Environmental Pollution 238 (2018) 663–676

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Effects of ozone (O_3) and ethylenediurea (EDU) on the ecological stoichiometry of a willow grown in a free-air exposure system☆

Evgenios Agathokleous [1, 2, *], Mitsutoshi Kitao [1], Chu Qingnan [2, 3], Costas J. Saitanis [4], Elena Paoletti [5], William J. Manning [6], Toshihiro Watanabe [2], Takayoshi Koike [2]

[1] Hokkaido Research Center, Forestry and Forest Products Research Institute (FFPRI), Forest Research and Management Organization, 7 Hitsujigaoka, Sapporo, Hokkaido, 062-8516, Japan
[2] Research Faculty of Agriculture, Hokkaido University, Kita ku Kita 9 Nishi 9, Sapporo, Hokkaido, 060-8589, Japan
[3] Institue of Agricultural Resources and Environment, Jiangsu Academy of Agricultural Science, Nanjing, 210014, China
[4] Lab of Ecology and Environmental Science, Agricultural University of Athens, Iera Odos 75, Athens, 11855, Greece
[5] Institute of Sustainable Plant Protection, National Council of Research, Via Madonna del Piano 10, Sesto Fiorentino, Florence, 50019, Italy
[6] Department of Plant, Soil and Insect Sciences, University of Massachusetts, Amherst, MA, USA

A R T I C L E I N F O

Article history:
Received 12 February 2018
Received in revised form
17 March 2018
Accepted 17 March 2018

Keywords:
Iron homeostasis
Nutrient cycling
Nutrient resorption
Ozone
Re-translocation

A B S T R A C T

Ground-level ozone (O_3) concentrations have been elevating in the last century. While there has been a notable progress in understanding O_3 effects on vegetation, O_3 effects on ecological stoichiometry remain unclear, especially early in the oxidative stress. Ethyelenediurea (EDU) is a chemical compound widely applied in research projects as protectant of plants against O_3 injury, however its mode of action remains unclear. To investigate O_3 and EDU effects early in the stress, we sprayed willow (*Salix sachalinensis*) plants with 0, 200 or 400 mg EDU L^{-1}, and exposed them to either low ambient O_3 (AOZ) or elevated O_3 (EOZ) levels during the daytime, for about one month, in a free air O_3 controlled exposure (FACE); EDU treatment was repeated every nine days. We collected samples for analyses from basal, top, and shed leaves, before leaves develop visible O_3 symptoms. We found that O_3 altered the ecological stoichiometry, including impacts in nutrient resorption efficiency, early in the stress. The relation between P content and Fe content seemed to have a critical role in maintaining homeostasis in an effort to prevent O_3-induced damage. Photosynthetic pigments and P content appeared to play an important role in EDU mode of action. This study provides novel insights on the stress biology which are of ecological and toxicological importance.

1. Introduction

The levels of tropospheric ozone (O_3) are nowadays elevated at wide areas of the Northern Hemisphere (Feng et al., 2015; Hendriks et al., 2016; Kalabokas et al., 2017; Sicard et al., 2017; Tian et al., 2016; Trieu et al., 2017), and are potentially phytotoxic at least to O_3-sensitive vegetation (Feng et al., 2008a,b; Hayes et al., 2007; Saitanis et al., 2015). When elevated O_3 doses are taken up by plants, plants undergo a responsive process which involves a series of physiological and biological adjustments ranging from single cell to whole plant level (Ashmore, 2005; Bussotti et al., 2007; Fiscus et al., 2005; Jolivet et al., 2016; Matyssek and Innes, 1999; Munne-Bosch et al., 2013). Exposure of vegetation to elevated O_3 doses poses a risk for food supplies, ecosystem health and biosphere sustainability (Andersen, 2003; Broberg et al., 2015; Felzer et al., 2004; Fuhrer, 2009; Lindroth, 2010; Lu et al., 2015; Peñuelas and Staudt, 2010; Tian et al., 2016; Wang et al., 2016; Wilkinson et al., 2012).

