HOKKAIDO UNIVERSITY

Title	Influence of elevated CO2 and ground-level O3 on native deciduous trees in Japan
Author(s)	Agathokleous, Evgenios
Degree Grantor	北海道大学
Degree Name	博士(農学)
Dissertation Number	甲第12714号
Issue Date	2017-03-23
DOI	https://doi.org/10.14943/doctoral.k12714
Doc URL	https://hdl.handle.net/2115/65632
Type	doctoral thesis
File Information	Evgenios_Agathokleous.pdf

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Influence of elevated CO_2 and ground-level O_3 on native deciduous trees in Japan

(北東アジアの落葉樹に対する地表付近の CO_2 とオゾンの影響力)

Evgenios Agathokleous
エフゲニオス　アガトクレウス
The Special Postgraduate Program in Biosphere Sustainability Science
Division of Environmental Resources
Graduate School of Agriculture
Hokkaido University

環境資源学専攻 博士後期課程

Evgenios Agathokleous

Doctor of Philosophy

TITLE:

Influence of elevated CO_2 and ground-level O_3 on native deciduous trees in Japan

Name: Evgenios Agathokleous

Approved, dissertation committee:

Committee chairperson: Professor, Dr. Kazunori Iwabuchi, Hokkaido University, Japan

Committee member: Professor, Dr. Ryusuke Hatano, Hokkaido University, Japan

Committee member: Professor, Dr. Takashi Hirano, Hokkaido University, Japan

Committee member: Professor, Dr. William J. Manning, University of Masacchusetts, USA

Committee member: Senior Researcher, Dr. Elena Paoletti, National Research Council, Italy

Committee member: Professor, Dr. Yuzo Sano, Hokkaido University, Japan

Committee member: Associate Professor, Dr. Toshihiro Watanabe, Hokkaido University, Japan

Committee member: Associate Professor, Dr. Masato Shibuya, Hokkaido University, Japan

Committee member: Lecturer Dr. Hideyuki Saito, Hokkaido University, Japan

Committee member: Assistant Professor, Dr. Tomomichi Kato, Hokkaido University, Japan

APPROVAL SHEET

GRADUATE SCHOOL OF AGRICULTURE

HOKKAIDO UNIVERSITY

I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION ENTITLED

Influence of elevated CO_2 and ground-level O_3 on native deciduous trees in Japan

BE APPROVED AND ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

SUPERVISOR

Professor, Dr. Takayoshi Koike

Hokkaido University

Sapporo, Japan

Contents

ABSTRACT 1

Chapter 1 5

1.1 Atmospheric carbon dioxide (CO_2) and plants 6

1.1.1 Background 6

1.1.2 Effects on plants 7

1.1.3 Limitations in CO_2 research 8

1.1.4 Roots 9

1.1.5 Soil as an additional factor 9

1.2 Tropospheric ozone and plants 10

1.2.1 Ozone 10

1.2.2 Effects of O_3 on plants 11

1.2.3 Plant protectants against O_3 injury 13

1.3 Contents of the present thesis 14

Chapter 2 16

2.1 Introduction 17

2.2 Materials and Methods 19

2.2.1 Experimental site 19

2.2.2 Experimental Design 19

2.2.3 Plant Materials 20

2.2.4 CO_2 treatment 22

2.2.5 Sampling and measurements 23

2.2.6 Statistics 23

2.3 Results 26

2.3.1 Four-year CO_2 exposure on the community 26

2.3.2 Eleven-year CO_2 exposure on beech 31

2.4 Discussion 36

2.4.1 Four-year CO_2 exposure on the community 36

2.4.2 Eleven-year CO_2 exposure on beech 41

Chapter 3 47

4.1 Study 1: the role of soil (larches as model trees) 48

4.1.1 Introduction 48

4.1.2 Materials and Methods 50

4.1.3 Results 56

4.1.4 Discussion 66
4.2 Study 2: The role of EDU (willow as a model tree) 70
4.2.1 Introduction 70
4.2.2 Materials and Methods 74
4.2.3 Results 81
4.2.4 Discussion 90
Chapter 4 93
4.3 Introduction 94
4.4 Materials and Methods 96
4.4.1 Experimental site 96
4.4.2 Plant material and Design of the Experiment 96
4.4.3 EDU treatments 97
4.4.4 Measurements & Samplings 98
4.4.5 Data handling and statistics 102
4.5 Results 103
4.6 Discussion 111
Chapter 5 115
5.1 Effects of elevated CO_2 levels on root traits 116
5.1.1 Four-year CO_2 exposure on the community 116
5.1.2 Eleven-year CO_2 exposure on beech 117
5.2 Effects of elevated O_3 levels on trees 117
5.2.1 Investigating the role of soil (larches as model trees) 117
5.2.2 Investigating the role of EDU (willow as a model tree) 119
5.3 EDU toxic and fertilization effects 120
5.4 Importance for the practice 124
REFERENCES 125
ACKNOWLEDGEMENTS 147
END 150

“As you set out for Ithaka

hope the voyage is a long one,

full of adventure, full of discovery.

….

And if you find her poor,

Ithaka won’t have fooled you.

Wise as you will have become,

so full of experience,

you will have understood by then what these Ithakas mean.”

C.P. Cavafy, *Collected Poems.* Translated by Edmund Keeley and Philip Sherrard. Edited by George Savidis. Revised Edition. Princeton University Press, 1992.

Dedication

The present dissertation is lovingly dedicated to my grandfather Andreas Aresti, whose dream was to have a scientist grandson, and to my mother, Christoula Aresti Agathocleous, and father, George Agathocleous, for all they have been deprived for the education and welfare of their children. Their continuous support and encouragement served as a motivating power.

ABSTRACT

The levels of ozone (O_3) and carbon dioxide (CO_2) are nowadays elevated throughout the Northern Hemisphere, and further elevation is projected for the future. Ozone and CO_2 are the major gases which may pose threat for vegetation, ecosystems, human welfare and finally the biosphere sustainability in the future. Therefore, a critical issue will be the reduction of their effects on plants. The expected depletion of the global phosphate resources may lead to increase in fine root production with a consequent increase of root-fungi symbiosis and mediation of CO_2 effects. However, mediation of O_3 effects through soil is not expected because O_3 is a strong oxidant which can also directly injure plant tissues. Although several substances have been studied as potential protectants of plants against O_3 injury, only ethylenediurea (EDU), a chemical compound, has been found to effectively protect plants against O_3 damage, via a currently unclear mode of action. In this study, I investigated the following topics:

1. **Long term effects of elevated atmospheric CO_2 levels on root traits of a community of native tree species and the role of soil as a driver of the CO_2 effects**

Long-term effects of elevated CO_2 levels on belowground structure of trees growing in different soils remain hitherto unexplored. Saplings of a beech (*Fagus crenata*), an oak (*Quercus mongolica* var. *crispula*) and three birches (*Betula ermanii*, *B. maximowicziana* and *B. platyphylla* var. *japonica*) were grown in immature volcanic ash soil (VA: Vitric Andosols) or brown forest soil (BF: Dystric Cambisols). Volcanic ash is a nutrient poor soil, especially phosphorus poor, broadly distributed in northern Japan. The saplings were further exposed to ambient CO_2 (375-395 ppm) or elevated CO_2 (500 ppm), during daylight hours; each treatment was replicated three times. Beech was exposed to the treatments for eleven years, whereas the other species were exposed for four growing seasons. For both beech and the other species, elevated CO_2 caused a significant increase ($P<0.05$) in the total root production of saplings

grown in VA but did not significantly affect (P>0.05) that of saplings grown in BF. It seems that impacts of elevated CO_2 levels expected in the future may vary among regions with different soils. Elevated CO_2 caused rhizo-morphogenesis through significant enhancement (P<0.05) of fine root production accompanied by an extensive foraging strategy of roots. These phenomena may have long-term implications in the biogeochemical cycles of ecosystems and the below ground biodiversity. The present study provides evidence showing that the soil is an important factor which affects the impacts of elevated CO_2 on roots.

2. Effects of elevated tropospheric O_3 levels on Japanese larch and its hybrid F_1 and the role of soil as a driver of the O_3 effects

Elevated O_3 impacts to economically and ecologically important larches (*Larix sp.*, Pinacaeae) are particularly concerning. I investigated the effect of two-year elevated O_3 exposure (≈66 nmol mol^{-1}) on Japanese larch (*L. kaempferi*) and its hybrid larch F_1 (*L. gmelinii* var. *japonica* × *L. kaempferi*) planted directly into either fertile brown forest soil (BF) or BF mixed with infertile volcanic ash soil (VA). Overall, photosynthetic pigmentation and the growth performance of the stem and crown were reduced in both taxa exposed to elevated O_3. Furthermore, hybrid larch, in both O_3 treatments, performed better than Japanese larch. This finding contradicts findings of prior experiments with potential experimental artifacts of O_3 exposure facilities and root restrictions. Elevated O_3 also disproportionately inhibited stem diameter growth and caused an imbalance in chlorophylls *a*/*b* and chlorophylls/carotenoids ratios. Hybrid and Japanese larches grown in BF and VA had a significantly lower drop of stem diameter over the run of stem height (from base to top) when exposed to elevated O_3, compared to ambient O_3. This finding indicates altered stem shape under elevated O_3. Among eleven response variables, there were no significant interactions between O_3 treatment and taxa. There was also no significant interaction of soil condition and taxa, suggesting that the two larches shared a similar response to O_3 and soil type. Understanding the performance of hybrid larch

in relation to its parent species has ramifications for breeding success in an soil-degraded and O_3-polluted environment.

3. Effects of elevated tropospheric O_3 levels on willow (*Salix sachalinensis* F.Schmidt) and the use of ethylenediurea (EDU) as protectant against O_3 damage

The willow *Salix sachalinensis* (=*S. udensis* Trautv. et C.A. Mey.) is a fast growing species native to wide areas of Asia and Russian Far East. As a hygrophilous and heliophilous species, *S. sachalinensis* could be sensitive to elevated O_3 levels. However, elevated O_3 effects on this species have not been previously studied. A two-year integrated study was carried out to provide new insights on this issue. Current-year cuttings were grown in commercial potting medium, a mixture (1:1) of Akadama (well-weathered VA) and Kanuma (well-weathered pumice) soils – free from organic matter. Plants were exposed to ambient O_3 (≈29 ppb) or to elevated O_3 (≈66 ppb) levels, with three replicates per treatment, during daylight hours. In addition, the plants were treated with 200 ml soil drench containing 0, 200 or 400 mg EDU L^{-1} or with foliar spray at 0, 200 or 400 mg EDU L^{-1}, every nine days. Elevated O_3 injured *S. sachalinensis* plants as it was evidenced by significantly lower (P<0.05) number of leaves, average leaf size and dry matter, plant leaf area and dry matter of root, shoots, foliage, aboveground and the total. The impacts of elevated O_3 were moderate in magnitude and practically significant. EDU soil drench was ineffective in protecting the plants against elevated O_3 injury. However, EDU foliar spray efficiently protected the plants when applied at 200-400 mg L^{-1}. It is concluded that: (i) *S. sachalinensis* plants are sensitive to elevated O_3; (ii) EDU foliar spray is more effective in protecting against O_3 phytotoxicity in this fast-growing species than EDU soil drench.

