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High doses of ethylene diurea (EDU) are not toxic to willow and act as nitrogen fertilizer

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ABSTRACT

Ethylene diurea (EDU) is synthetic chemical which protects plants against damage caused by ground level O₃ and is used experimentally as a biomonitoring tool at doses usually ranging from 200 to 400 mg L⁻¹ a.i. Although several studies have investigated the protective action of EDU, this mechanism remains unclear. Important uncertainties in EDU action are whether EDU acts as a source of nitrogen (N) to plants and whether high doses are phytotoxic. In order to answer these questions, we conducted an open-field experiment where potted willow (*Salix sachalinensis* Fr. Schm) plants were exposed to ambient O₃ conditions and treated with 0, 800 or 1600 mg L⁻¹ EDU as a soil drench, every nine days, for about 2.5 months. We examined approximately 50 response variables. Based on N content in different plant organs, we found that (a) all EDU was transferred to the leaves and (b) high doses of EDU increased the leaf N content. However, EDU did not affect the C content and distribution within the plant body. Still, even at the highest dose, EDU was not toxic to this fast-growing species (however such a high dose should not be applied in uncontrolled environments); and there was no EDU persistence in the soil, as indicated by soil N content. Notably, our soil was free from organic matter and N-poor.

Keywords: air pollution, antiozonant, C₄H₁₀N₄O₂, ethylenediurea, N-[-2-(2-oxo-1-imidazolidinyl) ethyl]-N'-phenylurea], ozone

Key message: EDU *per se* does not cause toxicity to willow plants when applied as drench to a soil with no organic matter, rather, high EDU doses may act as nitrogen fertilizer in a nitrogen-poor soil.

1. INTRODUCTION

Elevated ozone (O₃) concentrations at the lower troposphere (Doche et al. 2014; Paoletti et al. 2014a; Akimoto et al. 2015; Kalabokas et al. 2015) are phytotoxic to plant ecosystems (Agathokleous et al. 2015a, 2016a; Vaultier and Jolivet 2015; Sicard et al. 2016; Wang et al. 2016). Ambient O₃ is not only produced by photochemical reactions but also transported across regions, or even continents (Ganev et al. 2008; Kopanakis et al. 2015; Verstraeten et al. 2015). Because of the transboundary nature of O₃, the development of control measures is difficult. It is therefore critical to find measures for the protection of plants against O₃ toxicity.

Several substances have been tested as to their efficacy to protect plants against O₃-induced damage (e.g. Manning et al. 1973; Spotts et al. 1975; Gimeno et al. 1999; Agathokleous et al. 2014; Saitanis et al. 2015), including a few environmentally-friendly substances (e.g. Agathokleous et al. 2016b). Ethylene diurea (abbreviated EDU) is the most widely studied and effective phytoprotectant against O₃ oxidative stress (Agathokleous et al. 2015b). Almost four decades have passed since Carnahan et al. (1978) reported that the antiozonant EDU, which was described by Wat (1975) as N-[-2-(2-oxo-1-imidazolidinyl) ethyl]-N'-phenylurea], was effective in protecting plants against O₃. After the report by Carnahan et al. (1978), there was an initial increasing interest in EDU research in assessing O₃ effects on plants, while later the interest declined due to misconceptions and erroneous conclusions by part of the air pollution research community (as reviewed in Manning et al. 2011). Recently, this interest has increased again, with numerous studies conducted in Europe and Asia (Hoshika et al. 2013; Paoletti et al. 2014b; Carriero et al. 2015; Agathokleous et al. 2015b; Singh et al. 2015).

EDU has four nitrogen (N) atoms and thus almost 22 % of its molecule is N (Manning et al. 2011). The N free electron pairs may produce an affinity to the greatly electrophilic O₃ (Manning et al. 2011). Thus, there have been speculations about EDU contribution to plants as N fertilizer (see the review by Manning et al. 2011).

In addition, assertions were made that chronic exposure of plants to EDU soil drench may lead to phytotoxicity. However, such conclusions were based on experiments with growing substrates containing organic matter, which confounds the EDU applied doses (Manning et al. 2011; Agathokleous et al. 2015b; Pasqualini et al., 2016). A recent toxicological study with the hydrophyte *Lemna minor* L. in EDU water solution, revealed that EDU concentrations ≥ 600 mg L⁻¹ may be phytotoxic (Agathokleous et al. 2016c). Indeed, it remains unclear whether or not EDU *per se* is phytotoxic to higher terrestrial plants when applied as soil drench.

This experiment was designed to investigate if chronic soil-drenched EDU exposure can cause phytotoxicity and if high doses of EDU can act as N fertilizer after chronic treatment. We used willow (*Salix sachalinensis* Fr. Schm) as an experimental plant because it is a very fast growing species (Koike et al. 1995) and can grow on nutrient-poor substrates (Haruki and Tsuyuzaki 2001). We applied EDU as soil drench because spray application of EDU is impractical for fast growing species while soil drench application is more appropriate (Ainsworth et al. 1996; Paoletti et al. 2011).

