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Title	High doses of ethylenediurea (EDU) as soil drenches did not increase leaf N content or cause phytotoxicity in willow grown in fertile soil
Author(s)	Agathokleous, Evgenios; Paoletti, Elena; Manning, William J. et al.
Citation	Ecotoxicology and environmental safety, 147, 574-584 https://doi.org/10.1016/j.ecoenv.2017.09.017
Issue Date	2018-01
Doc URL	https://hdl.handle.net/2115/76455
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Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	Manuscript.pdf



High doses of ethylenediurea (EDU) as soil drenches did not increase leaf N content or cause phytotoxicity in willow grown in fertile soil

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ABSTRACT

Ground-level ozone (O₃) levels are nowadays elevated in wide regions of the Earth, causing significant effects on plants that finally lead to suppressed productivity and yield losses. Ethylenediurea (EDU) is a chemical compound which is widely used in research projects as phytoprotectant against O₃ injury. The EDU mode of action remains still unclear, while there are indications that EDU may contribute to plants with nitrogen (N) when the soil is poor in N and the plants have relatively small leaf area. To reveal whether the N content of EDU acts as a fertilizer to plants when the soil is not poor in N and the plants have relatively large total plant leaf area, willow plants (*Salix sachalinensis* Fr. Schm) were exposed to low background O₃ levels and treated ten times (9-day interval) with 200 mL soil drench containing 0, 800 or 1600 mg EDU L⁻¹. Fertilizer was added to a nutrient-poor soil, and the plants had an average plant

leaf area of 9.1 m^2 at the beginning of EDU treatments. Indications for EDU-induced hormesis in maximum electron transport rate (J_{max}) and ratio of intercellular to ambient CO_2 concentration ($C_i:C_a$) were observed at the end of the experiment. No other EDU-induced effects on leaf greenness and N content, maximum quantum yield of photosystem II (F_v/F_m), gas exchange, growth and matter production suggest that EDU did not act as N fertilizer and did not cause toxicity under these experimental conditions.

Keywords: air pollution, antiozonant, ethylenediurea, hormesis, ozone, soil fertility

Key message: EDU *per se* did not act as nitrogen fertilizer in willow plants.

1. INTRODUCTION

Surface ozone (O₃) levels are elevated in the Northern Hemisphere since preindustrial times, and, even if the levels decrease in some regions in the coming decades, they will remain potentially phytotoxic (Hendriks et al., 2016; Heue et al., 2016; Kopanakis et al., 2016; Paoletti et al., 2014; Sicard et al., 2016; Takigawa et al., 2009; Travis et al., 2016). Production of O₃ and its precursors is high at rapidly developing countries, such as China, from where O₃ can be exported to trans-boundary regions too (Verstraeten et al., 2015). Ozone-sensitive vegetation may be at a high risk for O₃ phytotoxicity in Asian and Mediterranean regions where O₃ occurs at high levels (Bermejo et al. 2003; Calatayud et al., 2011; Feng et al. 2015; Li et al., 2016; Mishra and Agrawal, 2014; Paoletti et al., 2014; Sicard et al., 2016, 2017; Wang and Mazuerall, 2004 ; Yamaguchi et al., 2011). Chronic exposure of plants to high O₃ levels may generate over-accumulation of reactive oxygen species, suppress photosynthesis and plant growth, and, thus, lead to reductions in the productivity and yields which account for a high economic value (Agathokleous et al., 2016e, 2015; Avnery et al., 2011; Feng et al., 2015; Oksanen et al., 2013; Paoletti, 2006; Vaultier and Jolivet, 2015; Wang et al., 2007). Several studies suggested that even current ambient O₃ levels may accelerate autumn senescence and reduce plant productivity in many areas of the world (Carriero et al., 2015; Kitao et al., 2016; Manning et al., 2011a; Paoletti et al., 2009).

Ethylenediurea (EDU) is a synthetic chemical (with formula: C₄H₁₀N₄O₂) which has been reported to protect plants against O₃ injury since late 70s (Carnahan et al., 1978). There was an initial interest in the air pollution research community which was followed by a decline and again a rise within the last decade. More than 100 research articles about EDU role in plants exist in Science Citation Index (Thomson Reuters Co., New York). The mechanism through which

69 EDU protects plants against O₃ injury has not been elucidated yet (Agathokleous, 2017;
70 Agathokleous et al., 2015; Manning et al., 2011a; Oksanen et al., 2013; Paoletti et al., 2009;
71 Singh et al., 2015).

72 One open question is whether EDU protects against O₃ phytotoxicity by adding nitrogen (N) into
73 the leaf tissue. EDU has four N atoms, accounting for almost 22 % of its molecule mass, which
74 may produce a chemical affinity to the electrophilic O₃ (Manning et al., 2011a). Still, there is an
75 evidence showing interactive effects of N availability and O₃ pollution on tree species (Nakaji et
76 al., 2004; Yamaguchi et al., 2010, 2007a). However, interaction between N availability and O₃
77 pollution is not a rule of thumb, and, still, low N loads to the soil which are much greater than
78 those that would be loaded through EDU applications do not necessarily affect aboveground
79 physiological functioning or productivity in tree species (Nakaji and Izuta, 2001; Watanabe et al.,
80 2007; Yamaguchi et al., 2010, 2007a). Furthermore, although the tree response to O₃ under
81 increasing N load seems to be species specific (Yamaguchi et al., 2011), O₃ injury may be even
82 more severe under higher N availability or excess leaf N content (Izuta and Nakaji, 2003; Lippert
83 et al., 1996; Tjoelker and Luxmoore, 1991; Utriainen and Holopainen, 2001; Yamaguchi et al.,
84 2011). Such an effect would contrast the EDU protection against O₃ injury. Earlier short-term
85 experiments suggested that N addition by EDU is not the mechanism through which EDU
86 protects against O₃ phytotoxicity (Godzik and Manning, 1998; Manning et al., 2011a).

87 Nonetheless, it is unclear if EDU adds N after chronic exposure of plants where EDU application
88 is repeated over time. In an experiment with willow (*Salix sachalinensis* Fr. Schm), EDU high
89 doses increased the foliar N content and contributed as plant nutrient whereas did not cause
90 phytotoxicity (Agathokleous et al., 2016b), when the soil was lacking N and the plants had
91 relatively small leaf area.

It remains unknown if EDU high doses would contribute with N and cause toxicity when the plants have relatively large leaf area and the soil does not lack N, and this is an important issue (Agathokleous et al., 2015; Manning et al., 2011b; Singh et al., 2015). One important aspect is that EDU amounts in soil may vary over time, when EDU is applied as soil drench, because EDU may be adsorbed onto soil organic matter and released gradually through resolubilization by irrigation water, thereby potentially leading to greater amounts in the soil than the applied ones at random times (Pasqualini et al., 2016). The amount of leaf area is an additional factor affecting the EDU effectiveness and its potential N contribution (Agathokleous et al., 2016c; Ainsworth et al., 1996; Bortier et al., 2001; Paoletti et al., 2008, 2007), through an imbalance in the equilibrium of EDU amount per leaf area (i.e. importantly larger EDU amounts in a smaller leaf area).