Since EDU contains nitrogen (N), there is a speculation that EDU protects plants against O_3 injury via contributing with N to plants. To further test if EDU at high doses is phytotoxic and

if it acts as a N source to this species, an additional open-field experiment was conducted. Willow plants, from the same source as the prior experiment, were exposed to ambient O_3 atmosphere and treated with soil drench of 0, 800 or 1600 mg EDU L^{-1}, every nine days, for approximately 2.5 months. These EDU concentrations are 0, 2 and 4 times the common concentration of 400 mg EDU L^{-1}, which effectively protected different plant species against O_3 injury. After examining about fifty response variables, among them N content in different plant organs, it was found that EDU was transferred up to the leaves and high doses significantly increased (P<0.05) the N content in leaves. EDU had no effect on the carbon contents in the plant and was not toxic to this fast-growing species even at the highest dose. Furthermore, based on soil N content, EDU did not persist in the soil which was free from organic matter and poor in N content. Based on estimations, EDU is not expected to act as N source when applied in the appropriate low doses and when the soil does not lack N, and it is unlike N level being responsible for the O_3 protection effect of EDU.

Based on the findings of all topics, I discuss specific responses of deciduous trees to the atmospheric conditions as whole-tree level. My findings may contribute to silviculture techniques for protection and conservation in the future changing environment.

Keywords: CO_2, EDU, Ethylenediurea, O_3, soil

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Chapter 1

GENERAL INTRODUCTION

1.1 Atmospheric carbon dioxide (CO_2) and plants

1.1.1 Background

Carbon dioxide (CO_2) is an anthropogenic greenhouse gas. Limb viewing spaceborne sounders, *in situ* aircraft measurements and simulations from air transport models show that the CO_2 vertical profiles (5-25 Km) range within 3 ppm deviation (Foucher *et al*. 2011), meaning that the variation among atmosphere layers is little. Carbon dioxide level has already risen up to ≈

400 ppm, continues increasing and is projected to reach 500 ppm with the possibility to elevate even further until 2040, as a result mainly of fossil fuel consumption along with intensive deforestation (IPCC 2007; Meehl *et al*. 2007; NOAA 2014). As a naturally occurring compound, CO_2 is the principal source of carbon on Earth. It is thus essential for sustaining life in Earth. However, 'all substances are poisons, there is none which is not a poison. The right dose differentiates a poison and a remedy' (Paracelsus 1525). Therefore, when CO_2 level exceeds some particular thresholds is expected to negatively affect biota.

1.1.2 Effects on plants

It is known that elevated CO_2 alters above- and below-ground functions of plants (Koike *et al*. 1995, 2015; Beerling 1996; Karnosky 2003; Ozanne *et al*. 2003; Heath *et al*. 2005; Cheng *et al*. 2012; Slattery *et al*. 2013; Guo *et al*. 2015; Wang *et al*. 2016a). Trees accumulate carbon (C) via photosynthesis in their stem, branches, and roots (Bazzaz, 1996; Schulze *et al*., 2005). Increases in CO_2 levels may affect the growth of forest trees by altering physiological traits, such as photosynthesis, with further changes in forest productivity and ecosystem functioning (Koike *et al*. 2015; Resco de Dios *et al*. 2017). As CO_2 provides C for the metabolic needs of the plants, it may be speculated that photosynthesis linearly increases as CO_2 level increases. However, this is not the case. It is rather demonstrated that the CO_2-induced stimulation of photosynthesis shifts to down-regulation over time, i.e. CO_2 has negative effect in the long term (Tissue and Oechel 1987), as reviewed by Koike *et al*. (2015). Similarly, stimulation of growth in some tree species may lead to changes in light conditions in forest floor and thereby negatively affect understory vegetation and ecological processes (Oikawa 1986; Koike *et al*. 2015).

The CO_2 effects on plants are complex and highly dependent on the environmental conditions, such as the space given to plans, water availability, soil fertility and stand stage (Körner, 2006). Elevated CO_2 may lead to alterations in qualitative properties of plants, and thus threaten

human nutrition (Loladze, 2002; Myers *et al*. 2014; Shi *et al*. 2016), or quantitative shifts in tree chemical composition (Lindroth *et al*. 2010; Shi *et al*. 2016). It is therefore fair to hypothesize that further increases in CO_2 level can be critical in biosphere sustainability (Lindroth *et al*. 2010; Terashima *et al*. 2014), despite a potential increase in net primary productivity at an extent (Leuzinger *et al*. 2011; Norby and Zak, 2011; Nie *et al*. 2013).

1.1.3 Limitations in CO_2 research

A hitherto weak point in knowledge on effects of future CO_2 levels on trees is the lack of long-term studies; most studies have dealt with seedlings at a juvenile stage and for a short-term of exposure (e.g. Körner 2009; Norby and Zak 2011). Phytochemistry research on several aspen genotypes revealed that the effects of elevated CO_2 levels on forest trees are temporally dynamic over decadal time periods, and underlined the need for long-term research (Couture 2014). Such long-term studies should be conducted in different regions with different edapho-climatological conditions in order to shed light on CO_2 effects on trees after canopy closure and mature stage and to quantify the effect (Leuzinger *et al*. 2011).

The importance of studies conducted in different regions is also highlighted by the latitude-dependency of the effect of atmospheric changes on forest productivity (Silva and Anand 2013). Additionally, another weakness is the wide spacing of plants which has been implemented in several experiments (e.g. Körner 2006). Wide spacing leads to artifacts due to the influence of nutritional resources: If a first year effect is induced by offering open space as a surrogate for ample nutrients, that signal will propagate into the future (even if CO_2 exposure is terminated), which is even worse than short experimental duration (Körner 2006). Furthermore, the evidence from long-term experiments remains little. Short-term experiments might not represent the actual responses of tree root system to CO_2 (Norby and Zak 2011; Kostiainen *et al*. 2014) due to physiological age and size dependency, stand development, community composition, nitrogen deposition, ground-surface ozone, etc. (Asshoff *et al*. 2006; Körner

2006; Pregitzer *et al*. 2008; Kostiainen *et al*. 2009, 2014; Bader *et al*. 2013; Yan *et al*. 2014; Agathokleous *et al*. 2016a). As such, most data do not truly represent the effects of future elevated CO_2 levels on tree roots, the responses of which to elevated CO_2 levels are still not well understood (e.g. Körner 2011; Wang *et al*. 2016a).

1.1.4 Roots

Roots are the hidden half of plants and play a crucial role in forest sustainability under the changing environment (e.g., Eshel and Beeckman 2013; Matyssek *et al*. 2012; 2013; Noguchi and Koike 2016). Fine roots (i.e. production and turnover) partly adjust the biogeochemical cycles of ecosystems and form their response to global change (Norby *et al*. 2004). Moreover, fine root data along with models contribute to the understanding of the global belowground diversity and biogeochemical processes in the terrestrial biosphere (McCormack 2015). Root dynamics can explain elevated-CO_2-induced differences among ecosystems (Norby *et al*. 2004); however, fine-root biomass can vary across years (Pregitzer *et al*. 2008; Wang *et al*. 2016a), as fine root production is also related to biotic factors, such as soil fauna (Lipson *et al*. 2014). In addition, future changes in allocation to belowground of trees in response to elevated CO_2 levels are likely to alter the fungal community (Lipson *et al*. 2014; Wang *et al*. 2016a).

Despite the importance of roots, knowledge about their response to future elevated CO_2 concentrations remains meager (e.g. Körner 2011; Wang *et al*. 2016a). What is also surprising is the prevailing unawareness in the role of soils in belowground responses of trees to atmospheric CO_2 despite that soils have greater influences on responses of plants to other applied treatments (Spinnler *et al*. 2002, 2003; Körner 2011; Sigurdsson *et al*. 2013).

1.1.5 Soil as an additional factor

As it is mentioned above, soil is of utmost importance in driving CO_2 effects on plants. Soil fertility is one of the factors affecting the biomass production of the loblolly pine (Oren *et al*. 2001) and several other tree species (e.g. Norby and Zak 2011; Pregitzer and Tlhelm 2013)

under elevated CO_2 conditions. Departing from Holocene and entering the Anthropocene epoch (Waters *et al*. 2016), makes more than ever the influence of soil fertility essential to be critically studied along with CO_2 treatment.

According to estimations, more than 50% of the global potential arable lands are acidic (von Uexkull and Mutert 1995) and usually Phosphorous (P)-deficient (Zheng 2010; Cordell and Neset 2014; Reijnders 2014; Ulrich and Frossard 2014). Specifically, the pH in Japanese forests ranges from 4.8 to 5.2, except for red soil and podzolic soil (e.g. Hashimoto *et al*. 2012). Soils of Northern Japan are partly dominated by nutrient-poor immature volcanic ash or pumice soils, where P is usually impoverished (Eguchi *et al*. 2005a; Kim *et al*. 2010; Hashimoto *et al*. 2012). Many plants grown under such nutrient limited conditions are usually suffering from nutrient deficiency, and are consequently forced to develop fine roots (Hermans *et al*. 2006) in order to establish symbioses with ectomycorrhizae (e.g. Smith and Read 2008; Qu *et al*. 2010; Qu 2016).

1.2 Tropospheric ozone and plants

1.2.1 Ozone

Unlike CO_2, two types of ozone can be found in the atmosphere of Earth: ozone in the upper atmosphere (stratospheric), which protects the biota against under ultraviolet (UV) radiation, and the ozone in lower atmosphere, at ground level (tropospheric) (O_3), which degrades materials and is highly harmful for the biota. Ozone is formed through reactions between O_3 precursor substances (mainly volatile organic compounds (VOCs), and nitrogen oxides (NOx)), under UV radiation (Kalabokas and Reparis 2004; Kleanthous *et al*. 2014).