2. MATERIALS AND METHODS

2.1. Experimental site

The experiment was conducted at Sapporo Experimental Forest of Hokkaido University, Japan (43°04' N, 141°20' E, 15 m a.s.l.), in 2014. The snow-free period lasted from early-May to mid-November. Meteorological data were recorded at a station located in Sapporo (WMO, ID:

47412) at 43°03.6'N 141°19.7'E (Japan Meteorological Agency, 2016). For the period May-October, the main meteorological conditions (mean $\pm$ s.e. in parenthesis) were: mean monthly average of air temperature = 17.83 ($\pm$ 1.83) °C; daily maximum temperature = 22.52 ($\pm$ 1.75) °C; daily minimum temperature = 14.07 ($\pm$ 2.02) °C; wind speed = 3.52 ($\pm$ 0.17) m s⁻¹; relative humidity = 68.83 ($\pm$ 1.58) %; mean monthly total sunshine duration = 186.25 ($\pm$ 9.98) h; and mean monthly precipitation 120.50 ($\pm$ 23.19) mm, respectively. Ambient O₃ concentration at the experimental area was continuously monitored by an ultraviolet (UV) absorption O₃ analyzer (TUV-1100; Tokyo Industries Inc., Tokyo, Japan), which was recording one observation per minute for the period July 29th to October 17th. Data were averaged per hour, and the 8-hour, 08:00 am – 16:00 pm, JST, data were further averaged per day. The mean O₃ concentration for the 80 days was 23.6 $\pm$ 0.7 nmol mol⁻¹. For the same period, the index of Accumulated exposure to O₃ Over the Threshold of 40 nmol mol⁻¹ (AOT40, Mills et al. 2007) was 104.9 nmol mol⁻¹ h.

2.2. Plant material & Design of the Experiment

One hundred fifty current-year uniform cuttings of *S. sachalinensis* (= *S. udensis* Trautv. et C.A. Mey.), originated from the river basin of Ebetsu City, near Sapporo, were obtained from the Hokkaido Horti-Tree Planting Center, Co. Ltd. Height and basal diameter were 12.09 ($\pm$ 0.25) and 1.90 ($\pm$ 0.05) cm, respectively. The cuttings were stored in an incubator, at 0-4 °C, for one 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; commonly found in Hokkaido (Schmincke 2004; Kam et al. 2015); see also Eguchi et al. (2008) for chemical and physical properties of these soils, but from different origin. Cuttings were planted for rooting on May 13th, and kept under field conditions. On June 19th, when the cuttings were

well rooted, 40 of them were selected for uniformity based on total leaf number (45 ± 3) and height (13.5 ± 0.2 cm), transplanted into 15 L pots filled with the same substrate mixture, and left in the field until establishment and full adaptation. On July 28th, a day before the first EDU application, the 40 potted plants were transferred to three different plots (13-14 pots per plot) scattered across the experimental forest, and four to five plants were randomly assigned to each EDU treatment in each plot. All the pots within each plot were subjected to a monthly rotation and the plots were interchanged three times. The plants were neither fertilized nor treated by agrochemicals during the experiment. Visible injury by pests or pathogens was very rarely observed. Irrigation with tap water was carried out on June 1, 2 and 3, due to unusual increases of the air temperature for the season (>30 °C), on June 19 (just after transplantation) and on June 23 and 28 (to ensure the plant establishment).

The characteristics of the morphology of this species when grown from cuttings are schematically represented in Koike et al. (1995).

2.3. EDU treatments

Three concentrations of EDU were selected for testing: 0 (control), 800 and 1600 mg L⁻¹. According to a meta-analysis, soil-drenched EDU has the highest positive effect on plants grown in the field when applied at the concentrations range of 200-400 mg L⁻¹ (Feng et al. 2010). The two selected concentrations were 2-fold and 4-fold the maximum concentration of 400 mg L⁻¹. EDU was always prepared 15-30 minutes prior to its application by gently warming and continuously stirring until full dilution of 100% a.i. EDU (source: W.J. Manning, University of Massachusetts, Amherst, MA, USA) in pH 6.5 water. Surfactant was not used.

The first EDU soil drench was applied on July 29th (6 weeks after the transplanting), and repeated at 9-day intervals. This interval was selected because EDU may persist in the leaf

apoplast for more than eight days (Paoletti et al. 2009). The last EDU application was on October 10th, for a total of 9 applications. The applied doses were 200 mL solution plant⁻¹ per application, and the applications were always conducted during afternoon hours. The total amount of EDU applied was $\approx 4.68 \text{ kg ha}^{-1}$ for the 1600 mg EDU L⁻¹ treatment which, based on Manning et al. (2011), equals to $\approx 1.02 \text{ kg N ha}^{-1}$; half of these amounts were applied for the 800 mg EDU L⁻¹ treatment.

2.4. Measurements & Samplings

2.4.1. Pre-treatment

A day before the first EDU application (July 28th), measurements were taken for baseline (pre-treatment) assessment. The mean plant height (from soil surface to the top of canopy), the mean crown spread (the distance of the two remotest points as observed vertically from above the canopy) and the mean shoot diameter (measured at the point where shoot is attached on the cutting) were measured.