The vast majority of EDU studies were conducted under high O₃ levels, without toxicologically assessing EDU and confirming its mode of action independently from O₃ first. We aimed at clarifying whether chronic exposure of *S. sachalinensis* plants to high EDU doses would translate into a N-like fertilization or cause toxicity under low O₃ levels. In contrast to an earlier experiment with willow treated with the same EDU doses (Agathokleous et al., 2016b), the present study used a soil that did not lack N and plants that did not have relatively small leaf area. The N amount that would be applied by an EDU treatment of 1600 mg L⁻¹ is approximately 1 kg ha⁻¹ yr⁻¹, an amount which is by far smaller than the N amounts which can be deposited from the atmosphere (Ban et al., 2016; Liu et al., 2011; Wright et al., 1995; Xu et al., 2015; Yamaguchi et al., 2010, 2007b). We hypothesized that EDU would not add N and would not cause toxicity to willow plants under these experimental conditions because the N amount contained in EDU would be biologically negligible relative to plant leaf area. To answer our research question, we

monitored plant growth and leaf N over time and measured biomass and potential physiological biomarkers of N fertilization based on leaf gas exchange and chlorophyll *a* fluorescence (Archontoulis et al., 2012; Coskun et al., 2016; Ji et al., 2015; Jin et al., 2015; Kitajima and Hogan, 2003; Nakaji and Izuta, 2001; Novriyanti et al., 2012).

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.). The experimental area was selected because of relatively low background O₃ levels (Agathokleous et al., 2017; Hoshika et al., 2013). The free of snow period was around early-May to mid-November. Meteorological conditions were monitored and data were recorded by Japan Meteorological Agency (<http://www.jma.go.jp/jma/indexe.html>, accessed on 7th February 2017; Electronic Supplementary Materials, Table 1S), at a nearby station (WMO, ID: 47412, 43°03.6'N 141°19.7'E).

Background O₃ mixing ratio at the experimental area was continuously monitored (one recording per minute) by an ultraviolet absorption O₃ analyzer (TUV-1100; Tokyo Industries Inc., Tokyo, Japan), from June 1st to September 6th, 2016. Data were averaged per hour, and the 24-hr mean was calculated. The daily mean O₃ mixing ratio for the monitored period (June 1st to September 6th) was 12.5 (± 4.91 s.d.) 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 practically 0 μmol mol⁻¹ h.

2.2. Plant material & Design of the Experiment

Sixty current-year uniform cuttings of *S. sachalinensis* (= *S. udensis* Trautv. et C.A. Mey.), originated from the river basin of Ebetsu city, were obtained from the Hokkaido Horti-Tree Planting Center, Co. Ltd, Japan in 2015. Plant growth containers were filled with freshly opened commercial soil, free from organic matter. Akadama (well-weathered volcanic ash) and Kanuma (well-weathered pumice) soils were mixed at a ratio of 1:1. Akadama soil is naturally occurring and has high potential to retain water and nutrients along with efficient porosity and free drainage.

Cuttings were planted for rooting on May 14th 2015, irrigated, and kept under field conditions. On June 11th, when well rooted, the cuttings were transplanted into 15 L pots filled with the same substrate mixture, irrigated, and placed at the field, on a completely randomized design. Irrigation with tap water was repeated on June 15th and 20th 2015, for plant establishment. From July to October, the plants were subjected to monthly rotation. The plants left at the field until the next growing season (2016), without any human impact (no fertilizers, agrochemicals or other treatments).

On May 19th, 2016, each plant was watered with 300 ml of a water solution containing 1.2 ml (1:250 v/v) of liquid balanced fertilizer (N:P:K=6:10:5, Hyponex, Japan). Two days later, 39 of them were selected for uniformity, and the remaining twenty were placed around the experimental plot to minimize potential edge effects for the experimental plants. The application of liquid balanced fertilizer was repeated twice, May 27th and July 4th, at the same dosage as in the first application.

On May 27th 2016, the 39 experimental plants were transferred to three different plots (13 potted plants per plot) scattered within the experimental forest; four to five plants in each plot were randomly assigned to each EDU treatment. Three plants per EDU treatment per plot were randomly selected and marked in order to be harvested at the end of the experiment. All the pots within each plot were subjected to a monthly rotation and the plots were interchanged three times in total. Visible injury by pests or pathogens was rarely observed and thus the plants were not treated with agrochemicals during the experiment.

The morphological characteristics of this species when grown from cuttings were illustrated in earlier articles (Agathokleous et al., 2016c; Koike et al., 1995).

2.3. EDU treatments

Three concentrations of EDU were selected for testing: 0 mg EDU L⁻¹ (EDU0), 800 mg EDU L⁻¹ (EDU800) and 1600 mg EDU L⁻¹ (EDU1600). Based on prior studies, EDU effectively protects plants against O₃ injury when applied in a concentration range of approximately 200-400 mg L⁻¹ (Agathokleous et al., 2015; Feng et al., 2010; Paoletti et al., 2009); this applies to this willow too (Agathokleous et al., 2016c). The selected concentrations are higher than the threshold for toxicity to sensitive organisms based on a toxicological bioassay with biological standardization (Agathokleous et al., 2016a), as they are two and four times higher than the maximum concentration needed for protection against O₃ injury (Agathokleous, 2017), and cover a wide range that cannot be exceeded in practice; importantly higher concentrations than those we used cannot be sufficiently diluted (Agathokleous et al., 2016a).

EDU was freshly prepared 15-30 minutes prior to each application by continuously stirring and gently warming until full dilution of 100% a.i. EDU in pH 6.5 water (source: William J.

Manning, University of Massachusetts, Amherst, MA, USA). The required EDU amount was dissolved in partitions of 500 mL, and the target concentration was achieved in the final desired volume by adding cool water. Surfactant was not added in the solutions.

The first EDU soil drench was applied on May 31st 2016, and repeated every nine days, for a total of ten applications. The 9-day interval was selected because EDU persists in the leaf apoplast for more than eight days (Paoletti et al., 2009; Pasqualini et al., 2016). The applied amount for each application was 200 mL solution plant⁻¹, and the applications were conducted during afternoon hours. The total amount of EDU applied was 2.6 and 5.2 kg ha⁻¹ for the 800 and 1600 mg EDU L⁻¹ treatments, equaling to 0.57 and 1.14 kg N ha⁻¹, respectively (Manning et al., 2011a).

2.4. Measurements & Samplings

2.4.1. Leaf nitrogen and greenness

Before the first EDU application, leaf N was non-destructively assessed (May 31) in all the plants, as estimated N content per leaf area, using the portable device Agriexpert PPW-3000 (Satake Corp., Hiroshima, Japan) (Agathokleous et al., 2016b; Eguchi et al., 2006; Ichie et al., 2002; Mizusaki et al., 2015, 2013). In this species, soil plant analysis development (SPAD) value and leaf N value linearly correlate with measured area-based leaf chlorophyll *a* content and measured dry matter-based leaf N content (Agathokleous et al., 2016b). For each plant, two fully sun-exposed leaves from the upper crown (starting 3rd or 4th from the top) and two leaves from the lower crown (from the 3rd or 4th lateral shoot from the base of a main shoot, the 4th or 5th leaf from the base) were measured (at least two measurements per leaf). SPAD (SPAD-502; Konica-Minolta, Osaka, Japan) was measured as described for leaf N (Chang and Robison, 2003; Xiong

et al., 2015; Yang et al., 2014) and in the same leaves. The leaf N measurements were repeated on June 1, 2, 5 and 9, July 15, 16, 17 and 23, August 28 and 31 and September 6 (11 times). The SPAD measurements were repeated on July 15 and September 6.

At the end of the exposure to EDU, four mature sunlit leaves located at the upper level of the crown were randomly sampled from each plant. These samples were taken after gas exchange measurements (explained below). The samples were dried in an air-dry oven at 80 °C air temperature, until a constant dry mass, and grounded into fine powder. The four samples per plant were pooled so as to have one robust sample per plant for analysis. Content of N and C was analyzed by a third generation Vario EL III Element Analyzer (Elementar Analysensysteme GmbH, Hanau-Germany).

