelerated senescence and this coincides with the findings of the present study. However, the fact that EDU soil drench protected against EOZ-induced accelerated senescence while did not protect against EOZ damage to all the other response variables (which are not related to the leaf number) indicates that either the EDU mode of action in protecting against O_3 injury is not upon protecting against O_3-accelerated senescence –which is in agreement with suggestions by Eckardt and Pell (1996)- or EDU protection against EOZ injury was not complete – as reported also by Paoletti et al. (2007). The higher biomass production of plants treated with foliar spray of 200 or 400 mg EDU L^{-1} than those treated with 0 mg EDU L^{-1} and the indifferent biomass production of plants treated with foliar spray of 200 mg EDU L^{-1} and those treated with 400 mg EDU L^{-1} in EOZ, suggest that EDU can reduce O_3-induced damage to plants of this species in the range of EDU doses 200-400 mg L^{-1}.

In our case, the amount of EDU was the same when applied as spray and as soil drench and this evidences that no more EDU is needed when applied as foliar spray to current-year plants of fast growing species grown under conditions like those in our experiment (Q5). When the plant leaf area was relatively low, *i.e*. at the final EDU application, the amount of EDU needed for foliar spray was 2.3 times lower than that needed for soil drench, showing that EDU foliar spray is more appropriate –in terms of financial cost- than EDU soil drench for plants with small leaf area.

5. CONCLUSIONS

We conclude that EDU *per se*, at the studied dosages and doses, did not affect *S. sachalinensis* plants grown in infertile and organic-matter-free soil, while exposure to EOZ did cause an overall moderate negative effect which is of practical significance.

Ten EDU soil-drench applications at a dosage of 200 ml with 200 or 400 mg L^{-1} every nine days, apart from delaying O_3-induced accelerated senescence, did not protect this species against EOZ impact. On the other hand, ten EDU spray applications at a dosage of 200 or 400 mg L^{-1} every nine days protected this species against EOZ impact. Thus, foliar applications in the range of concentrations 200-400 mg EDU L^{-1} at the used dosage can be used for biomonitoring purposes with efficient protection against EOZ-caused phytotoxicity and without effects on plants of this fast-growing species.

Salix sachalinensis, in contrast to previous EDU literature, can be found both in remote (e.g. forests, across rivers etc.) and urban areas. Thus, it can be effectively used as an ecological indicator for O_3 biomonitoring purposes and O_3 risk assessment in Japan, north-east China, North Korea and Russian Far East. We present all the necessary information for such use, from EDU application method to EDU doses.

When EDU is used as a research tool, it is recommended to be applied as foliar spray instead of soil drench to plants of small size (small plant leaf area as in our case at the final application) for economy and for minimizing the error that could be caused due to the influence of soil since EDU should cycle from soil up to the leaves. However, for adult trees of larger size and with more foliage while more EDU is expected to be needed when applied both as foliar spray and soil drench (Paoletti et al. 2011), much more time would be needed for foliar spray application and it could be practically prohibitive to tall trees, unless motorized vehicles are available, which increases the financial cost in turn.

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Captions

Table 1 Results of statistical hypotheses testing. Six contrasts (Q1, Q2, Q3a, Q3b, Q4a, Q4b) were applied to answer six out of seven questions regarding comparisons which were defined *a priori*. The questions were: Is the mean of *Salix sachalinensis* plants treated with 200 or 400 mg EDU L^{-1} different from those treated with 0 mg EDU L^{-1} in ambient ozone (AOZ)? (Q1); Is the mean of elevated ozone (EOZ) plants different from the mean of AOZ plants in the absence of EDU treatment? (Q2); Is the mean of plants treated with 200 ml soil drench of 200 or 400 mg EDU L^{-1} comparable to those treated with 0 mg EDU L^{-1} in EOZ? (Q3a); Is the mean of plants treated with 200 ml soil drench of 400 mg EDU L^{-1} comparable to those treated with 200 mg EDU L^{-1} in EOZ? (Q3b); Is the mean of plants treated with foliar spray of 200 or 400 mg EDU L^{-1} comparable to those treated with 0 mg EDU L^{-1} in EOZ? (Q4a); Is the mean of plants treated with foliar spray of 400 mg EDU L^{-1} comparable to those treated with 200 mg EDU L^{-1} in EOZ? (Q4b); Which application method is more appropriate for protecting this fast growing species against O_3 phytotoxicity? (Q5) The last question was not statistically tested due to no protection of EDU soil drench.