In addition, the soil plant analysis development (SPAD) value, which indicates leaf greenness, and the estimated N content per leaf area (N_{PPW}) were non-destructively measured, using the portable chlorophyll meter SPAD-502 (Konica-Minolta, Osaka, Japan) and Agriexpert PPW-3000 (Satake Corp., Hiroshima, Japan) devices, respectively (Ichie et al. 2002; Eguchi et al. 2006). For each plant, two fully sun-exposed leaves from the upper canopy (3rd or 4th from the top) of two different shoots were measured (totally 4 leaves per plant) and averaged so as to give one more robust value. Based on DMSO extracts (as described below), SPAD correlated with measured chlorophyll *a* content ($y = 0.5234x - 0.4106$, $R^2 = 0.5147$, $\text{adj}R^2=0.504$, $p<0.001$) but not with chlorophyll *b* content ($y = 0.0221x + 4.3437$, $R^2 = 0.0034$, $\text{adj}R^2=-0.019$, $p=0.697$) (data not shown). Therefore, SPAD indicated the chlorophyll *a* content in the present study.

2.4.2. Post-treatment

A week after the last EDU application (October 17th) and for two days, measurements of growth and samplings were carried out. SPAD and N_{PPW} measurements were taken earlier, on October 8th.

Growth

The growth characteristics measured were the same as those of the baseline. In addition, the dimensions of each leaf (length and width) were measured by a caliper. Each leaf was destructively collected and put into an envelope for dry mass measurement.

From the total number of collected leaves, 47 of various sizes were selected and directly photographed on a fixed base. Then, the leaf length (L), the leaf width (W) and the leaf area of each of the 47 leaves were measured using image analysis software (Adobe Photoshop CS4 Extended v.11, Adobe Systems Incorporated, CA, USA). Leaf area was plotted against the L*W product (predictor). Finally, the regression line was drawn and the regression equation ($y=0.5786x+1.6913$; $R^2 = 0.907$, $_{adj}R^2=0.905$, $p<0.001$) was calculated and used as a model to estimate the size of all the collected leaves. Mean leaf size per plant, mean leaf dry mass (mean leaf DM, g), leaf area per plant (sum of the individual leaves) and specific leaf area (SLA = leaf area / leaf dry mass) were calculated. The leaf area index (LAI, unitless) was calculated for each plant as leaf area per ground area. For the SLA ($m^2 g^{-1}$) calculation, the first 5 leaves from the base were excluded and only the next mature 15 leaves were included.

Shoot height (from stem to the apical meristem), diameter (at the base) and the number of formed buds were measured. Photographs of the shoots were used to calculate the angle between each shoot and the stem (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA,

Schneider et al. 2012). The plant height gain, crown spread gain and shoot diameter gain were calculated by subtracting the post-treatment data from the pre-treatment data. Finally, from the dimensions (diameter and height) of stem, the volume of the stem of each plant was calculated as $\pi \times r^2 \times h$, where $\pi = 3.14$, r = stem radius and h = stem height.

Elemental concentrations

Shed leaves were collected from each plant (shoots were gently shaken and the fallen leaves were collected) to measure the elemental concentrations of P, K, Mg, Ca, Mn, Fe, Ni and Cr. Leaf samples were manually grounded into powder (50 mg) and digested by nitric acid (HNO₃) and hydrogen peroxide (H₂O₂). The analyses were conducted with an Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (Elan, DRC-e; PerkinElmer, Waltham, MA, USA), which provides ultratrace-level detection limits in virtually any sample.

Photosynthetic pigments

From each plant, two fully functioning (mature and well green but not over-mature or senescing) leaves - the 3rd or 4th from the base from two different shoots - were selected for pigment analysis. A circular sample of 0.8 cm diameter was taken from each leaf, immediately stored in liquid nitrogen, and transferred to deep freezer until measurements. Extracts were prepared using DMSO and stored in dark condition at 65 °C until the appearance of a “ghost-like” thallus (Barnes et al. 1992). Measurements were taken for the optical densities (OD) 415, 435, 470, 648, 665 nm (GeneSpec III; Hitachi Genetic Systems; MiraiBio, Alameda, CA). The ODs 435 and 415 were measured to serve as a potential index of phaeophytinization by estimating chlorophyll degradation to phaeopigments (OD₄₃₅/OD₄₁₅), according to Ronen and Galun (1984). The OD 470 was measured to check alterations in total carotenoid content (TCar) following the equation

189 $TCar = (1000A_{470} - 1.90Chl_a - 63.14Chl_b)/214$ by Lichtenthaler (1987); however, it was
190 given for acetone 100 % and therefore the actual values of carotenoid contents may differ. The
191 ODs 648 and 665 were measured to estimate the chlorophyll *a* content (Chl_a), chlorophyll *b*
192 content (Chl_b) and chlorophylls *a+b* content ($TChl$), according to Barnes et al. (1992). The
193 fractions Chl_a/Chl_b and $TChl/TCar$ were calculated as well.

194 **Biomass Production**

195 The root system of each plant was perfectly excavated as a whole, gently washed with tap water
196 and dried in an air-dry oven at constant air temperature of 80 °C, until a constant dry mass.