2.4.2. Growth

Baseline (pre-treatment) plant size measurements were taken from the 27 plants selected for harvest, from 26th to 30th of May, 2016. The mean plant height (from the point the first main shoot is attached to the cutting to the apical meristem of the tallest shoot), the mean crown spread (the distance of the two remotest points as observed vertically from above the canopy) and the mean shoot diameter (at the point where the shoot is attached on the cutting) were measured. Furthermore, the number of main shoots, the number of first order lateral shoots (those attached on each main shoot) and the number of leaves were counted per plant. For the mean crown spread and the shoot diameter, two cross-wise measurements were taken to give one robust average value. Diameter was measured with digital caliper in inches and converted to mm, whereas plant height, mean crown spread and leaf dimensions were measured with a measuring tape with a 1-mm scale. For each plant, the length and width of each leaf was also measured (6804 leaves in total), and the product of the two dimensions was calculated. From the product of

leaf length $\times$ leaf width, the area of each leaf was calculated using an earlier predicting model (Agathokleous et al., 2016b). Finally, the average leaf size (cm^2) and the total plant leaf area (m^2) were calculated per plant.

For post-treatment assessment, plant height, crown spread and shoot diameter were measured on 11 September 2016, as described above for pre-treatment measurements. However, diameter was measured with a different digital caliper (mm) than in pre-treatment assessment. These traits were measured from the nine plants per EDU treatments, which were randomly selected *a priori*.

2.4.3. Chlorophyll fluorescence

On 4th of September (08:30 am to 12:30 pm), chlorophyll *a* fluorescence was measured by a pulse amplitude modulation 2D fluorometer (PAM, EM-FluorCam-800MF, Photon system inst., Drasov, Czech Republic). One mature leaf per plant (13 plants per EDU treatment) was measured. Each leaf was freshly detached, immediately wrapped with aluminium foil and adapted to dark for 20 min before the measurement. Minimum fluorescence in dark-adapted state (F_0) was measured using low measuring modulated light, whereas maximum fluorescence in dark-adapted state (F_m) was determined by applying a saturated light pulse of 2400 $\mu\text{mol}(\text{photons}) \text{m}^{-2} \text{s}^{-1}$. The maximum PSII quantum yield in dark-adapted state was estimated as $(F_m - F_0)/F_m (= \Phi_{exc})$, that in healthy leaves is usually 0.80-0.83 units.

2.4.4. Gas exchange

During morning hours on 4th September, photosynthesis was measured from the 27 plants, which were *a priori* selected to be harvested, one mature leaf per plant, using Licor instruments (LI-6400, Li-Cor Inc., Lincoln, NE, USA). Stomatal conductance (g_{s380}), net photosynthetic rate (A_{380}) and transpiration rate (E_{380}) were determined at 380 $\mu\text{mol mol}^{-1} \text{CO}_2$, 60 $\pm$ 5 % relative air humidity, and 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic active photon flux (Koike et al., 2012). The leaf

temperature was maintained at 25 °C. The ratio of intercellular to ambient CO₂ concentration ($C_i:C_a$) was also recorded. The A/C_i curve (net CO₂ assimilation rate vs. intercellular CO₂ concentration) was yielded after exposing the leaf to changing CO₂ levels in the leaf chamber fluorometer in fourteen steps (380, 300, 200, 100, 50, 200, 300, 380, 380, 500, 800, 1000, 1200, 1500 $\mu\text{mol mol}^{-1}$) while maintaining photosynthetic active photon flux, temperature and relative humidity constant; the AutoProgram of LI-6400 was used. From the A/C_i curve, the maximum rate of carboxylation ($V_{C_{max}}$), net photosynthetic rate at 1500 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ (A_{max}), and maximum rate of electron transport rate (J_{max}) were estimated using a biochemical model for C₃ species (Farquhar et al., 1980; Long and Bernacchi, 2003). The real EDU treatments were not revealed to the persons who took the fluorescence and photosynthesis measurements, whereas the principal investigator was present, supervising the measurements. Gas exchange and chlorophyll fluorescence were measured in mature sunlit leaves located at the upper level of the crown.

2.4.5. Biomasses

The plants whose growth was measured on 11 September 2016 were harvested the following day, separated into leaves, shoots and stem, and the fresh matter (FM) was measured. The samples were then dried in an air-dry oven at 80 °C air temperature, until a constant dry mass. The dry matter (DM) of each main shoot (including multi-order lateral shoots) and the foliage was measured per each plant. The total shoot matter was calculated by summing the values of all shoots per plant and the total aboveground matter was calculated by summing the foliage matter and shoots matter per plant. The fresh to dry matter ratio (FM/DM) was also calculated per plant.

2.5. Data handling & Statistics

The threshold for statistical significance was predefined at an α level of 0.05. Gas exchange and chlorophyll fluorescence parameters were measured in one leaf per plant (sampling unit = plant). Nitrogen content was measured in one composite sample per plant. For response variables where more than one measurement were taken per plant (i.e. SPAD and estimated N), the values were averaged per sampling unit (i.e. per plant). Prior to the analysis, data were transformed with BoxCox transformation (Box and Cox, 1964) according to prior description (Agathokleous et al., 2016d).

All the data, except SPAD and estimated N, were subjected to simple contrasts of Least Squares means (Agathokleous et al., 2016a). The two degrees of freedom ($k-1$) were partitioned to the contrasts (a) EDU0 vs. EDU800 and (b) EDU0 vs. EDU1600.

For leaf N, the targeted total number of recorded values was at least 8 per plant. The average recorded number of values was four (two per crown level) per plant per time, and the total number of recorded values was 3720. The values were averaged per leaf and then per crown level, thus resulting to one robust estimate per crown level, per plant (no missing values for the analysis). Transformation was also necessary for the leaf N data as the distribution significantly deviated (χ^2 test=15.22, $P<0.001$) from the standard Gaussian distribution. Because of the longitudinal nature of the dataset, the data were subjected to repeated measures analysis of variance (ANOVA) with type 6 sums of squares (effective hypothesis tests) straightforward computation method (Hill and Lewicki, 2006). Time was the within-subjects factor with 12 levels and EDU and Crown level the categorical predictors. When the overall effect of a main factor with more than two levels was significant, Bonferroni post hoc test was followed.

SPAD data were statistically analyzed by repeated ANOVA, as described for the leaf N data, but with Time having 3 levels.

To explain the biomass results and safely answer the research question, exploratory data analysis was followed. Regression analysis was conducted between the plant leaf area at the beginning of the experiment and the total FM or the total DM at the end of the experiment.

For the hypothesis testing of all the response variables, the minimum plant *n* of each treatment of each variable was 9 and the maximum was 13.

MS EXCEL 2010 (Microsoft ©) and STATISTICA v.10 (StatSoft Inc. ©) software were used for data processing and statistics.

3. RESULTS

3.1. Pre-treatment assessment

At the pre-treatment assessment, plants treated with EDU800 or EDU1600 had statistically indifferent plant height, crown spread, shoot diameter, number of main shoots, number of lateral shoots, number of leaves, average leaf size and plant leaf area from plants treated with EDU0 (Table 1).

It is assumed that pre-treatment plant leaf area was the lowest during the period of EDU treatments because plants had greater plant size and senescence did not initiate at the end of the experiment. Also, it is practically impossible for plants to uptake all the applied EDU into the soil. Based on these assumptions, the maximum applied amount of EDU was 0.20 g (CI: [0.14-0.33 g]) per m² plant leaf area for EDU800 and 0.41 g (CI: [0.32-0.59 g]) per m² plant leaf area for EDU1600 for the total of all the applications, or 19.56 and 41.24 mg EDU per m² plant leaf

area per application for EDU800 and EDU1600, respectively. In terms of number of leaves, the maximum applied amount of EDU was 7.16 mg (CI: [6.29-8.29 mg]) per leaf for EDU800 and 13.10 mg (CI: [11.29-15.62 mg]) per leaf for EDU1600 for the all the applications, or 0.72 and 1.31 mg EDU per leaf per application for EDU800 and EDU1600, respectively.