Ozone levels are currently elevated enough compared to preindustrial era, especially in the Northern hemisphere (Young *et al*. 2013; Akimoto 2003; Akimoto *et al*. 2015; Kalabokas *et al*. 2015; Saitanis *et al*. 2015a; Kopanakis *et al*. 2016; Sicard *et al*. 2016). This phenomenon is more severe in Asia, due to rapid population growth and industrialization (Ohara *et al*. 2007; Yamaji *et al*. 2008; Verstraeten *et al*. 2015). It is also shown that O_3 levels in European and

USA cities and remote sites are still increasing, although peak values are decreasing (Sicard *et al*. 2013; Paoletti *et al*. 2014a). Furthermore, O_3 levels are elevated in nonindustrial or remote areas, where plants are growing, due to its *in situ* formation and transportation through exchanges of the aerial mass among regions and countries (Ganev *et al*. 2014; Kleanthous *et al*. 2014; Saitanis *et al*. 2015a).

1.2.2 Effects of O_3 on plants

1.2.2.1 General

Nowadays, O_3 is a well-known threat to plants (e.g., Emberson *et al*. 2009; Matyssek *et al*. 2012; Agathokleous *et al*. 2015b,c), and it has been characterized as a novel plant pathogen (Lorenzini and Saitanis 2003). Current evidence shows that O_3 threatens both cultivated plants (Emberson *et al*. 2009; Tiwari and Agrawal, 2010; Feng *et al*. 2008a, 2015; Agathokleous *et al*. 2015a, 2017a) and wild plants (Temple 1989; Bermejo *et al*. 2003; Manning *et al*. 2003; Agathokleous *et al*. 2015b, 2016a). Ozone enters leaves through stomata, alters the membrane properties (Calatayud *et al*. 2003), inhibits guard-cell K^+ channels which mediate K^+ uptake -a key driver of stomatal opening- (Torsethaugen *et al*. 1999), and causes phytotoxicity in the internal leaf tissue (Calatayud *et al*. 2003). The phytotoxicity is possible to be induced by the generation of highly reactive oxygen species (ROS) -including peroxides and free radicals- (Hippeli and Elstner 1996), as oxygen is activated under normal metabolic steady-state conditions and during the plant disease development (here ozone oxidation) (Tzeng and Devay 1993). The inevitable result of such "chain states" over time is cell death (Agathokleous *et al*. 2015b).

1.2.2.2 Forest trees under elevated O_3

Dozens of reviews have documented that elevated O_3 dosages, adversely affect tree species (e.g. Karnosky *et al*. 2005; Paoletti and Grulke 2005; Valkama *et al*. 2007; Wittig *et al*. 2009; Ainsworth *et al*. 2012; Matyssek *et al*. 2013; Agathokleous *et al*. 2015b; Vaultier and Julivet 2015) by impacting plenty of phenological characteristics and physiological functions

(Karnosky *et al*. 2005; Yamaguchi *et al*. 2011; Matyssek *et al*. 2012; Koike *et al*. 2013). This impact is often carried-over, worsening, thus, the plant vigor over time (e.g. Muller *et al*. 1996; Andersen and Scagel 1997; Oksanen and Saleem 1999; Le Thiec and Manninen 2003). Agathokleous *et al*. (2015b,c) retrieved, from scientific databases, 195 papers dealing with the response to O_3 of 473 wild plant species (mostly forest trees) to ozone. It was found that 378 wild plant species were damaged and almost 100 response variables were affected by elevated O_3. Furthermore, some species, which have been characterized as "ozonophobic" (Agathokleous *et al*. 2015b), have been reported to be negatively affected by O_3, even at O_3 levels lower than the AOT40 (= Accumulated Ozone exposure over a Threshold), the sum of the hourly O_3 concentrations exceeding the threshold of 40 ppb during the daylight hours, usually, over the 3 (or 6 for forest trees) months of the growing season.

Ozone does not affect only the aboveground part of the plant but also indirectly—the belowground (Chappelka and Samuelson 1998; Andersen 2003; Karnosky *et al*. 2005; Grantz *et al*. 2006; de Bauer and Hernandez-Tejeda 2007; Matyssek *et al*. 2012; 2013; Huttunen and Manninen 2013; Wang *et al*. 2015). The O_3 effects on belowground of trees and also to other issues related with ecological processes have been recently reviewed (Agathokleous *et al*. 2016a; Wang *et al*. 2016a).

Agathokleous *et al*. (2016a) reviewed 143 SCI research articles which presented results of below-ground variables, including a total of 260 studied cases of which 101 (39 %) concern conifers, 157 (61 %) deciduous and 3 (1 %) shrubs. The total number of studied taxa was 73 (70 trees and 3 shrubs) belonging to 29 genera and 19 families. Twenty of the taxa (32 %) were conifers and 44 (60 %) were deciduous trees. The most reported O_3-induced responses were reduction of root biomass and alteration in the root/shoot ratio, via different strategies of plants to cope with O_3 stress. The imbalance in carbon allocation toward roots leads to a relatively greater reduction on root mass compared with shoot biomass. Such a reduction in root biomass

of urban trees may result in tree failure under strong winds, and may, thus, cause severe risks to citizens (Lorenzini and Nali 2014).

The imbalance in root/shoot ratio further leads to reduced supply of nutrients and water from roots to canopy, which, as a negative feedback effect, may amplify the O_3 direct effects on the leaves and enhance the plant susceptibility to other biotic or abiotic stressors. In addition, negative O_3 effects on the root nutrient quantity (and probably quality) may have unknown implications to soil biodiversity. The O_3-induced belowground effect may —in long term— reduce the net productivity of ecosystems.

All above and below ground disturbances occurring due to elevated O_3 and its interactions not only may have an impact on atmosphere in turn but also on hydrosphere, lithosphere, and biosphere (Earth) in long term. It is thus important to study protection methods of plants against O_3, both for protecting the plants against deleterious effects and biomonitoring (Agathokleous *et al*. 2014, 2016b; Saitanis *et al*. 2015b).

1.2.3 Plant protectants against O_3 injury

Soon after recognizing O_3 as a phytotoxic factor (Middleton *et al*. 1950) various researchers began trials to find a suitable protectant (Middleton *et al*. 1953; Freebairn 1960; Dugger *et al*. 1962; Manning *et al*. 1973; Elfving *et al*. 1976; and others). Many substances have been tested as antiozonants/phytoprotectants over the decades, however, none offered a satisfactory protection to plants. Tested protectants, belonging to various categories, are Bordeaux mixture (Middleton *et al*. 1953), diphenylamine (Elfving *et al*. 1976), di-1-*p*-menthene (Elfving *et al*. 1976), manganese (Middleton *et al*. 1953), peroxyacetyl nitrate (Dugger *et al*. 1962), vitamin C (Freebairn 1960), zinc ethylene-bis-di-thio-carbamate (Middleton *et al*. 1953), and many others. This was the case until 1978, when Carnahan *et al*. (1978) first reported an antiozonant with the common name ethylene-di-urea (EDU) which was described (only as a chemical – not antiozonate role) by Wat (1975) as N-[-2-(2-oxo-1-imidazolidinyl) ethyl]-N'-phenylurea].

EDU appeared to be very effective at protecting plants against O_3 and was included in many investigations carried out over the years (e.g. Weidensaul 1980; Lee *et al*. 1981; Roberts *et al*. 1987; Long and Davis 1991; Kostka-Rick and Manning 1993a,b,c,d; Tonneijck and van Dijk 1997; Tiwari *et al*. 2005; Szantoi *et al*. 2009).

EDU remains hitherto the only known method used by the air pollution research community for protecting plants against O_3 with sufficient outcome in the long term (Paoletti *et al*. 2009; Manning *et al*. 2011; Hoshika *et al*. 2013; Agathokleous *et al*. 2015a; Carriero *et al*. 2015; Basahi *et al*. 2016).

1.3 Contents of the present thesis

Sustainable production of deciduous trees under changing environment should be a goal to be set. With this in mind, ecophysiology of trees under elevated CO_2 and O_3 is covered in the present thesis (Fig. 1). How the soil influences the effects of elevated CO_2 on tree roots, and how EDU acts in plants under elevated O_3 are questions of particular interest. Up to this point, the current problems have been explained. In Chapter 2, the interactive effects of elevated CO_2 and soil on the roots of broad-leaved trees are explored. In Chapter 3, the interactive effects of elevated O_3 and soil on two conifers are covered. Furthermore, the interactive effects of elevated O_3 and EDU on a willow are covered. In Chapter 4, whether EDU is toxic or act as a fertilizer source is investigated. Finally, in Chapter 5, a general discussion of the findings is followed.