197 The dry masses of each leaf, shoot, root (Root DM) and stem (Stem DM) were measured. Apart
198 from the mean leaf dry mass (Mean leaf DM) and mean shoot dry mass (Mean shoot DM) per
199 plant, the total foliage dry mass (Foliage DM) and total shoot dry mass (Shoots DM) per plant
200 were calculated. The sum of Foliage DM, Shoots DM and Root DM constituted the total plant
201 dry mass (Plant DM). The Root DM/Foliage DM ratio was also calculated as a biomass
202 partitioning indicator.

203 **SPAD and N_{ppw}**

204 SPAD and N_{ppw} measurements were taken following the same methodology as described for
205 pre-treatment measurements.

206 **N/C analyses**

207 For N/C analyses, soil, fine roots (diameter < 1 mm), shoots and buds were analyzed. For the soil,
208 all the sampled soil from each pot (8 randomly selected pots per EDU treatment) was grounded.
209 From the grounded soil of each plant, two samples were separately analyzed (to give a mean per

plant/pot). To prepare each sample, small soil quantities were selected from 4-5 points of the pooled grounded soil. For fine roots, a sample was prepared from each plant by grounding fine roots from 4-5 different parts of the root system. For the shoots, one shoot was selected from each plant so as the selected shoots being of the same developmental stage. The buds were then removed with a metal needle. The whole shoot (with no buds) was turned into powder using an electrical grinder. From the total grounded sample, one sample was prepared by selecting powder from 4-5 different locations of the whole sample and analyzed for N and C content. The buds of each plant (of the shoot analyzed for N and C content) consisted one sample for N and C analysis, and therefore the mass of each buds sample represent the total dry mass of buds of that particular shoot. Content of N and C was analyzed by using a third generation Vario EL III Element Analyzer (Elementar Analysensysteme GmbH, Hanau-Germany).

2.5. Data handling & Statistics

For response variables where more than one measurement or sample was taken per plant, the values were averaged per plant. The independent applications of the treatments allowed us to consider the individual plant as statistical unit. Therefore, the data from the three plots were pooled and analyzed. Prior to the analysis, data were transformed with BoxCox transformation (Box and Cox 1964) using maximum iterations = 40, minimum $\lambda = -5$, maximum $\lambda = 5$ and $\varepsilon = 0.00001$; more explanation can be found in Agathokleous et al. (2016b). Transformed data were used only for the statistical analyses while for presentation purposes, in the manuscript, the untransformed data are shown.

Due to the experimental design, which employed focused questions about the data that are developed *a priori*, planned comparisons were examined using simple contrasts of Least Squares means. The two degrees of freedom ($k-1$) were partitioned to the contrasts (a) EDU800 vs. EDU0

and (b) EDU1600 vs. EDU0, which tested at an α level of significance that was corrected with Bonferroni technique ($\alpha = 0.017$) to control the experimentwise type I error, due to nonorthogonality, based on Boole's inequality. Orthogonality was sacrificed due to potential hormetic effects of EDU (Agathokleous et al. 2016c) which could lead to masked effects when using contrasts with pooled treatments. *P*-values were rounded to the next 0.001 and those in the range 0.0170-0.0179 were considered marginally significant.

The unbiased Cohen's δ (hereafter δ) was estimated (Hedges and Olkin 1985; Cohen 1988) to quantify the effect magnitude of the EDU800 and EDU1600 treatments by comparing each of them versus the control, EDU0. Yet, the 95% confidence interval of the δ estimate (hereafter CI) was estimated. Further explanations about the formula used for the δ estimation can be found in Agathokleous et al. (2016b). Cohen (1988) suggested that absolute δ values within the arbitrary segments [0.00, 0.20), [0.20, 0.50), [0.50-0.80) and 0.80+ indicate neutral, small, moderate and large *EM*, respectively, and Tallmadge (1977) and Wolf (1986) suggested that absolute δ values in the interval [0.25, 0.50) indicate educational significance while δ values >0.50 indicate practical significance (Tallmadge 1977; Wolf 1986). However, these suggestions were made based on, and applied to, behavioral sciences studies and not to ecophysiological studies. In plant ecophysiological studies, the variance can be small anyway due to factors such as the use of clonal plants and the high precision of assessing ecophysiological characteristics. According to the only available evidence (Agathokleous et al. 2016b; 2016d; 2016e) about the δ *EM* in ecophysiological studies, it is necessary to construct new arbitrary segments which will be more conservative, however, with cumulative evidence they should be changed if proved necessary. The new δ segments are: [0.00, 0.50), [0.50, 1.50), [1.50-3.00) and 3.00+, indicating neutral,

255 small, moderate and large *EM*, respectively, with absolute δ values in the interval [0.50-1.50]
256 indicating educational significance and δ values >1.50 indicating practical significance.

257 In order to test the efficiency in predicting the measured N content in leaves with the estimated
258 N_{ppw} or SPAD, regression analysis was performed at a level of significance $\alpha=0.05$, by using
259 only the data that were available for the same plants for both predictor and criterion variable.