3.2. Post-treatment assessment

SPAD was not significantly affected by EDU treatments (Fig 1). However, leaves located at the upper crown (Fig. 1a) had greater SPAD than those located at the lower crown (Fig. 1b). SPAD also varied significantly over time; leaves had greater SPAD in the post-treatment time than in the pre-treatment time. Upper crown leaves (Fig. 1a) had greater SPAD than lower crown leaves (Fig. 1b) and greater SPAD in the post-treatment time than in the pre-treatment time. There were no significant interactions of EDU×Crown level, Time×EDU and Time×Crown level×EDU.

Estimated leaf N remained also unaffected by EDU treatments and indifferent between crown levels (Fig 2). There were, however, significant fluctuations over time which were mainly observed at the last four time points (last week of August and first week of September). Similarly to SPAD, there were no significant differences in leaf N of lower crown leaves (Fig. 2a) between the pre-treatment time point and each post-treatment time, however leaf N of upper crown leaves (Fig. 2b) from the 6th time point onwards (mid. July to early September) was most of the time greater than in the pre-treatment time. As in SPAD, there were no significant interactions of EDU×Crown level, Time×EDU and Time×Crown level×EDU.

F_v/F_m was indifferent between EDU0 and EDU800 or between EDU0 and EDU1600 (Fig 3). Except J_{max} and $C_i:C_a$, there were no other significant differences in the other gas exchange response variables (Fig 3). J_{max} was increased and $C_i:C_a$ was decreased by EDU800 compared to

EDU0, but remained unaffected by EDU1600 (compared to EDU0). Such a phenomenon for differential effect between EDU800 and EDU1600 was observed in g_{s380} , E_{380} , $V_{c_{max}}$ and A_{max} too, however the EDU800 effect was statistically insignificant (high coefficient of variation compared to J_{max} and $C_i:C_a$). There was also no EDU-induced alterations in photosynthetic water balance, as indicated by E_{380}/g_{s380} , indicator of water loss outside of the stomata (Wang et al., 2015), photosynthetic water use efficiency (A_{380}/g_{s380}) and A_{380}/g_{s380} (Electronic Supplementary Materials, Fig 1S).

Plant height, plant crown spread and average shoot basal diameter were unaffected by EDU (Fig 4).

Unaffected by EDU also remained the measured leaf N content, C content and their ratio at the end of the experiment (Fig 4).

Regarding matter production (Fig 5), plants treated with EDU800 or EDU1600 had less FM and DM of foliage, shoots and total aboveground than plants treated with EDU0. However, FM/DM of foliage (2.95 ± 0.12 units, hereafter mean $\pm$ s.e.), shoots (2.15 ± 0.04 units) and total aboveground (2.23 ± 0.06 units) was indifferent ($P=0.165-0.563$) between EDU0 and EDU800 or EDU1600 (Electronic Supplementary Materials, Fig 2S). Total aboveground FM and DM at the end of the experiment were positively correlated (adjusted $R^2 < R^2$) with plant leaf area at the beginning of the experiment (Fig 6), indicating that FM and DM outcome was associated with the plant leaf area before the treatments began.

4. DISCUSSION

The SPAD and estimated N results suggest that EDU did not increase the leaf N content of the plants over time. This is verified by the measured N content in leaves at the end of the

experiment which remained unaffected by EDU. At that time, leaves from the three EDU treatments contained $1.55 \pm 0.08 \text{ g N } 10^2 \text{ g}^{-1}$ which is close to the $1.26 \pm 0.13 \text{ g N } 10^2 \text{ g}^{-1}$ contained in leaves of EDU1600 in the previous experiment with willow (Agathokleous et al., 2016b). This might be the optimum leaf N content for physiological functioning in this species. The only differences in SPAD occurred between upper crown and lower crown leaves, which can be explained by the developmental processes involved in leaf ontogeny (Kolb and Matyssek, 2001), with leaves at the lower crown being fully mature before treatments begin. These findings are consistent with previous findings where SPAD readings varied with leaf position and developmental stage (Yang et al., 2014; Yuan et al., 2016). As indicated by repeated measurements of N content, upper crown leaves reached the max N in mid-July.

It has been widely shown that N fertilization, depending on the dose, can alter chlorophyll fluorescence and gas exchange in leaves, which can be seen as increases in F_v/F_m , A_{380} and $V_{C_{max}}$ (Archontoulis et al., 2012; Coskun et al., 2016; Ji et al., 2015; Jin et al., 2015; Kitajima and Hogan, 2003; Nakaji and Izuta, 2001; Novriyanti et al., 2012). EDU high doses did not contribute to plants with N, thus photosynthetic parameters (F_v/F_m and A_{380}) also remained constant and C content in leaves was not increased by EDU. EDU1600 did not affect any physiological traits whereas EDU800 did affect some, especially J_{max} and $C_i:C_a$ which were significantly increased and decreased, respectively. This phenomenon where EDU800 increased J_{max} (or decreased $C_i:C_a$) whereas EDU1600 did not affect it indicates hormesis, a biological phenomenon where a biphasic response of an organism to an agent occurs in the framework of contrasting responses between low and high doses of an agent (Calabrese, 2016; Calabrese et al., 1999). That is, EDU800-induced stimulation was below the no-observed-adverse-effect level (NOAEL) and EDU1600 was the NOAEL. Hormesis is a widely occurring phenomenon which

has been reported for hundreds of chemical compounds across taxonomic groups and kingdoms (Calabrese, 2004, 2001; Calabrese et al., 1999; Calabrese and Baldwin, 2003a, 2003b, 2000). Several chemical compounds have been reported to induce hormesis in plants (Belz et al., 2008; Calabrese and Blain, 2005, 2009; Cedergreen et al., 2007; Poschenrieder et al., 2013), which can occur following either a direct stimulation or an initial disruption in homeostasis that leads to overcompensation (Calabrese, 2014; Agathokleous, 2017). The EDU800-induced stimulation of J_{max} indicates greater ribulose 1,5-bisphosphate (RuBP) regeneration, the last independent phase in Calvin cycle, which correlates with the cytochrome f contents of the thylakoid membranes (Onoda et al., 2005). The EDU800-induced decrease in $C_i:C_a$ indicates imbalance between the rates of inward CO_2 diffusion and CO_2 assimilation (Valletta et al., 2016). Since the ambient CO_2 was constant across EDU treatments, other supportive mechanisms may explain why $C_i:C_a$ changed by EDU800; one possible explanation is altered stomatal density (Ehleringer and Cerling, 1995), however there is no evidence to reveal if EDU800 altered stomatal density. A further more likely explanation is EDU800-induced decrease in gs_{380} , which was 128 % greater in EDU0 than in EDU800 plants (along with unchanged A_{380}), albeit statistically non-different due to large coefficient of variation. Significantly EDU800-induced lower C_i but no effect on A_{380} , suggests that g_s should be lower but $V_{C_{max}}$ should be higher in EDU800 than in control, despite of no statistical significance. Furthermore, it is widely shown that $V_{C_{max}}$ correlates well with J_{max} (Kitao et al., 2012). In support to the present findings for EDU-induced stimulation in physiological endpoints, EDU-induced hormesis was previously observed in non-photochemical quenching of fluorescence (q_N) and rate of non-photochemical quenching of fluorescence (NPQ) of a hydrophyte (*Lemna minor* L.) within the first 24 hours after exposing to several EDU concentrations up to 2370 mg L⁻¹; significant stimulation occurred at 148 mg EDU L⁻¹

(Agathokleous, 2017; Agathokleous et al., 2016a). In the present experiment, hormetic signs were observed a week after the final EDU application, whereas, in the previous experiment with willow, there were indications for EDU-induced hormesis in several morphological and reproductive endpoints at the end of the chronic exposure (Agathokleous et al., 2016b). The present and previous observations suggest EDU can induce hormesis in a variety of endpoints and time points, with a modest stimulatory response, similarly to hundreds of chemical agents that induce hormesis in plants (Calabrese and Blain, 2009, 2011).