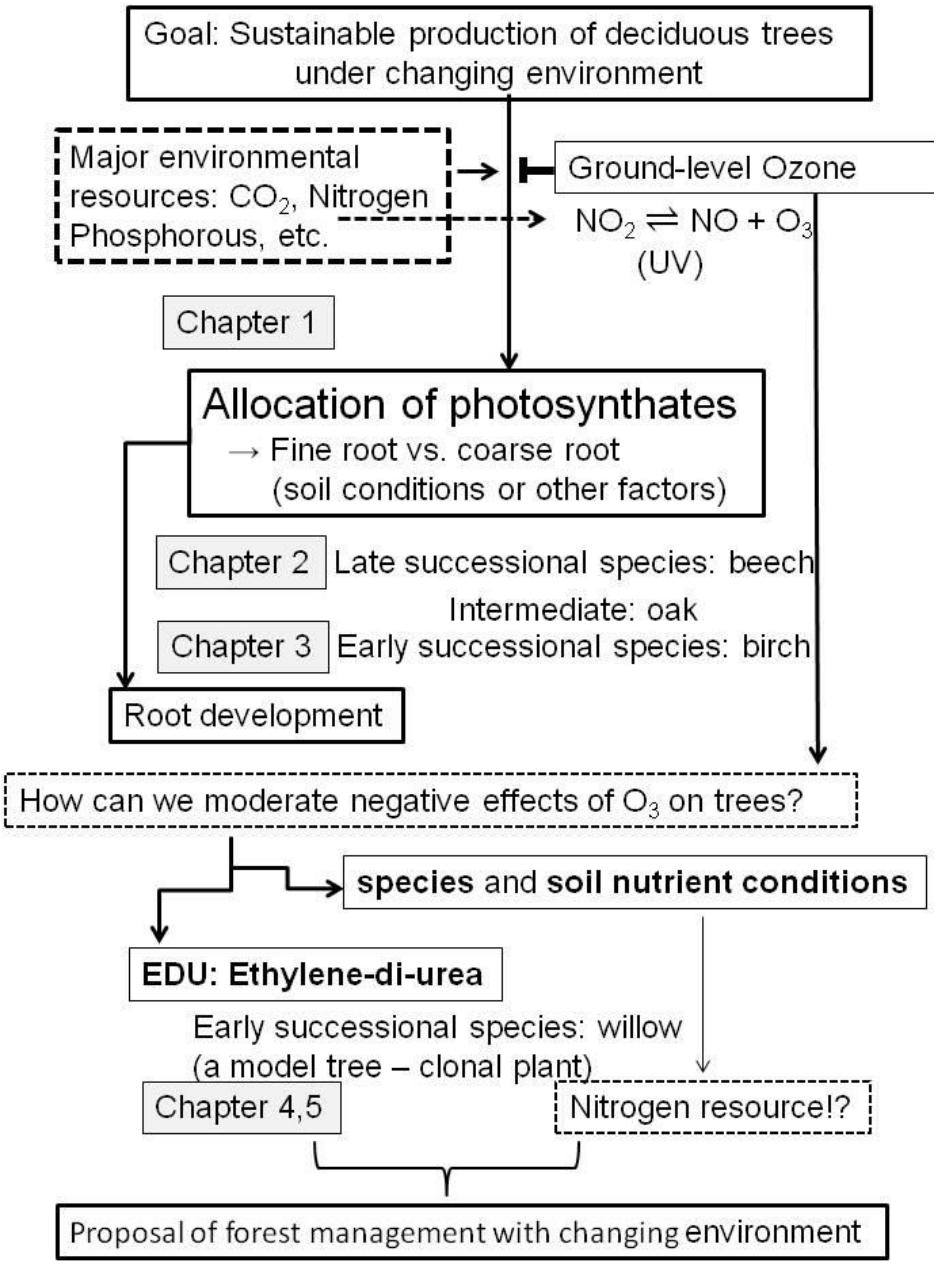

Fig 1 Graphical representation of the contents of the present thesis.

Chapter 2

EFFECTS OF ELEVATED CO_2 LEVELS ON ROOT TRAITS OF A COMMUNITY OF NATIVE TREE SPECIES AND THE ROLE OF SOIL

2.1 Introduction

In general, the allocation pattern of photosynthates to above- and below-ground differs broadly among deciduous tree species (e.g. Schulze *et al*. 2005). This pattern strongly depends on the successional traits of tree species; for example, early successional species (i.e. birch) versus mid-late successional species (i.e. oak) (Koike 1988; Sato 1995). Among birches, this tendency is typically found in mountain birch (Koike *et al*. 2003). The majority of the early successional species, such as birch, grow above- and below-ground simultaneously. Yet, late successional species, usually, first allocate photosynthates to their roots which store them, and then prepare for quick growth in the succeeding year (e.g. Koike 1988, 1995; Sato 1995).

Consequently, these woody plants flourish above ground in the early part of the growing season and then gradually develop their below ground structure. Under elevated CO_2, root growth is expected to increase, boosting the absorption of mineral nutrients and water which support an increased above ground body - if increased. A review of current literature (Agathokleous *et al*. 2016c) revealed that several studies have dealt with responses of individual species to elevated CO_2, while other studies tested the responses of communities to elevated CO_2. However, there is a lack of evidence on the response of communities to elevated CO_2 as a whole unit (e.g. Pregitzer *et al*. 2008).

The first aim of this study was to test if and how a 4-year exposure to elevated CO_2 and soil infertility could impact the belowground functioning, in terms of C allocation within plant body and productivity, of a community of trees of four deciduous, broad-leaved species, which are known to be physiologically (aboveground) affected by elevated CO_2 (Koike *et al*. 2015). This community consisted of three early successional birches and a mid-late successional oak, because these broad-leaved trees play an integral role in the forest functions mentioned above (Jensen and Anderson 1995: Matsuda *et al*. 2002).

Siebold's beech (*Fagus crenata* Blume; Fagaceae) is a late-successional, deciduous, broad-leaved tree native to Japan (Koike *et al*. 1998). It has a distribution from Kyushu (c. 30.5^{o} N) to southern Hokkaido (c. 42.8^{o} N) and a climatic threshold close to the cool-temperate zone (Horikawa, 1972; Fang and Lechowicz, 2006). Thus, it is a dominant species in the cool temperate zone of Japan (Asuka *et al*. 2004a). Among the beech species, Siebold's beech is adapted to and occurs in the most humid conditions of cool temprerate areas (Fang and Lechowicz 2006). Phenological events (*e.g*. flowering and autumnal surcease of growth) of late successional taxa, such as Siebold's beech, are primarily controlled by photoperiod and not temperature (Körner and Basler 2010). This species is vital to ecosystem functioning and biodiversity conservation (Asuka *et al*. 2004b; Hara 2010), and northern Siebold's beech forests (Shirakami-yama mountain range) are included in the World Heritage (UNESCO 2002).

The second objective of the present study was to quantify the belowground net primary production (NPP), in terms of root production, and carbon (C) allocation balance between above-ground and below-ground part of Siebold's beech saplings grown under ambient or elevated CO_2 levels and in fertile brown forest soil (BF: Dystric Cambisols) or infertile, immature volcanic ash plus pumice soil (VA: Vitric Andosols) for 11 years, in a free-air CO_2 enrichment (FACE) system. It was hypothesized that the response of saplings to elevated CO_2 levels would be affected by soil fertility due to shifts in the photosynthetic function and the nutritive demands. It was expected the soil nutrient deficiency to induce a differential impact of elevated CO_2 on the below-ground functioning of the community.

This FACE system was established in northern Japan, in a transition zone between cool temperate and boreal forests, a part of the Asian boreo-nemoral ecotone and sensitive to global climate changes (Uemura, 1992; Matsuda *et al*. 2002). Thus, this research will provide a new piece of information which can be used to determine the future C abundance in trees and NPP (Körner 2003, 2006; Leuzinger *et al*. 2011; Norby and Zak 2011).

2.2 Materials and Methods

2.2.1 Experimental site

The experiments were conducted at Sapporo Experimental Forest of Hokkaido University, Japan (43º.04' N, 141º.20' E, 15 m a.s.l.). 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 43º03.6'N 141º19.7'E (Japan Meteorological Agency 2016). The monthly means of air temperature, wind speed, and relative humidity and the monthly totals of sunshine duration and precipitation were averaged per year (Table 1). The mean values for the years 2003-2013 were 9.31 (± 0.08 se) ºC, 3.54 (± 0.04 se) m s^{-1}, 68.5 (± 0.39 se) %, 1709.86 (± 27.37 se) h, and 1150.14 (± 43.55 se) mm, for each variable, respectively.

Table 1 The yearly averages of the monthly average air temperature, average wind speed, average relative humidity, total sunshine duration, and total precipitation, for the experimental Julian years 2003-2013. Reprinted from Springer Water, Air, and Soil Pollution, Root production of *Fagus crenata* Blume saplings grown in two soils and exposed to elevated CO_2 concentration: an 11-year free-air-CO_2 enrichment (FACE) experiment in northern Japan, 227, 2016, 187. Agathokleous E, Watanabe M, Eguchi N, Nakaji T, Satoh F, Koike T, "With permission of Springer"

Julian Year	**Air Temperature (ºC)**	**Wind Speed (m s^{-1})**	**Relative Humidity (%)**	**Sunshine Duration (h)**	**Precipitation (mm)**
2003	8.8	3.6	68	1787.1	0916.0
2004	9.7	3.6	66	1668.4	1130.5
2005	8.9	3.6	68	1700.5	1236.5
2006	9.1	3.8	68	1725.4	1145.5
2007	9.4	3.4	68	1730.1	1028.5
2008	9.5	3.5	68	1844.5	0843.0
2009	9.4	3.6	68	1604.4	1147.0
2010	9.8	3.4	69	1526.9	1325.0
2011	9.3	3.3	69	1753.6	1253.5
2012	9.3	3.5	70	1819.6	1279.0
2013	9.2	3.6	71	1647.9	1347.0
Average	**9.3**	**3.5**	**69**	**1709.9**	**1150.1**

2.2.2 Experimental Design

The present research was conducted in the FACE system located in the Sapporo Experimental Forest of Hokkaido University, Japan (43º06' N, 141º20' E, 15 m a.s.l), with a split-plot factorial design and employing the randomized block method (Filion *et al*. 2000). The CO_2 treatments were ambient and elevated CO_2, with three site replicates for each treatment. The

design of these FACE facilities was based on the system used at the Stillberg, Davos, in the Swiss Alps (Hättenschwiler *et al*. 2002). The soil treatments were BF, a Dystric Cambisol, (Matsui 2001) and VA, a Vitric Andosol, (Kato 1983), both at each site with a distance of 1.5 m between them. VA is a nutrient-poor soil (Masyagina *et al*. 2006) that was excavated and brought from Tomakomai Experimental Forest of Hokkaido University (42°40’ N, 141°37’ E, 30 m a.s.l.); this soil is widespread in Hokkaido island Hokkaido and ideal for experiments mimicking soil-nutrient deficiency scenarios (e.g. Koike *et al*. 2015, Wang *et al*. 2016a). Since BF is native to the Sapporo Experimental Forest, half of each FACE rings was excavated to a depth of about 15 cm, and it was refilled by mixing the native BF (65 %) with VA (35 %). For the purpose of soil physical properties uniformity, the same process was followed for BF, *i.e.* the excavated soil was back-filled. Although roots can go much deeper, usually most of the nutrients accessed by plants are those found by exploring fine roots at top soil; in this case, most of the lateral and fine roots were distributed between the soil surface and a depth of 10–15 cm. Chemical and physical properties of BF and VA soils used in the present study are illustrated in Eguchi *et al*. (2008) and Watanabe *et al*. (2013). The VA soil had about 17 % lower P_2O_5 content than the BF soil. The sites were completed in autumn 2002.

2.2.3 Plant Materials

Seedlings of Siebold’s beech (*Fagus crenata* Blume), obtained from Hokkaido Hort-green Company Co. Ltd. (located near Sapporo city), were used as experimental subjects. These seedlings were originated from Kuromatsunai town (42°40.14'N 140°18.26'E), the northern boundary of beech stands in Japan (Koike *et al*. 1998). In order to limit tree growth and avoid compound interest effects (Körner 1995, 2006), 2-year-old seedlings (height = 15.3 cm ± 1.5 cm SD, stem basal diameter = 0.42 mm ± 0.08 mm SD) were planted in the FACE rings at a distance of 30 cm among them, after the snow had melted in 2003, with an equal number of 8 individuals in each research condition. In surrounding areas within the same plots (not between

beech saplings to avoid interspecific competition), plants of different species or families, such as alder, birches, larch and oak, were also planted at different times throughout the 11 experimental years (Eguchi *et al*. 2008; Watanabe *et al*. 2013; Agathokleous *et al*. 2016c, see Koike *et al*. 2015 for a complete list of references).