260 Log-transformed concentrations of EDU were used to plot EDU against measured N content in
261 mature leaves. From the data of the three EDU concentrations, data were estimated for 34 EDU
262 concentrations using interpolation on a scale of 50 mg EDU L⁻¹. The interpolation was done
263 based on a constrained version of the Bessel spline function that results to fewer overshoots and
264 oscillations, giving a more constrained smooth fit to data. Two lines were distinguished, one for
265 each of the two EDU intervals (*i.e.* 0-800 and 800-1600) of whose slopes were tested ($\alpha=0.05$)
266 using comparison of regression lines slopes from two independent samples based on pooled
267 variance (Howell 2010).

268 For the analyses of all the response variables, the minimum *n* of each treatment of each variable
269 was 6 and the maximum was 14.

270 Data processing and statistical analyses were conducted using MS EXCEL 2010 (Microsoft ©),
271 PASW Statistics 18 (formerly SPSS Statistics, IBM ©) and STATISTICA v.10 (StatSoft Inc. ©)
272 software.

3. RESULTS

3.1. Pre-treatment assessment

The mean plant height, the mean crown spread and the mean shoot diameter were 51.6 ± 1.2 cm, 28.4 ± 0.8 cm and 3.5 ± 0.1 mm, respectively, while the average number of lateral shoots, leaves per shoot, and leaves per plant were 3.4 ± 0.2 , 21.4 ± 1.2 and 66.6 ± 2.9 , respectively.

No significant differences were observed in SPAD values between EDU0 and EDU800 ($t=-1.510$, $p=0.147$) and between EDU0 and EDU1600 ($t=0.740$, $p=0.468$) and in estimated N content between EDU0 and EDU800 ($t=-0.453$, $p=0.655$) and between EDU0 and EDU1600 ($t=1.589$, $p=0.128$) before treatments (data not shown). However, the plants of EDU1600 had higher diameter (4.1 mm) than those of EDU0 (3.1 mm); $t= 2.673$, $p = 0.0136$.

3.2. Post-treatment assessment

EDU800, vs. EDU0, induced a large increase in plant height gain (Fig 1C), mean leaf DM (Fig 1E), LAI (Fig 1H), mean shoot DM (Fig 2C), shoots angle (Fig 2D), shoots DM (Fig 3C), foliage DM (Fig 3D), plant DM (Fig 3E) and N_{PPW} of mature leaves (Fig 2F). Similarly, EDU800 led to higher Ni content of shed leaves (Table 1, $\delta = 1.67$, CI [1.67, 1.68]) and N content of mature leaves (Fig 4E), at a moderate effect magnitude. On the other hand, EDU800 caused a decrease of large effect magnitude in SLA (Fig 1G) and root DM/foliage DM (Fig 3F).

EDU1600, vs. EDU0, affected the plants in a similar way. It caused an increase of large effect magnitude in plant height gain (Fig 1C), mean leaf DM (Fig 1E), mean shoot DM (Fig 2C), SPAD (Fig 2E), N_{PPW} (Fig 2F) and N content of shed leaves (Fig 4F). Further, EDU1600 caused an increase of moderate effect magnitude on Ni content of shed leaves (Table 1, $\delta = 1.67$, CI [1.67, 1.68]) and N content of mature leaves (Fig 4E). Finally, it lowered the SLA (Fig 1G) and

N/C fraction of shed (Fig 4F) and mature (Fig 4E) leaves at a large magnitude and the shoot diameter gain at a small magnitude (Fig 2A).

The coefficients of variation of element concentrations in shed leaves were very high (Table 1).

The measured N content in leaves was well correlated ($p < 0.001$) with the estimated N_{ppw} and with SPAD (Figure 1S, electronic supplementary material). Yet, spline interpolation showed a steeper slope of the no curvature shape of the collection of data points of EDU concentrations in the range [0, 800] and a shallower slope for the EDU concentrations in the range [800, 1600] outstretched in Euclidean space (Fig 5); the two slopes were statistically different ($t = -27.75$, $p < 0.001$).

4. DISCUSSION

The results show that N accumulated into leaves when plants were treated with very high doses of EDU. This phenomenon was responsible for higher leaf DM and lower SLA, a characteristic effect of N (e.g. Loomis 1997). The fact that N content of soil and all the other plant organs except leaves was not different between the contrasts suggest that all EDU was transferred up to the leaves and no EDU residual remained in the soil. We thus conclude that EDU, at concentrations usually applied in O_3 studies, is not expected to persist in soil containing no organic matter. In contrast, when soil contains organic matter, EDU may be absorbed onto organic matter and gradually re-solubilized by irrigation water (Pasqualini et al. 2016).

There was almost no available N in the soil used as substrate and therefore the findings should not be generalized across soil fertilities. It is unknown if high EDU doses would contribute as N fertilizer in soils with higher N availability (than the one we used) or with retention of

316 bioavailable N which as may, for instance, happen in volcanic soils of temperate evergreen
317 rainforests (Huygens et al. 2008).