Table 2 Monthly and experimental-period means of the main meteorological conditions at Sapporo, Japan, for the months August-October, of the years 2014-2015.

Fig 1 Arithmetic means (± s.e.) of leaf-level traits of *Salix sachalinensis* plants treated with 0, 200 or 400 mg EDU L^{-1} and exposed to ambient O_3 (A) or elevated O_3 (E) levels. In a growing season EDU was applied as soil drench and in the next growing season, following the same protocol, EDU was applied as foliar spray, to different plants.

Fig 2 Arithmetic means (± s.e.) of shoot-level traits of *Salix sachalinensis* plants treated with 0, 200 or 400 mg EDU L^{-1} and exposed to ambient O_3 (A) or elevated O_3 (E) levels. In a growing season EDU was applied as soil drench and in the next growing season, following the same protocol, EDU was applied as foliar spray, to different plants.

Fig 3 Arithmetic means (± s.e.) of plant-level dimensions and dry masses (DM) of *Salix sachalinensis* plants treated with 0, 200 or 400 mg EDU L^{-1} and exposed to ambient O_3 (A) or elevated O_3 (E) levels. In a growing season EDU was applied as soil drench and in the next growing season, following the same protocol, EDU was applied as foliar spray, to different plants.

Table 1

	Q1	Q2	Q3a	Q3b	Q4a	Q4b
Leaf traits (leaf level)						
Number of leaves	*t*=2.112, *P*=0.074	*t*=14.418, *P*<0.001	*t*=14.235, *P*<0.001	*t*=0.866, *P*=0.420	*t*=4.092, *P*=0.006	*t*=1.376, *P*=0.218
Leaf size	*t*=1.707, *P*=0.101	*t*=6.328, *P*<0.001	*t*=0.404, *P*=0.700	*t*=1.100, *P*=0.314	*t*=3.337, *P*=0.016	*t*=0.355, *P*=0.735
Plant leaf area	*t*=1.293, *P*=0.208	*t*=7.059, *P*<0.001	*t*=2.338, *P*=0.058	*t*=0.619, *P*=0.559	*t*=4.339, *P*=0.005	*t*=0.057, *P*=0.956
Leaf DM	*t*=1.683, *P*=0.105	*t*=4.444, *P*<0.001	*t*=0.075, *P*=0.943	*t*=1.087, *P*=1.319	*t*=12.367, *P*=0.006	*t*=0.691, *P*=0.516
Shoot traits (shoot level)						
Number of shoots	*t*=2.181, *P*=0.039	*t*=0.402, *P*=0.700	*t*=0.333, *P*=0.750	*t*=2.887, *P*=0.028	*t*=0.007, *P*=0.995	*t*=0.105, *P*=0.920
Shoot DM	*t*=2.688, *P*=0.013	*t*=0.882, *P*=0.386	*t*=0.901, *P*=0.402	*t*=1.028, *P*=0.344	*t*=0.727, *P*=0.540	*t*=1.270, *P*=0.251
Shoot length	*t*=2.015, *P*=0.055	*t*=0.546, *P*=0.604	*t*=0.862, *P*=0.422	*t*=1.072, *P*=0.325	*t*=0.513, *P*=0.626	*t*=0.293, *P*=0.779