Table 2 The mean (± SD) values of the initial height and diameter (at 2 cm) of the juveniles. Reprinted from Springer Trees, Impact of elevated CO_2 on root traits of a sapling community of three birches and an oak: a free-air-CO_2 enrichment (FACE) in northern Japan, 30, 2016, 353-362, Agathokleous E, Watanabe M, Nakaji T, Wang X, Satoh F, Koike T, "With permission of Springer"

	Ambient CO_2		Elevated CO_2	
	Height (cm)	Diameter (mm)	Height (cm)	Diameter (mm)
Betula ermanii	30.6 (0.8)	4.2 (0.1)	29.4 (1.2)	4.1 (0.2)
B. maximowicziana	64.4 (1.1)	6.1 (0.2)	61.3 (1.4)	6.3 (0.3)
B. platyphylla* var. *japonica	45.1 (0.9)	4.1 (0.2)	44.1 (1.0)	3.9 (0.3)
Quercus mongolica* var. *crispula	55.1 (0.9)	9.3 (0.4)	54.2 (1.1)	9.8 (0.5)

Seedlings of three birch species (*Betula ermanii* (Be: mountain birch), (*B. maximowicziana* (Bm: Monarch birch), *B. platyphylla* var. *japonica* (Bp: white birch)), and a deciduous oak (*Quercus mongolica* var. *crispula* (Qm: oak). All the seedlings were obtained from Hokkaido Hort-green Co. Ltd. near Sapporo, and planted in the FACE rings with a distance of 50 cm among them, in 2010 (after the snow had melted). Equal numbers of individuals among species were planted in each research condition. The mean values (± SD) of the height and trunk basal diameter (at 2 cm from the starting point of root system) of the seedlings were measured for the 3 birches and the Oak; the variability between the two gas treatments within each species was minimal (Table 2). The oak had been suppressed by the birches (i.e. restricted relative light conditions) after 1.5 years with CO_2 treatment (data not shown).

Similarly, data from leaf area per ground area have shown that the mountain birch was suppressed by the white and monarch birches (Hara *et al*. 2014). This was assumed to be a phenomenon that could have occurred in pristine conditions and thus can be considered as a

realistic growth of a community. Nonetheless, the obtained growth data could have been different if the trees had grown independently or in an uncompetitive environment

2.2.4 CO_2 treatment

Treatment with CO_2 was carried out in eleven consecutive Julian years (2003-2013) for beech and in four consecutive Julian years (2010-2013) for the community of the other species. Saplings were fumigated in each growing season during daytime, when the photosynthetic photon flux (PPF) exceeded the 70 μmol m^{-2} s^{-1} (i.e. light compensation point of photosynthesis; Koike 1988), from leaf emergence to leaf senescence (from May until late November). This FACE system consists of six rings, three of which were enriched with additional CO_2 in order to reach the targeted concentration of 500 μmol mol^{-1} (hereafter "eCO_2"); this concentration corresponds to the predicted CO_2 concentration for the Julian years 2040–2050 (Stocker *et al*. 2013).

The diameter and height of each FACE ring are 5 m. The other three ring plots remained under ambient CO_2 (ca. 370-390 μmol mol^{-1}) (hereafter "aCO_2") as noted in researches by Eguchi *et al*. (2008) and Watanabe *et al*. (2013). In order to control the CO_2 concentration, the Vaisala CARBOCAP® Carbon Dioxide Probe GMP343 (Vaisala ©), an accurate and rugged probe-type instrument for ecological measurements, was used (Eguchi *et al*. 2005b). This FACE regime was in accordance with other FACE regimes for studying trees (Karnosky *et al*. 2005; Liberloo *et al*. 2009; Norby *et al*. 2010; Ellsworth *et al*. 2012). More information can be found in previous publications (see Koike *et al*. 2015 for a complete list of references). The mean daytime CO_2 concentrations, as measured in the center of each FACE site, during treatment period 2003-2012 was 498 μmol mol^{-1}. The CO_2 concentration was 500 ± 50 or 500 ± 100 μmol mol^{-1} for 64 or 89 % of the treatment period, respectively.

2.2.5 Sampling and measurements

At the end of the last growing season (2013), the trunk basal diameter was measured for each grown sapling, and all the roots were excavated using a small bulldozer. This mechanistic method was previously compared with the manual method (by hand) and it was revealed that excavation by this method causes destruction of the roots during the excavation process: the final excavated root system had less mass; approximately 70~90 % of the whole roots (Matsunami 2008). Nevertheless, we assume there could be equal amounts of error across all the subjects.

After the excavation, the following procedure was carried out: a) the roots of small classes were sampled, and dried at 75 ^{o}C, for more than 5 days, to constant mass. The dry mass of intermediate roots (ø 2 - 4 mm) and fine roots (ø < 2 mm) – including hyphae of ectomycorrhizae – were determined; b) the whole root systems were left on the field to physically dry; this was unavoidable because of the large number and size of the root systems. The next summer, dry mass measurements were taken for the total root systems (TDM). Due to harsh natural conditions, the identities of some root systems were lost, therefore these samples were not measured. Forty one beech saplings and were sampled and measured, with an average number of 3 (± 1 CI) randomly selected saplings from each soil in each experimental unit. Yet, 168 saplings of the mixed other species community were sampled and measured, with an average number of 3.5 (± 1 CI) randomly sampled saplings per species, from each soil in each experimental unit

2.2.6 Statistics

The accepted threshold of significance was defined at α=.05. Trunk diameter data were transformed to area ($Area = \pi \times \left(\frac{d}{2}\right)^2\ cm^2$). In order to treat the heterogeneity (Saitanis *et al.* 2015b), the data of each variable were subjected to T-scoring standardization using the formula $T = \left(\frac{X-\mu}{\sigma}\right) \times 10 + 50$, where X is the raw score, μ the mean, and σ the standard deviation

(Agathokleous *et al*. 2016c). Consequently, the mean became equal to 50 and the standard deviation equal to 10. The average T-score of each treatment in each experimental unit constituted the real replicate in the overall analysis (n = 3). All the data were subjected to split-plot general linear model randomized by block (GLM), based on Kuehl (1999), and, if needed, Tukey range, posthoc test was followed. For data presentation purpose, the untransformed, instead of transformed, values are presented.

In order to find the effect magnitude of the treatments, the unbiased Cohen's δ (Cohen 1988; Hedges and Olkin 1985) was calculated (using T-scores) for each pair of treatments. The formula used for calculation was (Agathokleous *et al*. 2016b):

$$\delta_{unbiased} = \left(\frac{[C_{i(TRT)} - C_{i(CTRL)}]}{\sqrt{[(n_{i(TRT)} - 1) * (SD_{i(TRT)})^2 + (n_{i(CTRL)} - 1) * (SD_{i(CTRL)})^2]/(n_{i(TRT)} + n_{i(CTRL)} - 2)}} \right) \times \left(1 - \frac{3}{4(n_{i(TRT)} + n_{i(CTRL)} - 2) - 1} \right)$$

where the numerator is the difference between the two means, the denominator is the pooled standard deviation and C_i, n_i, SD_i, stand for the mean score, the sample size, and the standard deviation, respectively, in the plants of an experimental condition (*TRT*) or control group (*CTRL*), of the *i* case.

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). These characterizations should be interpreted with caution

since the segments are arbitrary and their practical and biological importance is case-specific (i.e. a small *EM* could be more practically important than a large *EM*) (Agathokleous *et al*. 2016b). These characterizations were made based on, and applied to, behavioral sciences studies and not to ecophysiological studies (Agathokleous *et al*. 2016f). 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 (Agathokleous *et al*. 2016f). According to the only available evidence (Agathokleous *et al*. 2016b,c,d) 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, small, moderate and large *EM*, respectively, with absolute δ values in the interval [0.50-1.50] indicating educational significance and δ values >1.50 indicating practical significance (Agathokleous *et al*. 2016f).

Finally, in order to find the percentile gain in experimental conditions, the Cohen's U_3 index (Cohen 1977) was calculated, from the δ using the following formula:

$$U_3 = \Phi(\delta)$$

In addition, $\delta_{unbiased}$ was converted to overlapping coefficient (OVL) (Reiser and Faraggi 1999) using the formula

$$OVL = 2\Phi(-|\delta|/2)$$

where, in both U_3 and OVL formulas, Φ is the cumulative distribution function of the standard normal distribution and δ the population $\delta_{unbiased}$. Unbiased δ, U_3 and OVL were calculated only for the pairs with statistically significant difference in order to quantify the size of the difference.

Data processing and statistical analyses were conducted using the MS EXCEL 2010 (Microsoft ©) and STATISTICA v.10 (StatSoft Inc. ©) software.

2.3 Results

2.3.1 Four-year CO_2 exposure on the community

Elevated CO_2 appeared a main effect on all variables with the exception of Area:TDM; this effect ranged from small to large effect magnitude. Soil, however, had only a moderate effect on Fine:Area and a small effect on fine root biomass (Tables 3 and 4). The CO_2 × Soil interactions was insignificant only for Area:TDM and intermediate roots.

Table 3 Summary of the GLM results and mean untransformed values (± sd) of the measured variables trunk basal area (Trunk Area), total belowground dry mass (TDM), Area/TDM rate, coarse, fine and intermediate roots dry masses, ratio of fine root biomass to intermediate root biomass (Fine/Intermediate) and fine root to trunk basal area rate (Fine/Area). The lowercase letters above the mean values indicate the significant differences among the 4 combination treatments. The results of each variable obtained by a GLM analysis or a Tukey range, post-hoc test, after significant results of the GLM analysis, based on standardized data. Means within each variable marked with different lowercase letters differ statistically significantly at a level of significance α=0.05. Data obtained from Siebold's beech (*Fagus crenata*) saplings exposed to ambient (370–390 µmol mol^{-1}) or elevated (500 µmol mol^{-1}) CO_2 and grown either in brown forest soil or immature volcanic ash plus pumice soil for 11 consecutive years. Three real replicates were used for each experimental condition.