318 Plants have different mechanisms to cope with O₃-induced stress according to the available
319 nutrient supply (Utriainen and Holopainen 2001). Potential impacts of elevated foliar N
320 concentration on the leaf responses to O₃ worth further consideration. Although some reports
321 have shown no interactive effect of N availability with O₃ (Watanabe et al. 2007; Yamaguchi et
322 al. 2007b), O₃-induced damage to aboveground physiological functioning or production of plants
323 is possible to be higher when N availability or leaf N content is higher (Tjoelker and Luxmoore
324 1991; Lippert et al. 1996; Utriainen and Holopainen 2001; Izuta and Nakaji 2003; Yamaguchi et
325 al. 2007a). In such a case, elevated leaf N content due to EDU would contrast with EDU
326 prevention of O₃ damage.

327 Godzik and Manning (1998) applied 300 mg EDU L⁻¹ and corresponding amounts of N as urea
328 (70 mg L⁻¹) or phenylurea (159 mg L⁻¹) to leaves of Bel-W3 tobacco plants (*Nicotiana tabacum*
329 L.), and suggested that N was not responsible for the EDU protection of plants: EDU and
330 phenylurea were equally effective in preventing O₃ injury, whereas urea was not effective.
331 Manning et al. (2011) also reported that a foliar application of 300 mg EDU L⁻¹ to the O₃-
332 sensitive bean line 156 (*Phaseolus vulgaris* L.) resulted to a slight increase of N and ¹⁵N only
333 one day after the application, followed by a decline. In contrast, three spray applications
334 (biweekly) of 300 mg EDU (50 % a.i.) L⁻¹ for one growing season increased the leaf N content of
335 *Pinus taeda* L. seedlings late in the study but not early in the study (Kuehler and Flagler 1999).
336 Paoletti et al. (2007, 2008) found that six gravitational trunk infusions (every 21 days) with 450
337 mg EDU L⁻¹ did not contribute to leaf N content of adult ash (*Fraxinus excelsior* L.) trees; the
338 amount of EDU applied was 13-26 mg m⁻² leaf area, i.e. 2.85-5.69 mg N m⁻² leaf area. At the last

EDU application of our experiment, the given EDU amounts were ≈ 49 and 117 mg m^{-2} leaf area for EDU800 and EDU1600, respectively, equaling to ≈ 11 and 49 mg N m^{-2} leaf area, respectively. If we consider that the total amount of N applied was approximately 1 kg N ha^{-1} for EDU1600, the N amount that would be applied with the usual treatment of $400 \text{ mg EDU L}^{-1}$ would be as little as $0.25 \text{ kg N ha}^{-1}$ (75% lower). Such a low amount of N may be used by plants only for their functional needs (*e.g.* RuBisCO activity) when growing in soils poor in N, especially when the plants have high leaf area. Adult trees grown in nature are expected to have much higher leaf area and a fertilizer effect of EDU is expected to have much lower or no impact on growth even at high doses like the ones used in this study. Further studies should investigate whether EDU contribute to plants as a N source when they grow in substrates with optimum N-fertilization.

Nitrogen partitioning depends on irradiance and temperature, while the adjustment of Chla/Chlb ratio indicates acclimation to low N availability (Hikosaka and Terashima 1995; Kitajima and Hogan 2003; Hikosaka 2005). EDU did not significantly affect the Chla/Chlb ratio (despite a 10 % difference of EDU1600 vs. EDU0). *Inductive reasoning* may hint to the conclusion that N abundance remained low even at the highest EDU dose. It is possible that the EDU800 or EDU1600 plants had grown in more suitable conditions than the EDU0 ones; namely, plants of this species may not perform well physiologically under N-poor conditions as the case of EDU0 where the soil was N-impoverished.

The shoots DM was lower in EDU1600 plants than in EDU800 plants and this difference was caused by a lower pre-treatment number of shoots per plant in EDU1600 (2 ± 0 shoots) compared with EDU800 (3 ± 0 shoots) or EDU0 (3 ± 0 shoots). The number of shoots did not change among treatments at the final measurements. In addition, the plants of EDU1600 had 24 % higher

diameter than those of EDU0 at the initial assessment (perhaps due to fewer shoots), and this may explain why the plants of EDU1600 had less diameter gain, *i.e.* they reached a maximum growth stage (for the given conditions) earlier. Yet, the EDU800 plants had 11 and 17 % more leaves than EDU0 plants and 9 and 33 % more leaves than EDU1600 plants at pre-treatment and post-treatment measurements, respectively (data not shown).

EDU800 plants had increased shoot-stem angle compared to EDU0 plants. The fact that the shoot angle in the EDU1600 plants was not increased in comparison to the EDU0 plants is attributed to the fewer shoots per plant and therefore to the absence of the need in increasing the angle.

Carbon metabolism is crucial when dealing with O₃ stress (Agathokleous et al. 2016a). EDU did not affect the carbon distribution within plant and between plant and soil (Table 1). Therefore, in EDU-O₃ studies, alteration in C accumulation or distribution within plant would be caused by O₃ and not by EDU. N/C ratio could be used in the same manner as C content, but only when EDU is applied at concentrations < 800 mg L⁻¹ (or < 600 mg L⁻¹ for safety; Agathokleous et al. 2016c). The same can be argued for the photosynthetic pigments as well.