Shoot diameter	*t*=2.040, *P*=0.071	*t*=1.902, *P*=0.069	*t*=0.033, *P*=0.975	*t*=2.244, *P*=0.066	*t*=0.489, *P*=0.642	*t*=0.434, *P*=0.680
Shoot angle	*t*=0.612, *P*=0.546	*t*=1.064, *P*=0.298	*t*=0.087, *P*=0.933	*t*=0.930, *P*=0.388	*t*=0.245, *P*=0.815	*t*=1.834, *P*=0.116
Number of buds	*t*=0.792, *P*=0.436	*t*=0.428, *P*=0.673	*t*=0.345, *P*=0.742	*t*=0.679, *P*=0.522	*t*=0.069, *P*=0.947	*t*=0.894, *P*=0.406
Plant traits (plant level)						
Crown length	*t*=1.750, *P*=0.093	*t*=0.380, *P*=0.707	*t*=0.468, *P*=0.657	*t*=1.292, *P*=0.209	*t*=0.808, *P*=0.450	*t*=1.175, *P*=0.284
Crown width	*t*=1.395, *P*=0.176	*t*=5.287, *P*<0.001	*t*=0.881, *P*=0.412	*t*=2.895, *P*=0.028	*t*=2.392, *P*=0.054	*t*=0.719, *P*=0.499
Root DM	*t*=1.780, *P*=0.123	*t*=3.060, *P*=0.042	*t*=0.836, *P*=0.435	*t*=1.336, *P*=0.230	*t*=5.180, *P*=0.002	*t*=1.000, *P*=0.423
Stem DM	*t*=0.867, *P*=0.395	*t*=1.599, *P*=0.123	*t*=0.947, *P*=0.380	*t*=0.615, *P*=0.561	*t*=0.200, *P*=0.848	*t*=1.139, *P*=0.298
Shoots DM	*t*=1.331, *P*=0.196	*t*=3.145, *P*=0.004	*t*=0.389, *P*=0.711	*t*=1.189, *P*=0.279	*t*=1.884, *P*=0.109	*t*=0.507, *P*=0.630
Foliage DM	*t*=0.897, *P*=0.379	*t*=7.855, *P*<0.001	*t*=3.112, *P*=0.021	*t*=0.810, *P*=0.449	*t*=3.561, *P*=0.012	*t*=0.308, *P*=0.768
Aboveground DM	*t*=0.847, *P*=0.406	*t*=4.442, *P*<0.001	*t*=1.007, *P*=0.353	*t*=0.693, *P*=0.514	*t*=2.169, *P*=0.137	*t*=0.698, *P*=0.511
Plant DM	*t*=0.462, *P*=0.658	*t*=5.337, *P*<0.001	*t*=1.037, *P*=0.340	*t*=0.685, *P*=0.519	*t*=3.515, *P*=0.013	*t*=0.533, *P*=0.613

Note: Data were collected from *Salix sachalinensis* plants treated with 0, 200 or 400 mg EDU L^{-1} and exposed to ambient or elevated O_3 levels (N=144). In a growing season EDU was applied as soil drench and in the next growing season, following the same protocol, EDU was applied as foliar spray.

Table 2

	2014				2015			
	August	September	October	Mean	August	September	October	Mean
Daily average air temperature (^oC)	22.4	18.1	11.3	17.3	22.4	18.4	10.8	17.2
Daily maximum air temperature (^oC)	26.6	22.8	15.7	21.7	26.4	22.5	15.2	21.4
Daily minimum air temperature (^oC)	19.0	14.1	7.0	13.4	19.4	14.9	6.7	13.7
Daily wind speed ($m\ s^{-1}$)	3.1	3.3	3.2	3.2	3.0	2.8	4.0	3.3
Daily relative humidity (%)	73	68	64	68.3	73	71	61	68.3
Total sunshine duration (h)	178.9	188.8	145.4	171.0	158.6	151.8	150.9	153.8
Total precipitation (mm)	217.5	146.0	124	162.5	131.5	198.0	98.0	142.5