	GLM results			Means (± se) & Tukey's range test results (CO_2 × Soil)			
	CO_2	**Soil**	**CO_2 × Soil**	**aCO_2×BF**	**eCO_2×BF**	**aCO_2×VA**	**eCO_2×VA**
Trunk Area (cm^2)	**F = 22.56, p < 0.010**	F = 01.83, *p* = 0.213	**F = 21.75, *p* < 0.010**	6.064^{a} (0.501)	6.159^{a} (0.323)	4.736^{b} (0.371)	6.931^{a} (0.400)
TDM (kg)	**F = 14.67, p < 0.010**	F = 01.76, *p* = 0.221	**F = 14.68, *p* < 0.010**	0.774^{a} (0.085)	0.796^{a} (0.039)	0.678^{a} (0.074)	1.040^{b} (0.113)
Area/TDM (cm^2 g^{-1} ×10)	F = 00.18, *p* = 0.681	F = 00.75, p = 0.413	F = 04.42, *p* = 0.069	0.337^{a} (0.020)	0.358^{a} (0.021)	0.352^{a} (0.021)	0.319^{a} (0.029)
Coarse root (kg)	**F = 09.72, p < 0.050**	F = 01.64, *p* = 0.236	**F = 11.27, *p* < 0.010**	0.740^{ab} (0.089)	0.744^{a} (0.067)	0.647^{a} (0.080)	0.989^{b} (0.121)
Fine root biomass (g)	**F = 79.15, *p* < 0.001**	**F = 10.65, *p* < 0.050**	**F = 18.91, *p* < 0.010**	14.822^{a} (0.807)	17.242^{b} (0.885)	10.812^{c} (1.099)	17.816^{b} (0.844)
Intermediate root biomass (g)	**F = 73.00, *p* < 0.001**	F = 04.19, *p* = 0.075	F = 01.23, *p* = 0.300	24.855^{a} (3.217)	36.015^{b} (2.146)	21.769^{a} (1.291)	34.754^{b} (2.257)
Fine/Intermediate (g/g)	**F = 05.75, *p* < 0.050**	F = 04.62, *p* = 0.064	**F = 16.75, *p* < 0.010**	0.613^{a} (0.045)	0.479^{b} (0.036)	0.485^{b} (0.029)	0.524^{b} (0.047)
Fine/Area (g cm^{-2} ×10)	**F = 22.04, *p* < 0.010**	**F = 66.02, *p* < 0.001**	**F = 10.71, *p* < 0.050**	0.227^{a} (0.038)	0.263^{ab} (0.032)	0.338^{b} (0.039)	0.513^{c} (0.043)

Table 4 The unbiased Cohen *δ*, Cohen U_3 index and overlapping coefficient (OVL) of the measured variables trunk basal area (Trunk Area), total belowground dry mass (TDM), Area/TDM rate, coarse, fine and intermediate roots dry masses, ratio of fine root biomass to intermediate root biomass (Fine/Intermediate) and fine root to trunk basal area rate (Fine/Area). The effect size (*ES*), for each pair with statistically significant difference, is indicated by the letters M and L for Moderate and Large effect, respectively. "n/a" shows that there was no statistically significant effect and therefore the values are not available.

	aCO_2 vs. eCO_2			BF vs. VA			aCO_2×BF vs. eCO_2×BF			aCO_2×VA vs. eCO_2×VA		
	δ	**U_3**	**OVL**	***δ***	**U_3**	**OVL**	***δ***	**U_3**	**OVL**	***δ***	**U_3**	**OVL**
Trunk Area (cm^2)	1.560(M)	0.941	0.435	n/a	n/a	n/a	n/a	n/a	n/a	5.573(L)	1.000	0.005
TDM (kg)	1.430(S)	0.924	0.475	n/a	n/a	n/a	n/a	n/a	n/a	3.720(L)	1.000	0.063
Area/TDM ($\mathrm{cm}^2/kg \times 10$)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Coarse root biomass (kg)	1.259(S)	0.896	0.529	n/a	n/a	n/a	n/a	n/a	n/a	3.262(L)	0.999	0.103
Fine root biomass (g)	2.680(M)	0.996	0.180	0.585(S)	0.721	0.770	2.800(M)	0.997	0.162	7.004(L)	1.000	0.000
Intermediate root biomass (g)	4.306(L)	1.000	0.031	n/a	n/a	n/a	3.999(L)	1.000	0.046	6.919(L)	1.000	0.001
Fine/Intermediate (g/g)	0.817(S)	0.793	0.683	n/a	n/a	n/a	3.203(L)	0.999	0.109	n/a	n/a	n/a
Fine/Area ($\mathrm{g}/\mathrm{cm}^2 \times 100$)	0.942(S)	0.827	0.638	2.350(M)	0.991	0.240	n/a	n/a	n/a	4.201(L)	1.000	0.036

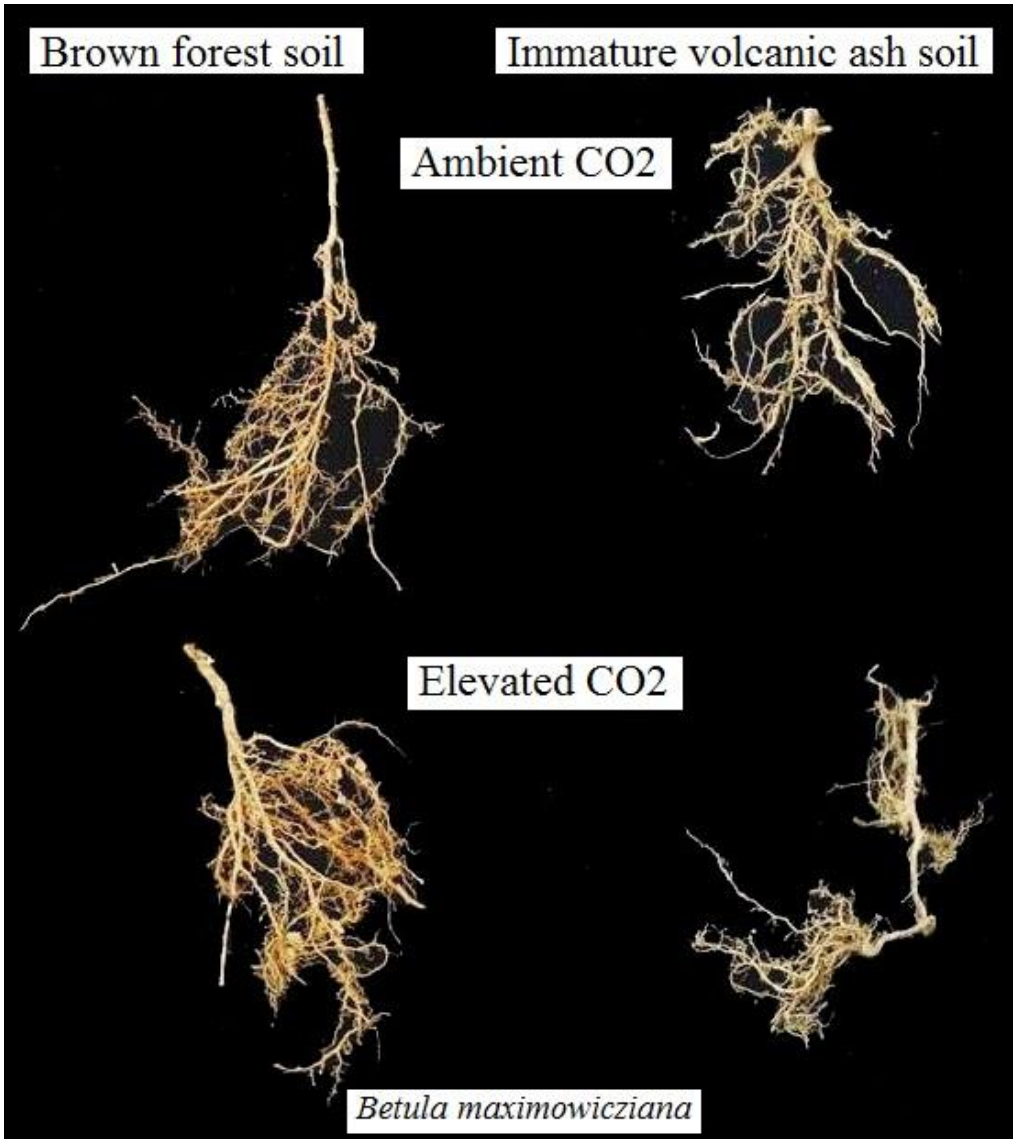

Fig 2 Typical difference of 10 cm root tips. Samples were obtained from Monarch birch (*Betula maximowicziana*) saplings which were grown in two different types of soil (BF: brown forest soil or VA: volcanic ash soil including pumice) and exposed either to ambient CO_2 (370–390 μmol mol^{-1}) or to elevated CO_2 (500 μmol mol^{-1}) for four growing seasons (2010-2013).

Regarding the Area, eCO_2 was responsible for a moderate effect, which appeared as smaller Area compared to aCO_2 (Table 4). There was a convergent interaction in Area due to a higher (ns) Area of the communities under eCO_2×VA, compared to eCO_2×BF, and a significantly smaller Area of the community grown under aCO_2×VA, compared to aCO_2×BF (Table 3). Under eCO_2×VA, there was not such a reduction. Instead, eCO_2×VA led to a larger Area than

that of plants in aCO_2×VA. There was no significant effect of CO_2 on the communities grown in BF soil.

Similarly, the greater TDM induced by eCO_2 at a small magnitude can be attributed to the communities grown in VA soil and not in BF soil. Under VA soil and eCO_2, the TDM was higher than under aCO_2×VA, and, unexpectedly, it was also higher than that under eCO_2×BF.

Likely to TDM, the coarse root biomass was increased by eCO_2 at a small magnitude, regardless of the soil treatment. This caused by a large increase of coarse root biomass in eCO_2×VA, which also led to a divergent interaction. Under eCO_2×VA, coarse root biomass was largely higher than under aCO_2×VA and eCO_2×VA. It is revealed that there was no effect of eCO_2 when the communities were grown in BF soil. On the other hand, none of the factors had a significant effect on Area:TDM (Table 3).

Fine root biomass was superior in eCO_2 than in aCO_2, as a main effect, and the magnitude was moderate (Table 4, Fig. 2). Furthermore, it was suppressed by VA soil, compared to BF soil, but the magnitude was small. The interaction of the two factors was significant and could be characterized as divergent with differences in sizes as well. When the communities had grown in BF and VA soils, eCO_2 caused moderate or large increases of fine root biomass. The small main effect of the VA soil was produced by a lower fine root biomass under aCO_2×VA, compared to aCO_2×BF.