According to the problem-solving principle of *lex parsimoniae*, it can be suggested that the observed differences were upon EDU *per se* and not upon prevention of ambient O₃ damage by EDU: No characteristic O₃ symptoms were observed on the EDU0 plants. Although such symptoms are not always present under O₃ stress (Agathokleous et al. 2015a), differences in C content or C distribution within plant, which is a classical O₃ effect (Agathokleous et al. 2016a), were not observed between the EDU contrasts.

Finally, Ni content in fallen leaves of EDU-treated plants was higher than that in fallen leaves of EDU0 plants, however, it is unclear if this was due to an increased content in the attached leaves or a decreased content in the shed leaves. Excess Ni accumulation was found to be related with reduced biomass production in birch, an early successional tree species (Kayama and Koike 2015). However, in contrast to some side or toxic effects of applications of arbitrarily selected doses of EDU on biomass of some plant species which have been previously reported (Cannon et al. 1993), the high doses used in the present study did not result to lower biomass of the treated plants. Since exposures of sensitive organisms to EDU doses above a threshold may result to toxic effects (Agathokleous et al. 2016c), high EDU doses may be toxic to root symbionts as well: Mycorrhizal fungi protects plants against toxic metals, such as Ni, by excluding the metals from uptake by plants (Jones and Hutchinson 1988).

5. CONCLUSIONS

In this study we investigated the potential of very high doses of EDU to contribute as a N source or to cause toxicity to a fast growing tree species.

EDU was not toxic to this willow species in a soil with no organic matter and thus no EDU persistence in the soil; there were, however, side effects on N/C ratio of attached mature leaves and N/C ratio and Ni content of shed leaves. Although EDU1600 was not toxic to the plants *per se*, it may affect decomposition process and N/C cycles through side effects on Ni content of shed leaves and N to C ratio of mature and shed leaves.

High doses of EDU above 800 mg L⁻¹ increased N content in willow leaves. Lower doses, as those usually applied in O₃ protection studies, are not expected to significantly increase foliar N

content. Yet, EDU did not affect C distribution within plant even after chronic exposure to high EDU doses.

Overall, EDU was beneficial to plants even at such high concentrations, which are much higher than those used experimentally for plant protection against O₃ impact. It is however recommended to avoid applications of EDU concentrations $\geq 600 \text{ mg L}^{-1}$ in uncontrolled environments because of potential EDU toxicity to sensitive organisms (Agathokleous et al. 2016c).

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Captions

Table 1 Mean $\pm$ se of post-treatment pigment characteristics of mature leaves and elemental composition of shed leaves of willow (*Salix sachalinensis*) plants grown in ambient O₃ atmosphere and treated with 0 mg EDU L⁻¹ (EDU0), 800 mg EDU L⁻¹ (EDU800) or 1600 mg EDU L⁻¹ (EDU1600). Chl_a is chlorophyll *a*, Chl_b is chlorophyll *b*, TChl is total chlorophyll, Tcar is total carotenoids and OD₄₃₅/OD₄₁₅ is the ratio of the absorbance between the optical densities 435 and 415. Data analyzed with the contrasts EDU800 vs. EDU0 and EDU1600 vs. EDU0. *P*-values marked with bold were statistically significant. The tests were conducted at a level of significance $\alpha=0.017$.

Fig 1 Post-treatment growth characteristics (A, B, C) and leaf traits (D, E, F, G, H) of willow (*Salix sachalinensis*) plants grown in ambient O₃ atmosphere and treated with 0 mg EDU L⁻¹ (EDU0), 800 mg EDU L⁻¹ (EDU800) or 1600 mg EDU L⁻¹ (EDU1600). Asterisk above the error bar of an EDU800 or EDU1600 mean indicates statistical significance. Data analyzed with the contrasts EDU800 vs. EDU0 (a) and EDU1600 vs. EDU0 (b). *P*-values marked with bold were statistically significant. The tests were conducted at a level of significance $\alpha=0.017$. The unbiased Cohen's δ (δ) and its confidence interval (CI) were estimated when there was a significant EDU effect.

Fig 2 Post-treatment shoot traits (A, B, C, D), leaf greenness (E) and estimated leaf nitrogen content (F) of willow (*Salix sachalinensis*) plants grown in ambient O₃ atmosphere and treated with 0 mg EDU L⁻¹ (EDU0), 800 mg EDU L⁻¹ (EDU800) or 1600 mg EDU L⁻¹ (EDU1600). Asterisk above the error bar of an EDU800 or EDU1600 mean indicates statistical significance. Data analyzed with the contrasts EDU800 vs. EDU0 (a) and EDU1600 vs. EDU0 (b). *P*-values marked with bold were statistically significant. The tests were conducted at a level of

significance $\alpha=0.017$. The unbiased Cohen's δ (δ) and its confidence interval (CI) were estimated when there was a significant EDU effect.