The plant size remained indifferent between EDU treatments at the end of the experiment, in agreement with earlier experiments where EDU did not affect plant morphology under quite low O₃ exposures (Agathokleous et al., 2014, 2016c; Ainsworth and Ashmore, 1992; Ashrafuzzaman et al., 2017). These results may suggest an indirect EDU-induced stimulation of plants at dose-specific thresholds (Agathokleous et al., 2016a; Kostka-Rick and Manning, 1993a,b) through disruptions in homeostasis that lead to overcompensation (Calabrese, 2014, 2016; Agathokleous, 2017). FM and DM were lower in EDU800 and EDU1600 plants than in EDU0 plants. However, this decrease is associated with the pre-treatment plant leaf area, and is unlikely being upon EDU-induced toxicity because there were no signs for EDU-induced toxicity in: (a) SPAD and estimated N, which would detect EDU-induced chlorosis; (b) plant crown size; (c) the assessed physiological traits; and (d) FM/DM in whole plant, which would indicate imbalance in water relations. Even if the decrease in FM and DM was upon EDU, it should be mentioned that the applied EDU doses were multi-fold the common EDU doses applied to plants against O₃ toxicity (Agathokleous, 2017; Agathokleous et al., 2015; Feng et al., 2010; Paoletti et al., 2009). Such high doses should be applied only in controlled environments (e.g. the soil to be disposed

according to the national regulations for disposing chemicals) as they are potentially toxic to sensitive organisms (Agathokleous et al., 2016a).

In the previous experiment, where EDU contributed to willow plants with N, the soil was N-poor (Agathokleous et al., 2016b). Furthermore, the plant leaf area was on average 2.5 and 2.8 times lower in EDU800 and EDU1600 plants of the earlier experiment than in EDU800 and EDU1600 plants of the present experiment. Plant leaf area is an important factor in EDU research as EDU amounts should be translated as a function of plant leaf area. The average leaf size of willow is small (linear, lanceolate) compared to other broad-leaved plants and, thus, a small number of leaves equals to just a small plant leaf area. The plant leaf area in the present experiment was more realistic than in the previous experiment (Agathokleous et al., 2016b) in the sense that small-size broad-leaved plants are more likely to have a similar leaf area. While the mechanism of N contribution in the earlier experiment remains unknown, it was estimated that low EDU doses in the common applied range were unlikely to contribute with N under the same experimental conditions (Agathokleous et al., 2016b). This suggestion was confirmed by a further experiment with willow under the same experimental conditions (Agathokleous et al., 2016c). The present study cancels the possibility that EDU adds N to willow plants even after chronic exposure to high EDU doses when the soil does not lack N and the plants have not relatively small leaf area. Absence of apparent toxic effects that would be reflected in N-related physiological traits of plants are in agreement with the previous study with willow (Agathokleous et al., 2016b).

In conclusion, we investigated the potential of high EDU doses to contribute with N and cause toxicity to a fast growing tree species in a fertile soil. No signs justifying N fertilization by EDU throughout the experiment were found. Decreased matter production was observed in EDU-

treated plants, however it was not upon EDU toxicity. These differences were associated with plant leaf area at the beginning of the treatments. Also, there were no signs for EDU-induced toxicity in all the other plant physiological traits which have been assessed. We thus conclude that EDU does not act as N fertilizer and does not cause toxicity in willow when the soil fertility is adequate for physiological functions and the plant leaf area is large. Potential hormetic effects of EDU on higher plants need further examination.

ACKNOWLEDGEMENTS

The authors are grateful to Mr. Tetsuto Sugai and Mr. Kyohsuke Hikino for assistance in the experimental part. Evgenios Agathokleous is an International Research Fellow (ID No: P17102) of the Japan Society for the Promotion of Science (JSPS). This study was financially supported by research funds of JSPS to Takayoshi Koike (Type B: 26292075). JSPS is a non-profit organization. The authors declare that there are no conflicts of interest.

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Table 1

Table 1 Means $\pm$ s.e. of plant traits at the beginning of the experiment, prior to treatments with 0 mg EDU L⁻¹ (EDU0), 800 mg EDU L⁻¹ (EDU800) or 1600 mg EDU L⁻¹ (EDU1600). Data were analyzed with the contrasts EDU0 vs. EDU800 and EDU0 vs. EDU1600 at a level of statistical significance $\alpha=0.05$. Each mean is the average of 9 values.

	EDU0	EDU800	EDU1600	EDU0 vs. EDU800	EDU0 vs. EDU1600
Plant height (cm)	67.64 $\pm$ 22.55	61.26 $\pm$ 20.42	72.80 $\pm$ 24.27	$T=0.91, P=0.372$	$T=-1.06, P=0.230$
Crown spread (cm)	29.42 $\pm$ 8.04	26.50 $\pm$ 6.85	24.25 $\pm$ 6.05	$T=0.44, P=0.663$	$T=0.86, P=0.398$
Shoot diameter (mm)	8.85 $\pm$ 0.97	7.49 $\pm$ 0.44	8.74 $\pm$ 0.75	$T=1.07, P=0.295$	$T=0.03, P=0.976$
Number of main shoots	1.56 $\pm$ 0.24	1.89 $\pm$ 0.11	1.56 $\pm$ 0.24	$T=-1.41, P=0.172$	$T<0.01, P=1.000$
Number of lateral shoots	23.22 $\pm$ 2.90	18.33 $\pm$ 1.36	23.67 $\pm$ 3.44	$T=1.14, P=0.266$	$T=0.02, P=0.988$
Number of leaves	288.2 $\pm$ 33.5	223.6 $\pm$ 30.6	244.2 $\pm$ 39.3	$T=1.21, P=0.240$	$T=1.0, P=0.306$
Leaf size (cm ²)	3.73 $\pm$ 0.37	3.55 $\pm$ 0.36	3.28 $\pm$ 0.25	$T=0.52, P=0.605$	$T=0.85, P=0.405$
Plant leaf area (m ²)	11.37 $\pm$ 2.05	8.18 $\pm$ 1.69	7.76 $\pm$ 1.20	$T=1.1, P=0.305$	$T=1.2, P=0.251$

Fig 1

Fig 1 Soil plant analysis development (SPAD, units=procedure defined unit (p.d.u.)) means ($\pm$ s.e) of leaves located either at the upper (a) or the lower (b) level of the crown of willow (*S. sachalinensis*) plants treated every nine days with 0 mg EDU L⁻¹ (EDU0), 800 mg EDU L⁻¹ (EDU800) or 1600 mg EDU L⁻¹ (EDU1600). Measurements were taken three times: 1) May 31st; 2) July 15th; and 3) September 6th. Realistic time interval (in days) between measurements is simulated along the x-axis. Measurements were taken on the same day for all the EDU treatments, however EDU treatments are separated on a 5-day interval along x-axis for presentation clarity. Data were analyzed by repeated ANOVA at a level of significance $\alpha=0.05$. Different lowercase letters above time points 1-12 (Time means) show statistically significant differences within the interaction Time $\times$ Crown level. Different uppercase letters A and B above time points 1-12 along x-axis show statistically significant difference within Time (EDU and Crown level pooled). Differences are marked according to Bonferroni test. Each mean is the average of 13 robust values.