Intermediate root biomass experienced a large positive effect by eCO_2, independent of soil treatment, however it was not affected by the main effect of soil. There was also no interaction between the two factors.

As a result, the Fine: Intermediate root dry mass ratio (Fine:Intermediate) was negatively affected by eCO_2 as a main effect, at a small magnitude, and there was a significant interaction

with soil that tended to mimic a directional interaction. It seems that both the eCO_2 and VA soil alone or in combination caused a higher increase of intermediate root than fine root biomass. Communities under aCO_2×BF had higher Fine:Intermediate than under eCO_2×BF, aCO_2×VA, and eCO_2×VA, respectively. Among the latter three treatments, there was no statistical difference.

Finally, Fine:Area was increased by eCO_2 and VA factors at a small magnitude, regardless of each other factor. The factor of the interaction of these two variables was also significant. Elevated CO_2 had no significant effect on the Fine:Area of the community grown in BF, however it had a large effect on the Fine:Area of the community grown in VA.

2.3.2 Eleven-year CO_2 exposure on beech

Elevated CO_2 had a significant impact to all the variables except trunk area (Table 5). Soil *per se* and its interaction with CO_2 had also significant impact on trunk area, TDM, intermediate root biomass, and ratio of fine root biomass to intermediate root biomass (Fine:Intermediate); the impact on trunk area to TDM rate (Area:TDM) and fine root biomass was insignificant. Particularly, eCO_2 led to an increase in TDM (small magnitude), fine root biomass (moderate magnitude), and Fine:Intermediate (small magnitude), and a decrease in Area/TDM (moderate magnitude) and intermediate root biomass (small magnitude) (Tables 5 and 6). The eCO_2-induced increase of fine root biomass was visible even to the naked eye (Fig 3). VA induced a moderate increase in the Fine/Intermediate and a decrease in trunk area (small magnitude), TDM (small magnitude), and intermediate root biomass (moderate magnitude) (Table 6).

According to Tukey range tests (n = 3), the only statistically significant difference between the aCO_2 and eCO_2 when the saplings had grown in BF was that of fine root biomass and Fine/Area, where eCO_2 caused large increases (Tables 5 and 6). Furthermore, variant results were obtained when the saplings had grown in VA: eCO_2 did not significantly alter the trunk area and the Area/TDM, but it did induce a large increase in TDM, fine root biomass, and Fine/Intermediate,

and a large decrease in intermediate root biomass. The largely reduced mass of intermediate root biomass, when saplings had grown in VA and exposed to eCO_2, was apparently accounted for the significant reduction in intermediate root biomass by eCO_2 as a main factor.

Under aCO_2, VA caused significant reductions of large size in the trunk area and TDM. On the other hand, under eCO_2, VA caused a large reduction in the intermediate root biomass and, consequently, a large increase in the Area:TDM (Tables 5 and 6). Although, under eCO_2, saplings grown in VA had increased TDM and fine root biomass (compared to those grown under eCO_2 and BF), these differences were not significant ($p>0.05$).

Finally, the Fine: Area ratio was largely increased by eCO_2, regardless of soil (Tables 5 and 6). Soil and the interaction between CO_2 and Soil were insignificant factors.

Table 5 Summary of the GLM results and mean untransformed values (± se) of the measured variables trunk basal area (Trunk Area), total belowground dry mass (TDM), Area/TDM rate, fine and intermediate roots dry masses, ratio of fine root biomass to intermediate root biomass (Fine/Intermediate) and fine root to trunk basal area rate (Fine/Area). The lowercase letters above the mean values indicate the significant differences among the 4 combination treatments. The results of each variable obtained by a GLM analysis or a Tukey range, post-hoc test, after significant results of the GLM analysis, based on standardized data. Means within each variable marked with different lowercase letters differ statistically significantly at a level of significance α=0.05. Data obtained from Siebold's beech (*Fagus crenata*) saplings exposed to ambient (370–390 μmol mol^{-1}) or elevated (500 μmol mol^{-1}) CO_2 and grown either in brown forest soil or immature volcanic ash plus pumice soil for 11 consecutive years. Three real replicates were used for each experimental condition. Reprinted from Springer Water, Air, and Soil Pollution, Root production of *Fagus crenata* Blume saplings grown in two soils and exposed to elevated CO_2 concentration: an 11-year free-air-CO_2 enrichment (FACE) experiment in northern Japan, 227, 2016, 187. Agathokleous E, Watanabe M, Eguchi N, Nakaji T, Satoh F, Koike T, "With permission of Springer"

	GLM results			Means (± se) & Tukey's range test results (CO_2 × Soil)			
	CO_2	**Soil**	**CO_2 × Soil**	**aCO_2×BF**	**eCO_2×BF**	**aCO_2×VA**	**eCO_2×VA**
Trunk Area ($cm^2/100$)	F = 00.55, *p* = 0.48	**F = 06.63, *p* < 0.05**	**F = 08.26, *p* < 0.05**	0.357^{a} (0.038)	0.306^{ab} (0.024)	0.227^{b} (0.003)	0.313^{ab} (0.015)
TDM (kg)	**F = 16.92, *p* < 0.01**	**F = 05.95, *p* < 0.05**	**F = 43.62, *p* < 0.01**	1.617^{a} (0.046)	1.421^{a} (0.104)	0.908^{b} (0.090)	1.748^{a} (0.060)
Area/TDM ($cm^2/kg \times 10$)	**F = 10.86, *p* < 0.01**	F = 01.16, *p* = 0.31	F = 00.07, *p* = 0.81	0.288^{a} (0.016)	0.233^{a} (0.014)	0.273^{a} (0.008)	0.209^{a} (0.028)
Fine root biomass (g)	**F = 42.48, *p* < 0.01**	F = 00.01, *p* = 0.93	F = 03.39, *p* = 0.10	0.122^{a} (0.021)	0.207^{b} (0.036)	0.090^{a} (0.013)	0.242^{b} (0.031)
Intermediate root biomass (g)	**F = 23.82, *p* < 0.01**	**F = 15.26, *p* < 0.01**	**F = 20.72, *p* < 0.01**	0.750^{a} (0.085)	0.730^{a} (0.051)	0.790^{a} (0.064)	0.209^{b} (0.033)
Fine/Intermediate (g/g)	**F = 20.90, *p* < 0.01**	**F = 20.27, *p* < 0.01**	**F = 19.58, *p* < 0.01**	0.249^{a} (0.036)	0.285^{a} (0.053)	0.268^{a} (0.059)	2.463^{b} (0.480)
Fine/Area ($g/cm^2 \times 100$)	**F = 42.20, *p* < 0.001**	F = 01.44, *p* = 0.26	F = 00.14, *p* = 0.72	0.347^{a} (0.012)	0.691^{b} (0.065)	0.394^{a} (0.028)	0.780^{b} (0.086)

Table 6 The unbiased Cohen *δ*, Cohen U_3 index and overlapping coefficient (OVL) of the measured variables trunk basal area (Trunk Area), total belowground dry mass (TDM), Area/TDM rate, fine and intermediate roots dry masses, ratio of fine root biomass to intermediate root biomass (Fine/Intermediate) and fine root to trunk basal area rate (Fine/Area). The effect size (*ES*), for each pair with statistically significant difference, is indicated by the letters M and L for Moderate and Large effect, respectively. "n/a" shows that there was no statistically significant effect and therefore the values are not available.

	aCO_2 vs. eCO_2			BF vs. VA			aCO_2×BF vs. eCO_2×BF			aCO_2×VA vs. eCO_2×VA		
	δ	**U_3**	**OVL**	***δ***	**U_3**	**OVL**	***δ***	**U_3**	**OVL**	***δ***	**U_3**	**OVL**
Trunk Area (cm^2)	n/a	n/a	n/a	1.159(S)	0.877	0.562	n/a	n/a	n/a	n/a	n/a	n/a
TDM (kg)	1.001(S)	0.842	0.617	0.544(S)	0.707	0.786	n/a	n/a	n/a	7.782(L)	1.000	0.000
Area/TDM ($cm^2/kg \times 10$)	2.003(M)	0.977	0.317	n/a	n/a	n/a	n/a	n/a	n/a	n/a		
Fine root biomass (g)	2.630(M)	0.996	0.189	n/a	n/a	n/a	6.395	1.000	0.001	4.490(L)	1.000	0.025
Intermediate root biomass (g)	1.359(S)	0.913	0.497	0.995(M)	0.840	0.619	n/a	n/a	n/a	8.013(L)	1.000	0.000
Fine/Intermediate (g/g)	1.220(S)	0.889	0.542	1.194(M)	0.884	0.551	n/a	n/a	n/a	4.537(L)	1.000	0.023
Fine/Area ($g/cm^2 \times 100$)	3.875(L)	0.100	0.053	n/a	n/a	n/a	5.223	1.000	0.009	4.238(L)	1.000	0.034