The protection of vegetation against O_3 adverse effects is thus an important matter. Plenty of potential agrochemicals have been tested as to their efficacy to protect plants against O_3 phytotoxicity (Agathokleous et al., 2016c; Manning et al., 1973a, 1973b; Runeckles and Resh, 1975; Saitanis et al., 2015; Taylor, 1974). The most effective phytoprotectant currently known is the synthetic chemical ethylenediurea (EDU, $C_4H_{10}N_4O_2$) (Agathokleous, 2017). EDU has

☆ This paper has been recommended for acceptance by Klaus Kümmerer.

* Corresponding author. Hokkaido Research Center, Forestry and Forest Products Research Institute (FFPRI), Forest Research and Management Organization, 7 Hitsujigaoka, Sapporo, Hokkaido, 062-8516, Japan.
E-mail addresses: globalscience@frontier.hokudai.ac.jp, evgenios@ffpri.affrc.go.jp (E. Agathokleous).

https://doi.org/10.1016/j.envpol.2018.03.061
0269-7491/

been a matter of study in dozens of investigations over the past four decades (Carnahan et al., 1978; Oksanen et al., 2013; Paoletti et al., 2009; Salvatori et al., 2017; Singh et al., 2015), and it is now recognized that EDU applied repeatedly at low concentrations of up to 400 mg L^{-1} sufficiently protects against O_3 phytotoxicity across plant species (Agathokleous et al., 2015; Manning et al., 2011; Oksanen et al., 2013; Paoletti et al., 2009; Singh et al., 2015). EDU can protect numerous biological or physiological endpoints; nonetheless, literature review studies suggest that the EDU mode of action in protecting against O_3 phytotoxicity remains unclear (Agathokleous et al., 2015; Manning et al., 2011; Oksanen et al., 2013; Paoletti et al., 2009; Singh et al., 2015). It was recently proposed that EDU may protect plants through hormesis, by activating plant defense at low doses via homeostatic disruptions, and the necessity for studying plant metabolism was highlighted (Agathokleous, 2017). Homeostatic re-adjustments after disruption may imply alterations in leaf metabolism and in quantitative and qualitative traits of leaf nutrients. However, there is hitherto no evidence on leaf stoichiometry under EDU treatments.

Ecological stoichiometry is critically important for predicting stress consequences in biochemical cycles and other ecological processes. Leaf stoichiometry and nutrient resorption efficiency (NRE) have been mainly studied in plants experiencing prolonged O_3 stress (Cao et al., 2016; Nakaji et al., 2004; Oksanen et al., 2005; Shang et al., 2018; Shi et al., 2016, 2017; Zhuang et al., 2017) but not at an early stage of the stress so as to be utilized as an early diagnostic tool. Leaf age is an important factor determining O_3 effects on stoichiometry. Younger or late leaves may be more resistant to O_3 than older, mature leaves (Li et al., 2017; Moreno Pina et al., 2017) as a result of adaptive responses which may take place at plant level. For example, relative to mature leaves, younger leaves may: emit greater concentrations of volatile organic compounds (Bison et al., 2018); take up less amount of O_3 (Craker and Starbuck, 1973); have greater carbon (C) assimilation rate and water use efficiency (Moreno Pina et al., 2017); and overall have greater efficiency in apoplastic O_3 detoxification as indicated by avoidance of accumulation of reactive oxygen species in the chloroplasts (Oksanen et al., 2005). Besides, shed leaves may be more affected than mature leaves as they experience O_3 pressure for longer time and the nutrient re-translocation may also be affected (Shang et al., 2018; Shi et al., 2017; Uddling et al., 2006). Despite NRE was studied at later stages of oxidative stress (Shang et al., 2018; Shi et al., 2017; Uddling et al., 2006), leaf nutrient cycling across the crown has not been explicitly investigated under O_3 pressure; none of the previous studies included all leaf classes (i.e. mature, young, shed). Effects of EDU on ecological stoichiometry, including NRE and leaf nutrient cycling across the crown, remain elusive too.