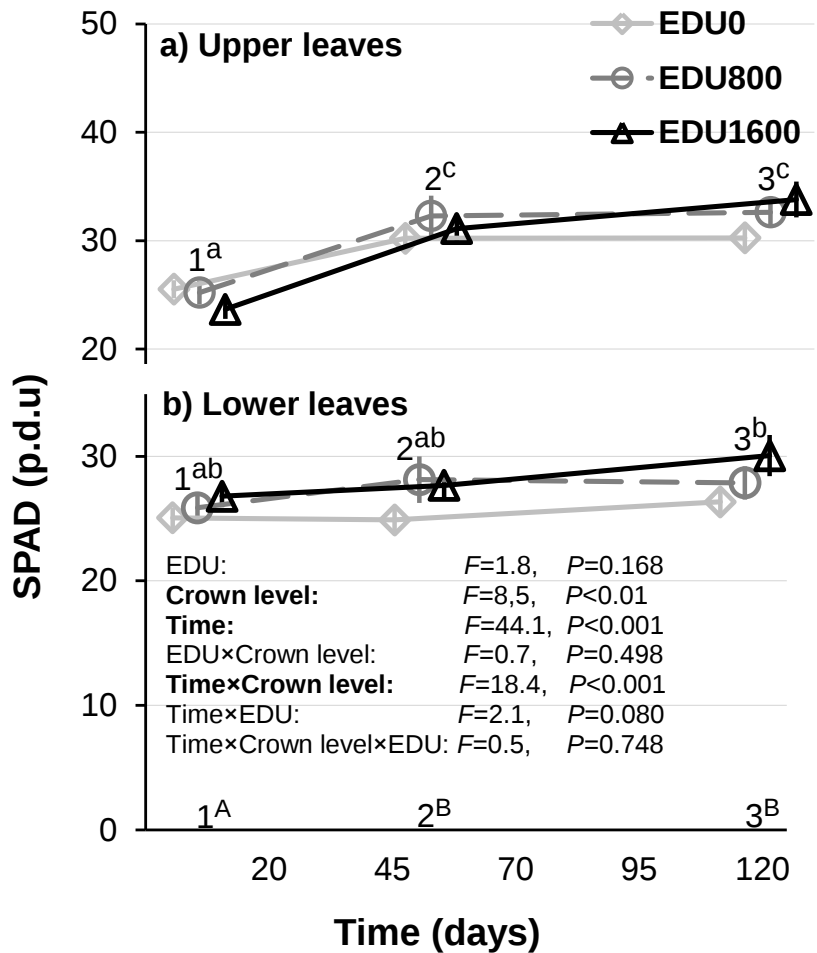


Fig 2

Fig 2 Leaf nitrogen (units=procedure defined unit (p.d.u.)) means ($\pm$ s.e) of leaves located either at the upper (a) or the lower (b) level of the crown of willow (*S. sachalinensis*) plants treated every nine days with 0 mg EDU L⁻¹ (EDU0), 800 mg EDU L⁻¹ (EDU800) or 1600 mg EDU L⁻¹ (EDU1600). Measurements were taken twelve times: 1) May 31st; 2) June 1st; 3) June 2nd; 4) June 5th; 5) June 9th; 6) July 15th; 7) July 16th; 8) July 17th; 9) July 23th; 10) August 28th; 11) August 31th; and 12) September 6th. Realistic time interval (in days) between measurements is simulated along the x-axis. Data were analyzed by repeated ANOVA at a level of significance $\alpha=0.05$. Different lowercase letters above time points 1-12 (Time means) show statistically significant differences within the interaction Time×Crown level; for presentation clarity, since there were no other biologically relevant differences, only the differences between pre-treatment and each post-treatment time points are marked. Different uppercase letters A, B, C and D above time points 1-12 along the x-axis show statistically significant difference within Time (EDU and Crown level pooled). Differences are marked according to Bonferroni test. Each mean is the average of 13 robust values.

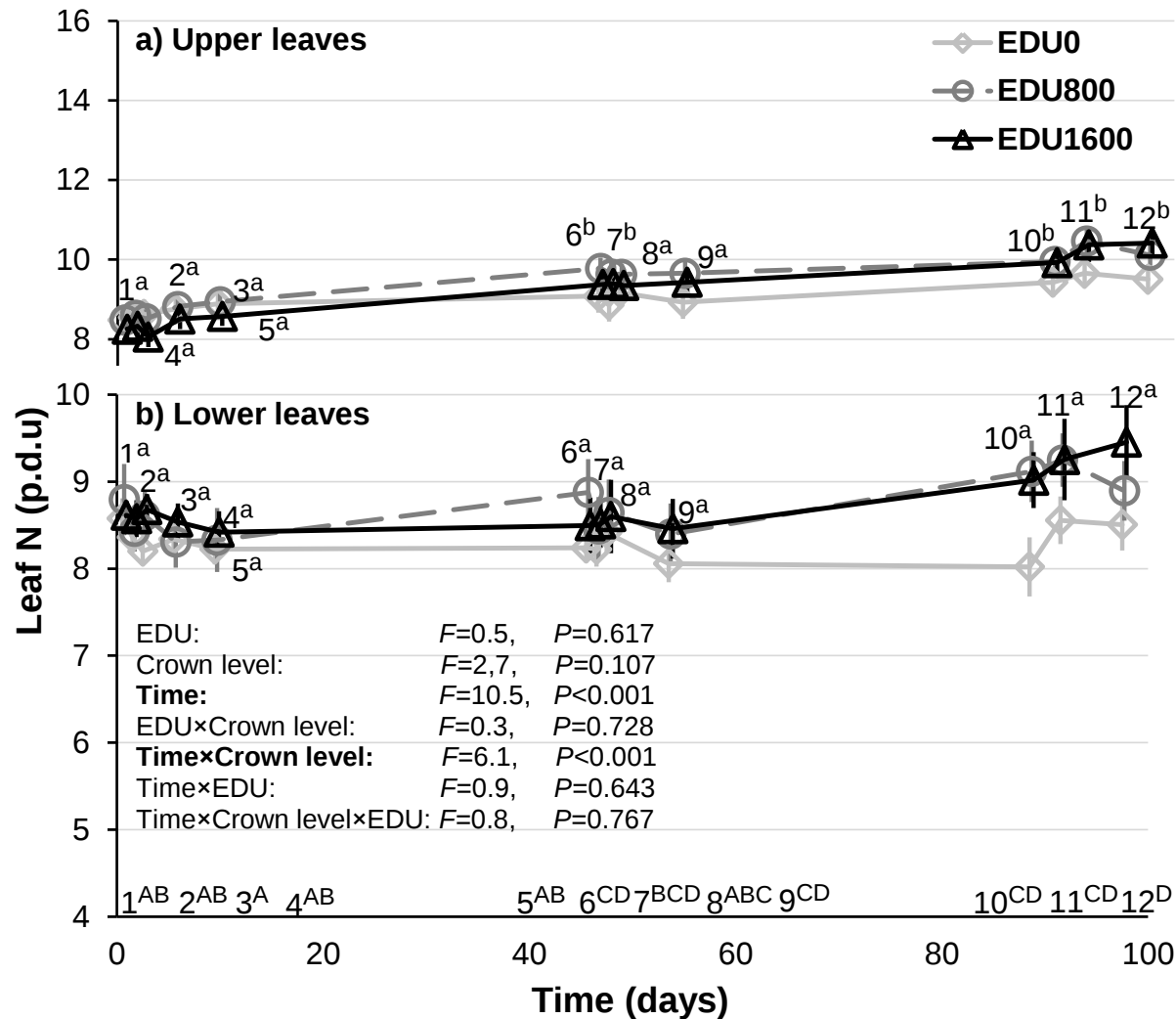


Fig 3

Fig 3 Chlorophyll fluorescence and gas exchange means ($\pm$ s.e) of mature sunlit leaves of willow (*S. sachalinensis*) plants treated ten times (9-day interval) with 0 mg EDU L⁻¹ (EDU0), 800 mg EDU L⁻¹ (EDU800) or 1600 mg EDU L⁻¹ (EDU1600). The response variables are net photosynthetic rate (A_{380}), stomatal conductance (gs_{380}), transpiration rate (E_{380}), maximum rate of carboxylation ($V_{c_{max}}$), maximum rate of electron transport rate (J_{max}), net photosynthetic rate at 1700 μ mol CO₂ mol⁻¹ (A_{max}), maximum PSII quantum yield (F_v/F_m) and ratio of intercellular to ambient CO₂ concentration (C_i/C_a). Asterisk above the error bar of an EDU800 or EDU1600 mean indicates statistical significance at a level of $\alpha=0.05$. Data analyzed with the contrasts EDU0 vs. EDU800 (a) and EDU0 vs. EDU1600 (b). *P*-values marked with bold were statistically significant. Each mean of F_v/F_m is the average of 13 values whereas each mean of gas exchange response variables is the average of 9 values.