Fig 3 Post-treatment biomass production of willow (*Salix sachalinensis*) plants grown in ambient O_3 atmosphere and treated with 0 mg EDU L⁻¹ (EDU0), 800 mg EDU L⁻¹ (EDU800) or 1600 mg EDU L⁻¹ (EDU1600). Asterisk above the error bar of an EDU800 or EDU1600 mean indicates statistical significance. Data analyzed with the contrasts EDU800 vs. EDU0 (a) and EDU1600 vs. EDU0 (b). *P*-values marked with bold were statistically significant. The tests were conducted at a level of significance $\alpha=0.017$. The unbiased Cohen's δ (δ) and its confidence interval (CI) were estimated when there was a significant EDU effect.

Fig 4 Post-treatment measured nitrogen (N) content, carbon (C) content, and N/C ratio in the soil (A) and different plant organs (B-F) of willow (*Salix sachalinensis*) plants grown in ambient O_3 atmosphere and treated with 0 mg EDU L⁻¹ (EDU0), 800 mg EDU L⁻¹ (EDU800) or 1600 mg EDU L⁻¹ (EDU1600). Asterisk above the error bar of an EDU800 or EDU1600 mean indicates statistical significance. Data analyzed with the contrasts EDU800 vs. EDU0 (a) and EDU1600 vs. EDU0 (b). *P*-values marked with bold were statistically significant. The tests were conducted at a level of significance $\alpha=0.017$. The unbiased Cohen's δ (δ) and its confidence interval (CI) were estimated when there was a significant EDU effect.

Fig 5 Interpolated nitrogen (N) content in mature leaves of willow (*Salix sachalinensis*) plants grown in ambient O_3 atmosphere vs. log-transformed concentrations of EDU which were repeatedly applied to the plants. The three original EDU treatments were 0, 800 and 1600 mg EDU L⁻¹ (black data points). From these concentrations, data were estimated for 34 EDU concentrations using interpolation on a scale of 50 mg EDU L⁻¹ (grey data points). The

670 interpolation was done based on a constrained version of the Bessel spline function. Two
671 regression lines are shown; one for the EDU range [0, 800] (a) and one for the EDU range [800,
672 1600] (b).

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687 **Table 1**

	EDU0	EDU800	EDU1600	EDU0 vs. EDU800	EDU0 vs. EDU1600
Pigments of mature leaves ($\mu\text{g cm}^{-2}$)					
Chl _a	14.827 ± 0.652	14.507 ± 1.022	14.830 ± 1.033	$t=-0.393$, $p=0.699$	$t=-0.105$, $p=0.918$
Chl _b	5.854 ± 0.331	5.728 ± 0.277	6.513 ± 0.227	$t=-0.351$, $p=0.729$	$t=1.589$, $p=0.128$
TChl	20.682 ± 1.074	20.235 ± 1.224	21.343 ± 1.120	$t=-0.368$, $p=0.717$	$t=0.438$, $p=0.667$
Chl _a /Chl _b	2.550 ± 0.141	2.599 ± 0.157	2.284 ± 0.148	$t=0.262$, $p=0.796$	$t=-1.242$, $p=0.229$
OD ₄₃₅ /OD ₄₁₅	0.765 ± 0.043	0.800 ± 0.044	0.687 ± 0.050	$t=0.553$, $p=0.586$	$t=-1.213$, $p=0.240$
Tcar	8.258 ± 0.465	8.096 ± 0.369	8.542 ± 0.558	$t=-0.234$, $p=0.818$	$t=0.454$, $p=0.655$
TChl/Tcar	10.305 ± 0.335	10.042 ± 0.582	10.226 ± 0.729	$t=-0.295$, $p=0.771$	$t=-0.029$, $p=0.977$
Elements of shed leaves (mg g^{-1} dry mass)					
P	3.826 ± 0.332	4.268 ± 0.412	4.085 ± 0.499	$t=0.711$, $p=0.487$	$t=0.317$, $p=0.755$
K	131.2 ± 19.7	100.8 ± 12.1	113.4 ± 12.5	$t=-1.135$, $p=0.271$	$t=-0.475$, $p=0.641$
Mg	3.245 ± 0.336	3.506 ± 0.412	4.135 ± 0.582	$t=0.385$, $p=0.705$	$t=1.261$, $p=0.223$
Ca	4.898 ± 0.519	4.870 ± 0.587	5.690 ± 0.764	$t=-0.130$, $p=0.898$	$t=0.902$, $p=0.379$
Mn	0.398 ± 0.031	0.431 ± 0.039	0.476 ± 0.053	$t=0.626$, $p=0.539$	$t=1.329$, $p=0.200$
Fe	0.455 ± 0.056	0.503 ± 0.049	0.541 ± 0.052	$t=0.828$, $p=0.419$	$t=1.334$, $p=0.199$
Ni	0.059 ± 0.004	0.081 ± 0.007	0.080 ± 0.008	$t=3.083$, $p<0.010$	$t=2.986$, $p<0.010$
Cr	0.125 ± 0.010	0.157 ± 0.015	0.156 ± 0.017	$t=2.160$, $p=0.044$	$t=2.012$, $p=0.059$

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