The principal aim of this study is to investigate the ecological stoichiometry in willow (*Salix sachalinensis* F. Schmidt) plants sprayed with 0, 200 or 400 mg EDU L^{-1} and exposed to ambient O_3 or an O_3-enriched atmosphere. The main research question is whether EDU and O_3 affect the leaf stoichiometry and leaf nutrient cycling across the crown (basal, top and shed leaves) early in oxidative stress, before leaves develop visible O_3 symptoms. Based on previous studies where O_3 affected leaf stoichiometry and NRE at later stages of oxidative stress (Shang et al., 2018; Shi et al., 2017; Uddling et al., 2006), it is hypothesized that leaf stoichiometry and nutrient cycling across the crown may be altered at early stages of oxidative stress when plants are under homeostatic disruption. An additional aim is to assess photosynthetic pigments and leaf mass per area (LMA) in mature leaves, as a reference of the stress status, and to assess essential and non-essential elements in an analysis of patterns in the dataset of the elements. Photosynthetic pigments and LMA are known as simple and effective indices for assessing health status in mature leaves (Agathokleous et al., 2017; Onoda et al., 2017; Ronen and Galun, 1984). Mature leaves experience O_3 exposure for longer time and are less affected by ontogenic adjustments than young leaves, and are widely assessed as to gas exchange and other traits under stress.

2. Materials and methods

2.1. Experimental site

The experiment was conducted at Sapporo Experimental Forest of Hokkaido University, Sapporo, Japan (43°.04′ N, 141°.20′ E, 15 m a.s.l.), in the year 2015. For the period June–September, mean monthly average of air temperature was 17.83 (±1.83 s.d.) °C; daily maximum temperature was 22.52 (±1.75 s.d.) °C; daily minimum temperature was 14.07 (±2.02 s.d.) °C; wind speed was 3.52 (±0.17 s.d.) m s^{-1}; relative humidity was 68.83 (±1.58 s.d.) %; mean monthly total sunshine duration was 186.25 (±9.98 s.d.) h; and mean monthly precipitation was 120.50 (±23.19 s.d.) mm, respectively (43°03.6′N 141°19.7′E; Japan Meteorological Agency, 2016; http://www.jma.go.jp/jma/indexe.html).

2.2. Plant material & design of the experiment

The plant materials were current-year cuttings of *S. sachalinensis* (=*S. udensis* Trautv. et C.A. Mey.), originated from the river basin of Ebetsu city, grown in 15-L pots filled with Akadama (well-weathered volcanic ash) and Kanuma (well-weathered pumice) soils at a ratio of 1:1 (DCM Homac CO., LTD., Sapporo, JP). This soil substrate is free from organic matter and poor in phosphorus (P) and nitrogen (N) (for further information please see Electronic Supplementary Material, Table 2S). This willow was selected as biological model due to prior knowledge with regards to its exposure to EDU and O_3 (Agathokleous et al., 2018, 2016a, 2016b). Eighty uniform plants were transplanted in the 15-L pots on June 9th, when they were well rooted. On August 14th, 72 pots were randomly allocated to six plots (12 pots per plot), of which three served as background O_3 (ambient) and three as elevated O_3 treatment. The pots in each plot were further randomly assigned three EDU treatments (four pots per EDU treatment). These plants were used for a different study and thus the full, detailed procedure has been previously described (Agathokleous et al., 2016a). The eight remaining plants were harvested on August 6th, for an approximate image of the initial leaf traits. The length and width of each leaf was non-destructively measured, and the leaf size was calculated using a simple linear model as described earlier (Agathokleous et al., 2016b). Leaves ($n = 549$) were air-dried at 65 °C until a constant mass, and the dry matter of each leaf was measured using a digital balance. The specific leaf area (SLA, or its inverse, LMA) was calculated as the one-side area of a fresh leaf to the dry matter of the leaf. The average leaf size was 7.20 ± 0.26 cm^2, average leaf dry matter 0.09 ± 0.01 g, and SLA 134.27 ± 11.78 cm^2 g^{-1}.

2.3. EDU treatments

The EDU treatments were 0 (EDU0), 200 (EDU200), and 400 (EDU400) mg L^{-1} (Agathokleous et al., 2016a). EDU (100% a.i., source W.J. Manning, University of Massachusetts, USA) was prepared using an electric hotplate 30 min before each application. The required EDU amount was dissolved in partitions of 500 mL, so as the target concentration to be achieved in the final desired volume by adding cool pure water. The initial 500 mL stock for each EDU concentration was prepared using gently-warmed water (Manning et al., 2011) with continuous stirring. The initial stocks were prepared with pure water, and surfactant was not added. EDU was applied using an electric sprayer using Venturi effect, with two