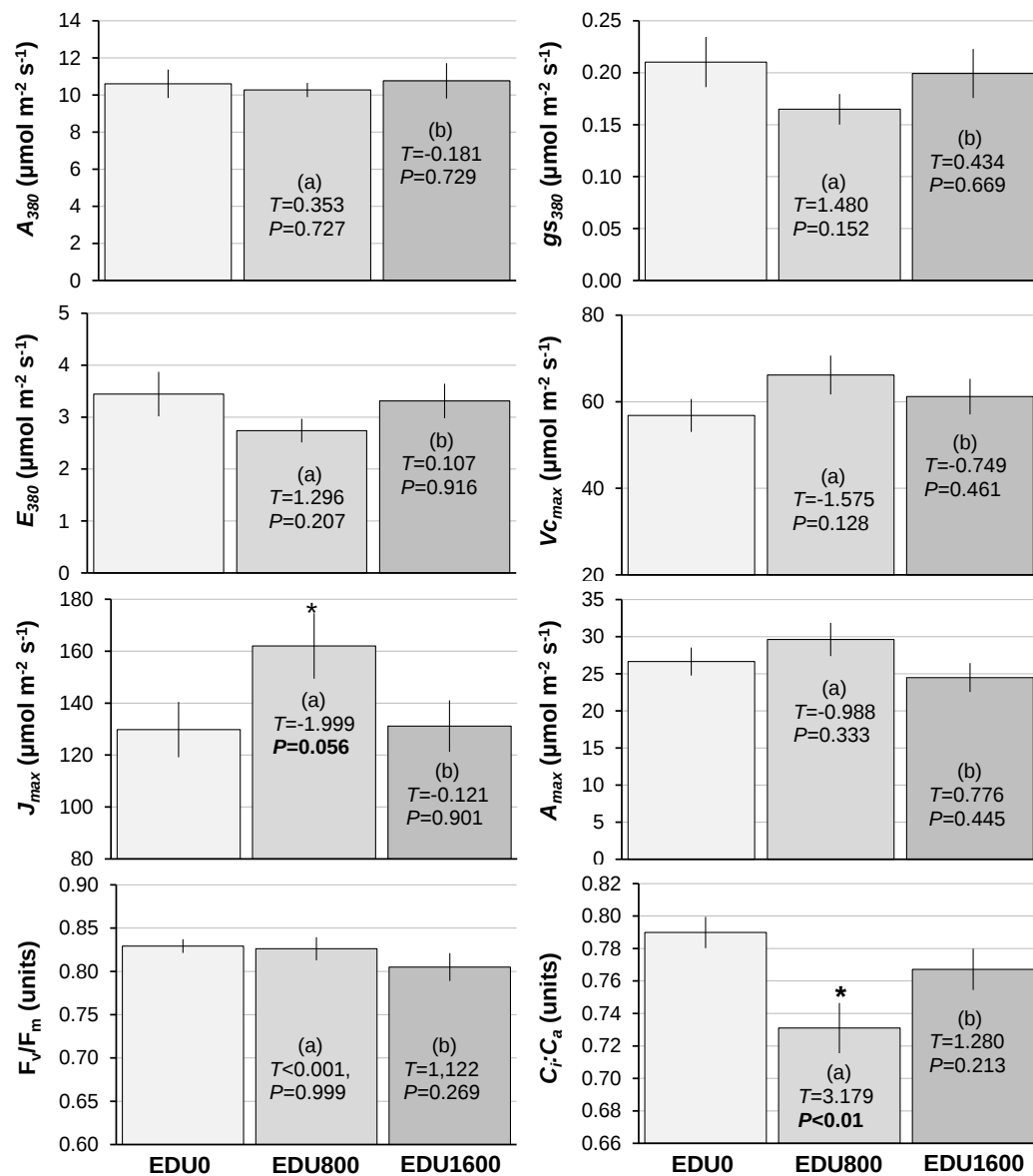


Fig 4

Fig 4 Plant height, plant crown spread, average shoot basal diameter, carbon (C) content, nitrogen (N) content and C/N ratio means ($\pm$ s.e) of willow (*S. sachalinensis*) plants treated ten times (9-day interval) with 0 mg EDU L⁻¹ (EDU0), 800 mg EDU L⁻¹ (EDU800) or 1600 mg EDU L⁻¹ (EDU1600). Data analyzed with the contrasts EDU0 vs. EDU800 (a) and EDU0 vs. EDU1600 (b) as a level of statistical significance $\alpha=0.05$. Each mean is the average of 9 values.

