Ethylenediurea (EDU) as a soil drench to reduce O_3 impact on willow (*Salix sachalinensis*) cuttings:

A preliminary observation using a free-air O_3 fumigation system

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Introduction

Ground-surface ozone (O_3) is a gaseous, anthropogenic air pollutant widespread at elevated concentrations (*1, 2*). O_3 has the potential to impact living organisms, including plants and ecosystems (*3, 4, 5, 6, 7*). More than 370 wild plant species are known to be impacted by O_3, and the situation is expected to worsen by increasing O_3 concentrations (*3*). In Asia, O_3 levels were increasing rapidly due to anthropogenic factors (*8, 9*), and they are expected to reach up to higher levels as the precursors will continue increasing (*10*). How can all these species facing the O_3 "nightmare" (*3*) to be protected?

The antiozonant Ethylenediurea (EDU) (*11, 12*) is the most effective protectant against O_3 phytotoxicity (*13, 14, 15*). Although many studies have been conducted to EDU through the decades, there is a big gap in understanding its mode of action (*13, 14, 15*). It is extensively applied on crop plants (*13*), mainly in Europe and U.S.A. (*15, 16*), but its application of forest species is limited. Hitherto, there is no evidence on EDU applications to woody species in Asia.

Taking into account the above, we established a free-air-O_3-fumigation experiment with willow (Onoe-yanagi, *Salix sachalinensis*) individuals subjected to elevated O_3 and 3 levels of EDU. This is an important species to Japan, suitable for energy production. Willows are considered as fast-growing species and usually can be found in places with high soil water content (*17*). Thus, we hypothesized that this species has a high O_3 uptake and may be damaged by chronic elevated O_3.

This preliminary research a part of a dose – response study aimed to find an effectiveness of EDU to protect this species against O_3.

Materials and methods

1. Experimental area

The experiment was conducted in 2014 at Sapporo Experimental Forest of Hokkaido University, Japan (43°.04' N, 141°.20' E, 15 m a.s.l.). The snow-free period in this area is usually from early-May to mid November.

2. Plant materials

Current-year clonal cuttings of *Salix sachalinensis* in the river basin of Ebetsu City, near Sapporo, were obtained from Hokkaido Horti-Tree Planting Center, Co. Ltd. The length and basal diameter of the cuttings were 12.09 ±0.25 (SE) and 1.90 ±0.05 cm, respectively. The cuttings were stored in an incubator at 0-4 ℃ for one month to break their dormancy. On May 13th containers were filled with a mixture (1:1) of Akadama (well-weathered volcanic ash) and Kanuma (well-weathered pumice) soils – free of organic matter-. Afterwards, the cuttings were planted for rooting, and they were kept on the field, under ambient conditions. Volcanic ash soils are phosphorus deficient; commonly found in Hokkaido (*18*). Willows are regarded as energy trees (*19*) and are also common pioneer tree species in contaminated soils of Central Europe (*20*).

Table 1. The major composition (%) of the two types of soil used as a substrate, according to the producing company.

Element	Composition (%)	
	Kanuma	Akadama
SiO_2	58.6	39.5
Al_2O_3	17.1	24.6
Fe_2O_3	1.93	9
MgO	0.076	2.2
CaO	0.044	0.88
MnO	0.041	0.14

More information on the soils nutrient content is given in Table 1. When the plants were well established each clonal plant was transplanted in a 15 L pot filled with the same substrate mixture. This was done on 9th June, when the plants had 39 ±4 leaves, and the 72 pots were randomly placed in six

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土壌を介した Ethylenediurea (EDU) のオノエヤナギ挿し木に対するオゾン影響の緩和、—予報—

free-air O_3 rings (ø=6.5 m and h=5 m at most). We placed 12 pots to each O_3 site –four for each EDU treatment. Due to the clonal nature of the cuttings, the variation among the plants was minimal allowing us to characterize them as uniform. However, all the plants within each O_3 ring were subjected to a monthly rotation. The plants were neither fertilized nor treated by agrochemicals during the experiment. Observation of visible injury by pests or pathogens was very rare.

Before the beginning of EDU and ozone treatments, the pH of the soil was measured for each treatment. Two plants were randomly selected per each ring site, and one sample of 50 ml soil was taken from each pot (n=12). In order to take 50 ml, two scoops of 25 ml were sampled from the depth of 0-10 cm, from two different points in the pot, and they were mixed. The pH measured at a rate of soil to water 1:2. Each sample was stirred for 1 min every 5 min, for 20 min, and then left for some time to stabilize. Then the pH was measured using a pH-meter (B-712, HORIBA, Kyoto, Japan); the average of pH value was 5.9±0.01. The average plant height (from soil surface to the top of canopy) and the canopy width (the distance of the two remotest points as observed vertically from above the canopy) before the treatments were 48.0 ±2.0 and 28.0±1.0 cm, respectively, with an average number of leaves per plant, and an average number of leaves per branch per plant being 52±4 and 20±1, respectively.

3. EDU treatments

Three concentrations of EDU were selected for testing: 0 (control), 200 and 400 mg dm^{-3}. According to a meta-analysis, soil drenched EDU has the highest positive effect on crops grown in the field in this range (*14*). Similar concentrations were also found to protect woody plants (*16*). EDU was always prepared 15-30 minutes prior to its application by gently warming and continuous stirring until the full dilution of pure EDU molecule in tap H_2O of about pH 6.5. Surfactant was not used.