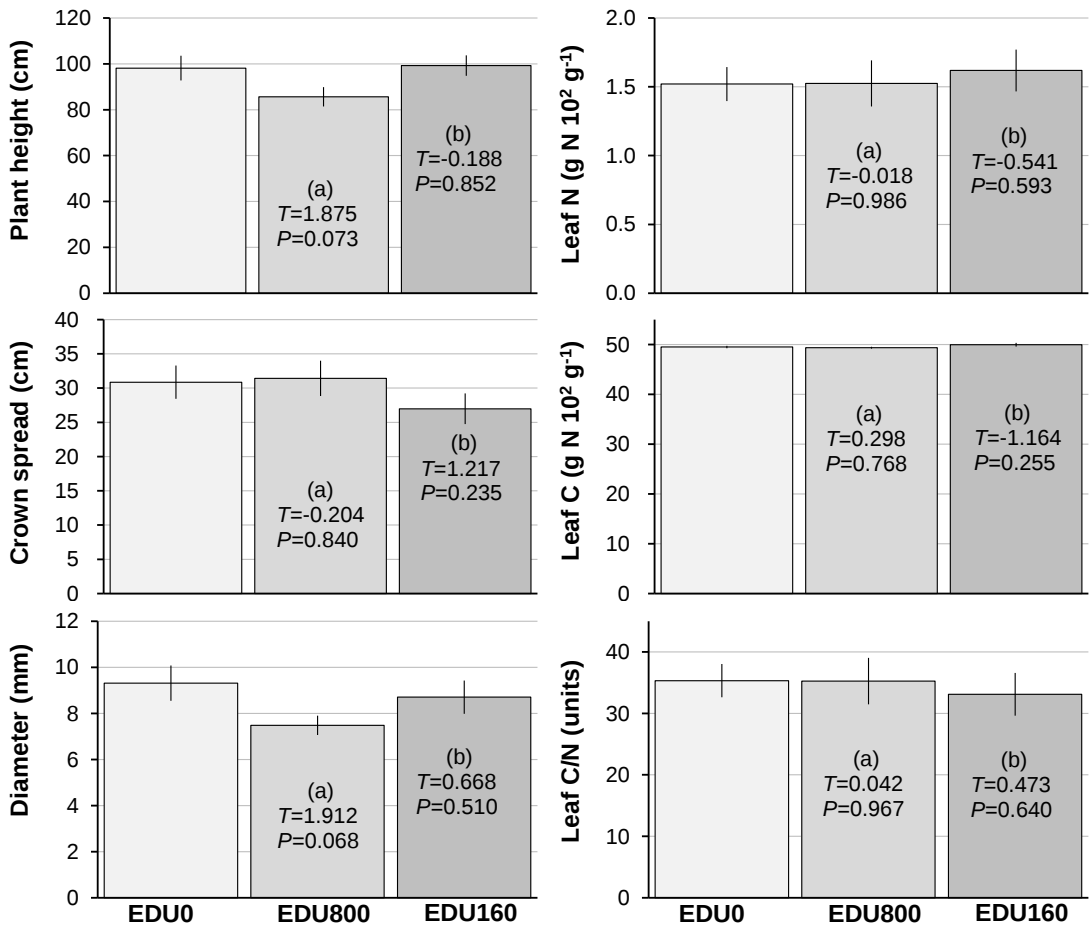


Fig 5

Fig 5 Fresh matter (FM) and dry matter (DM) means ($\pm$ s.e) of foliage, shoots (excluding leaves) and total aboveground of willow (*S. sachalinensis*) plants treated ten times (9-day interval) 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 at a level of $\alpha=0.05$. Data analyzed with the contrasts EDU0 vs. EDU800 (a) and EDU0 vs. EDU1600 (b). *P*-values marked with bold were statistically significant. Each mean is the average of 9 values.

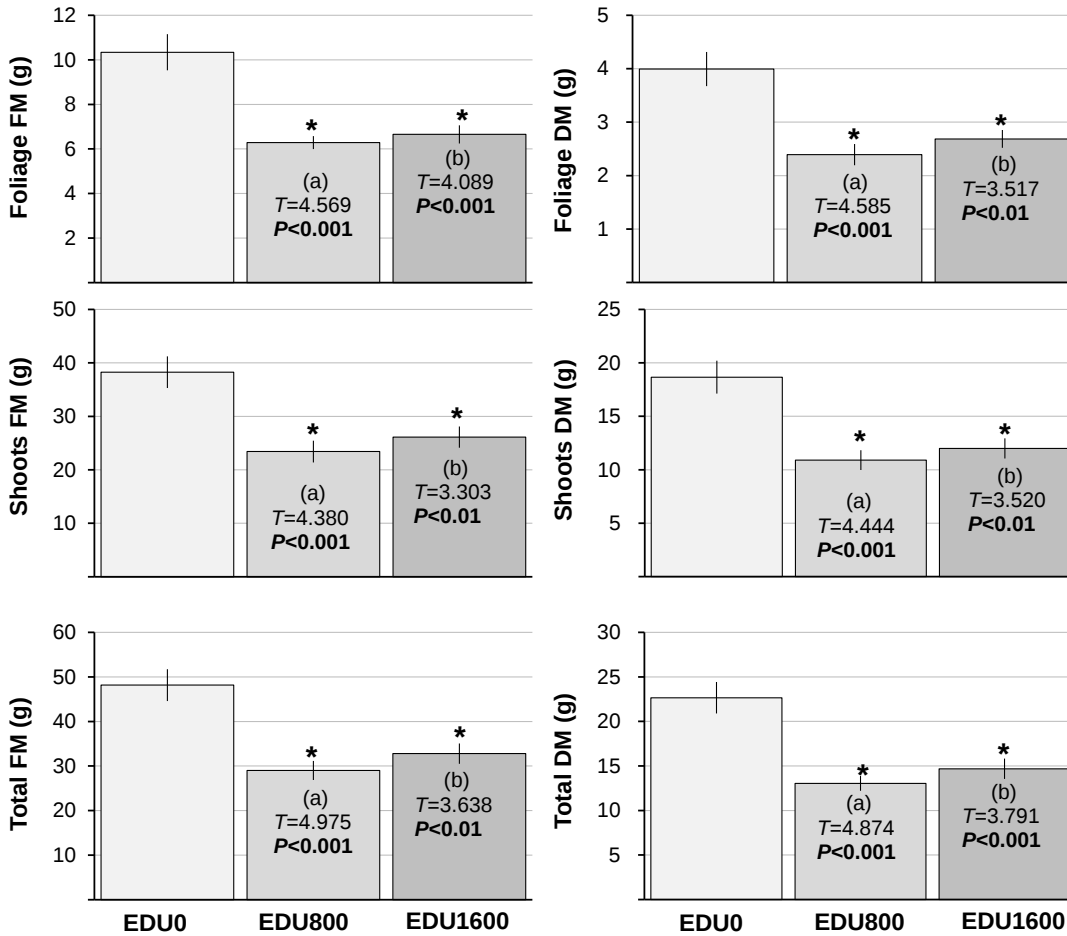
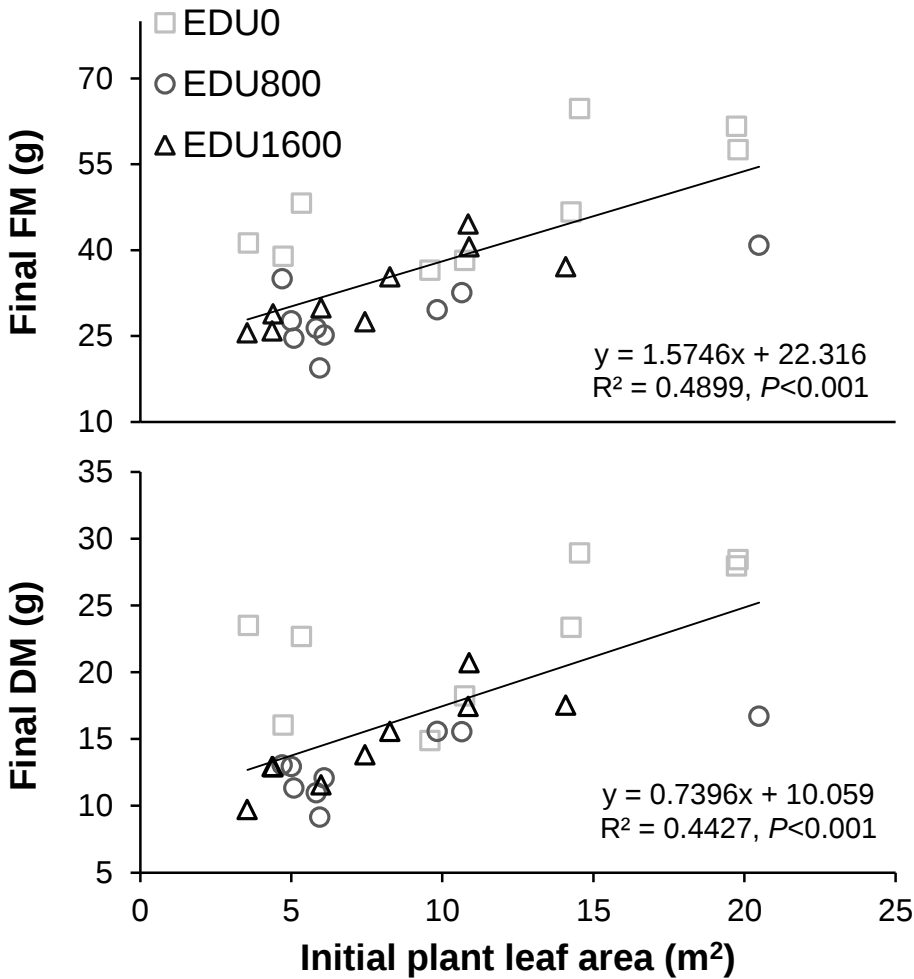


Fig 6

Fig 6 Regression analysis between the total plant leaf area at the beginning of the experiment (initial plant leaf area) and the total aboveground fresh matter (Final FM) or the total aboveground dry matter (Final DM) at the end of the experiment. Regression analysis was conducted at a level of statistical significance $\alpha=0.05$.



863 **HEREAFTER THE FIGURE FILES TO BE USED BY PRODUCTION EDITORS**

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