EDU soil drench was firstly applied on July 29th (7 weeks after the transplanting), and the targeted repetition interval was 9 days. The applied doses were 200 ml solution $plant^{-1}$, and the application was always performing at the same time, on the afternoon. Four plants were randomly assigned to each EDU treatment per O_3 ring.

4. Ozone treatments

O_3 fumigation began on Aug., 15th, 6 days after the 2nd EDU application, in a novel free-air-O_3-enrichment system consisted of 6 rings, and lasted until late October. The 3 rings served as control (ambient air) and the other 3 were enriched with O_3 at a targeted concentration, 80 nmol mol^{-1}, during daytime, when the photosynthetic photon flux (PPF) exceeded the 60 μmol m^{-2} s^{-1} (i.e. light compensation point of photosynthesis). Ozone generated from pure oxygen was diluted with ambient air in a pressurized tank and fumigated into the rings by 18 TEFLON tubes hanging down from a fixed nest above the plants (H=2.5m) and 2 TEFLON tubes fixed horizontally around the O_3 ring at 0.5 and 1.5 m from ground. Each tube had five holes (ø=1.5 mm), one every 50 cm, and an end-cup at the end. On the end-cup there was also a hole to avoid accumulation of humidity in the tubes. All the holes had a diameter of 1.5 mm.

5. Plant size, SPAD, and Chlorophyll fluorescence

At the end of the fumigation, measurements of plant height, branches diameter, and SPAD were taken from all the plants. A SPAD meter (Minolta, Tokyo) was used to assess the chlorophyll condition of 4 leaves, from 2 different branches, of each plant. To obtain an image of Chlorophyll fluorescence of O_3-fumigated leaves, we used a FluorCam- 800MF (Photon Systems Instruments, Czech Republic) closed system for detecting 2 D chlorophyll fluorescence of treated leaves. We sampled leaves after sunset to detect Chl fluorescence to maintain fresh leaves. The dark adaptation lasted 20 min. The measurement was accomplished by a continuous, non-modulated actinic light, driving photosynthetic reactions at physiological rates, and saturating flashes that are generated by version-dependent light sources.

6. *Statistics*

Data were checked for their normality and were transformed using a power transformation. Two-way ANOVA was used for the main effects and the interactions.

Results and Discussion

The EDU experiment will provide useful information on the protection of this important species against O_3 pollutant. We already have characterized some species as ozonophobic (*3*), but even though the O_3-sensitivity of important species is not known; among them willows (*7*).

The height of the plants was not significantly suppressed by O_3 ($p>0.05$). On the other hand, the diameter of the branches was significantly lower in the plants under elevated O_3 ($p<0.001$), compared with those under ambient. However, EDU had no significant effect on both growth parameters. No O_3 x EDU interaction seemed to be found ($P>0.05$). Leaf senescence was accelerated in all the plants exposed to the elevated O_3. No difference was found in plants height; possibly due to the rapid expansion of the branches to a maximum length. Thus, the maximum length reached when the O_3 uptake of the plants was less.

SPAD was also reduced significantly ($p<0.01$) by O_3. Again, EDU per se or in interaction with O_3 had no significant effects ($p>0.05$). We digitized the typical O_3-injury on willow clonal seedlings which followed approximately the same experimental procedure, but planted directly to the free-air O_3 systems soil. We observed severe O_3 injury (Fig. 1) in leaves of all the exposed plants, even if their growth seemed to be normal. Increased value of the right axis (0~4000) in Figure 1 indicates maintenance of the photosynthetic condition of PSII at a relatively healthy condition. This evidence can be used for field surveys since until now the O_3 symptoms of *S. sachalinensis* were unknown.

Most willows are growing rapidly and extremely vigorously making their research in closed or controlled chambers very

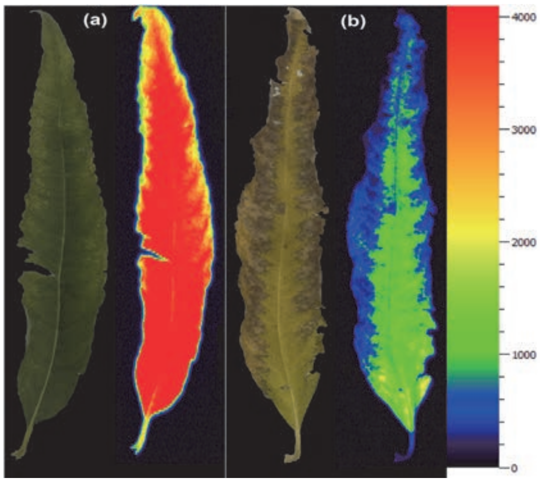

Figure 1. Typical ozone foliar visible-injury as shown by chlorophyll fluorescence images under ambient (a) and ozone enriched (b) air. Images were taken 71 days after the O_3 fumigation had begun. Vertical axis on the right hand stands for the physiological activity of PSII (The larger the value, the more active the PSII).

difficult. This could also be an explanation why no O_3 researches have been carried out until now (*7*).

Conclusion

This research will provide important information on *S. sachalinensis* sensitivity to O_3 and a possible countermeasure. The O_3 injury symptoms observed under the free-air O_3 fumigation system can be used in field surveys for verification and the future EDU results for assessment and verification of O_3 effects under natural conditions.

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