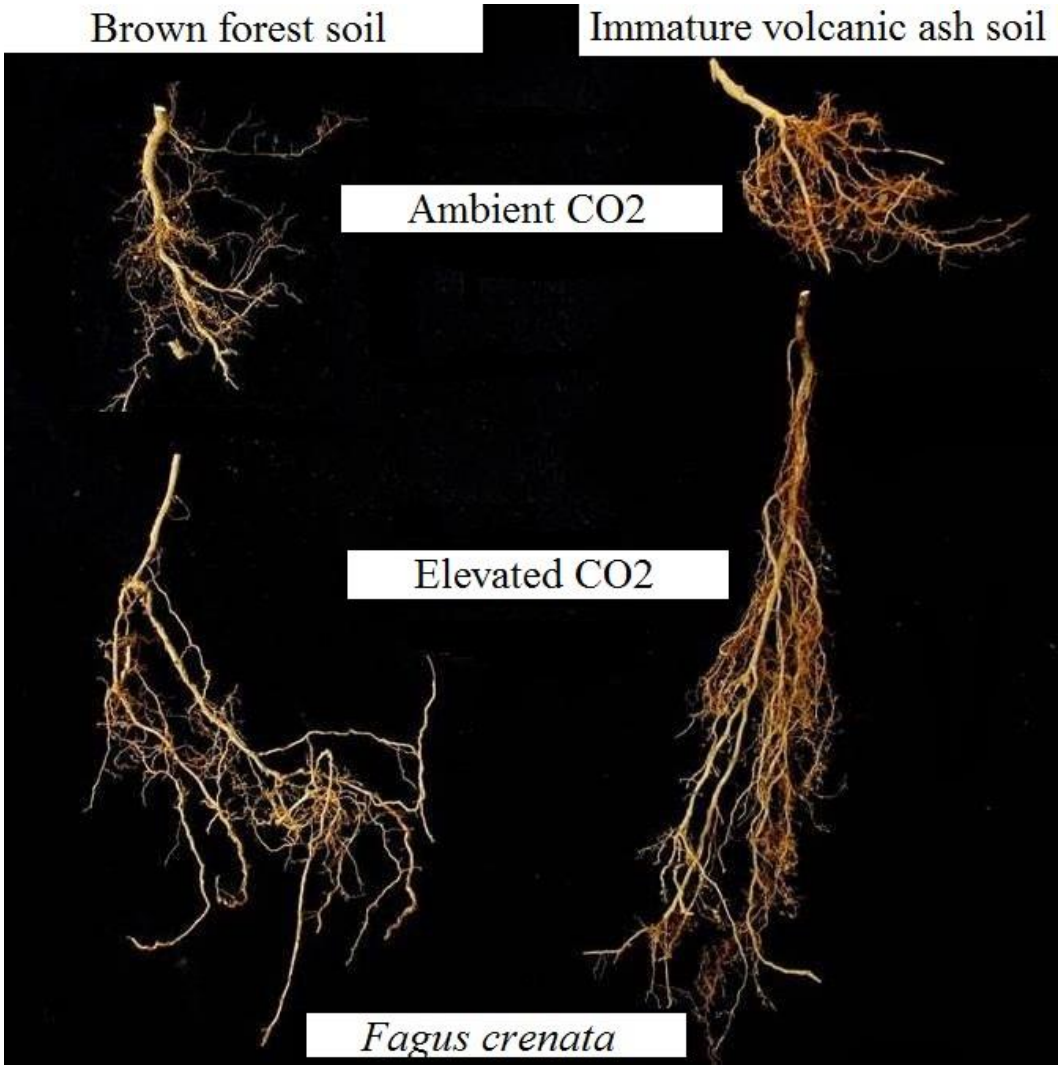

Fig 3 Typical difference of 10 cm root tips. Samples were obtained from Siebold's Beech (*Fagus crenata*) saplings which were grown in two different types of soil (BF: brown forest soil or VA: volcanic ash soil including pumice) and exposed either to ambient CO_2 (370–390 μmol mol^{-1}) or to elevated CO_2 (500 μmol mol^{-1}) for 11 growing seasons (2003-2013). Reprinted from Springer Water, Air, and Soil Pollution, Root production of *Fagus crenata* Blume saplings grown in two soils and exposed to elevated CO_2 concentration: an 11-year free-air-CO_2 enrichment (FACE) experiment in northern Japan, 227, 2016, 187. Agathokleous E, Watanabe M, Eguchi N, Nakaji T, Satoh F, Koike T, "With permission of Springer"

2.4 Discussion

2.4.1 Four-year CO_2 exposure on the community

It was predicted that root growth and root architecture of the sapling community would be influenced by eCO_2 and soil infertility and thus the response of the community to eCO_2 would depend on the soil fertility and species seral traits. Based on the results, and in contrast to the expectations, this 4-year treatment with eCO_2 resulted in neither a greater Area nor an increased TDM (or coarse root biomass) when the community had grown in the fertile BF soil. Elevated CO_2 also increased Area, coarse root biomass and TDM when the community had grown in the infertile VA soil. Moreover, no specific seral traits were found in final production of root biomass (P>0.05; data not shown), which may be due to only one final assessment of the production of root biomass. In addition, root growth may be restricted after canopy closure (Sato 1995; Hara *et al*. 2014; Wang *et al*. 2016b).

Area:TDM and TDM were assessed in order to evaluate the root responses to eCO_2. The former is involved in the tree functional traits, i.e. C allocation, and the latter is important for C sequestration capacity of the ground. Although VA suppressed the Area, it did not suppress the TDM under aCO_2. Meanwhile, it appeared that eCO_2 had a surprisingly large hostile effect on the VA soil, and thus the negative effects of the VA soil on Area were compensated. Although neither eCO_2 nor VA soil affected the Area:TDM, VA soil in combination with eCO_2 led to a marginally (P>0.05) higher belowground allocation than aboveground (i.e., trunk area) compared to aCO_2×BF, aCO_2×VA and eCO_2×VA. Despite this, under ambient CO_2, soil infertile conditions are usually expected to alter the C partitioning to favor root growth (Hermans *et al*. 2006), yet this was not the case under aCO_2.

The present results agree with the findings of quantitative reviews which state that, on average, eCO_2 does not alter the allometry of plants (Poorter and Nagel 2000; Poorter *et al*. 2012). However, the results of the statistical analyses differ to the general finding which notes that

soil infertility does alter the allometry (Poorter and Nagel 2000; Poorter *et al*. 2012) as explained by the *functional equilibrium* theory (Brouwer 1962; Poorter and Nagel 2000). The present findings pointed out that C allocation within a plant body was not altered in this community of trees by a long-term experimental fumigation with eCO_2, while the belowground productivity, in eCO_2, increased only in the community grown in VA soil but not BF, which may be due to physical structure of BF (e.g. Eguchi *et al*. 2005a).

The usual response of trees to elevated CO_2 is an increased root growth and root turnover of fine roots (e.g. Karnosky 2003; Pregitzer *et al*. 2008; de Oliveira *et al*. 2012; King *et al*. 2013; Cao *et al*. 2008). The results of this study support this response with the large effect of eCO_2 on TDM when the community was grown in VA soil. On the other hand, there was no effect of eCO_2 when the community was grown in BF. Insignificant increases of root biomass due to 2-growing-season treatment with eCO_2 were also found in *Larix gmelinii* var. *japonica* × *L. kaempferi* seedlings grown in well-homogenized BF soil in FACE (Watanabe *et al*. 2013) or OTCs (Wang *et al*. 2016b), in the same region. Furthermore, short-term treatment with elevated CO_2 had no significant effects on the root biomass of neither the *Betula papyrifera* seedlings grown in slight fertilization (Zhang *et al*. 2006) nor the *Fagus crenata* seedlings (Hirano *et al*. 2012). In general, root longevity is positively correlated with poor soil-nutrient status and well aeration of soils (Fitter and Hay 2002), which in this case may be attributed to high porosity of VA soil with pumice (e.g. Eguchi *et al*. 2005a; Masyagina *et al*. 2006).

The eCO_2 increased fine roots, of which the majority naturally colonizes A horizon (e.g. Jaloviar *et al*. 2009) in both the BF and VA soils. It seems fine roots are ecologically important components as they account for some 13 % of net primary production (NPP) (Ostonen *et al*. 2005) and a high proportion of total litter (e.g. Leppalammi-Kujansuu *et al*. 2014). Thus, increased production of fine roots may have an ecologically important benefit in nutrient cycling in a forest. As observed in the Area, although VA soil unexpectedly suppressed fine

roots under aCO_2, with the interaction of VA×eCO_2, the fine roots still reached levels similar to that of BF×eCO_2. It was expected that under the influence of either eCO_2 or VA soil, the saplings would undergo a higher need to seek nutrients thereby increasing the fine root-area:biomass in order to access more nutrients. Instead, this was the case only for eCO_2 since VA soil caused a reduction of fine root biomass, until the canopy closed (Hara *et al*. 2014). At present, however, a plausible reason for this phenomenon cannot be given.

There was an unexplained decrease in fine root biomass in VA soil under aCO_2. Yet the Fine:Area, which is an appropriate index to assess the belowground responses in relation to nutrient cycling, showed something different. Namely, VA soil induced a large (effect magnitude ≈3) increase when the community had been exposed to aCO_2 (cf. aCO_2×BF). Among the interaction levels, the largest Fine:Area was observed in eCO_2×VA: it was higher than aCO_2×VA and higher (effect magnitude >3) than eCO_2×BF. A different foraging strategy of fine roots among the research conditions is clear, in accordance with the findings of Ostonen *et al*. (2011) and Leppalammi-Kujansuu *et al*. (2014). Particularly, fine roots underwent extensive foraging strategy (Ostonen *et al*. 2011), i.e. higher Fine:Area, in VA soil, in both CO_2 conditions. Moreover, eCO_2 had a synergistic effect with VA soil leading to the highest Fine:Area. These results coincide with the results of Area:TDM, albeit insignificant, and indicating greater responses to eCO_2 in relation to nutrient cycling when the community was grown in VA soil.

Differences of intermediate root biomass were attributed only to eCO_2 which increased the biomasses of the communities grown in both the BF and VA soils, compared to the pairwise aCO_2. Consequently, there was a higher amount of intermediate roots than fine roots both in VA soil and eCO_2. The ratio of fine roots to intermediate roots indicates the balance of the two root classes, and its modification is dependent on the needs of the plants. For instance, plants in the need of increasing nutrient uptake are expected to modify a higher proportion of fine

roots compared to intermediate roots. However, this could be misleading in such cases which take into account single observations of only standing biomasses. A single observation, as in our case, of the living fine roots, does not stand for the total production of fine roots, but only a partial production of that given time. In this study, this ratio should be taken with a degree of caution as fine roots were increased by eCO_2 as well. One explanation for the higher amount of intermediate roots than fine roots could be the mortality and regeneration of fine roots that occur (Pritchard *et al*. 2008) more frequently throughout the growing season which deposit a significant amount of can. As such, it can only be speculated that there was an accelerated turnover of fine roots in all research conditions except aCO_2×BF.

Despite the expectations that soil infertility might lead to a maximum fine root production independent of the CO_2 treatment, CO_2 treatment did appear to have a combination effect with soil infertility, resulting in higher production of fine roots. Could this phenomenon be an ecological growth method of saplings in order to mitigate the negative effects via plasticity? In other terms, this phenomenon could be a kind of "*rhizo-morphogenesis*" as the root architecture is changing under these external stressors. This might be induced by a better communication between above- and below-ground under eCO_2, thus resulting in mitigation of the VA soil separated effects.

In general, eCO_2 increases root biomass and the demand for N, P, and K (Norby *et al*. 1986; Li *et al*. 2013), however, even if the concentrations of elements in the soil are adequate, a proportionally smaller root system accompanied by less microbial activity is quite hard to support the dissimilation organs. On the other hand, eCO_2 acts as an external force for plants in order to extract more nutrients from the soil: eCO_2 increased $NaHCO_3$- and NaOH-extractable organic P in the rhizosphere of wheat by 160 and 53 %, respectively (Jin *et al*. 2014).

It seems that the growing substance of plants is an important factor to which the plant response to CO_2 usually depends on (e.g. Conroy *et al*. 1990), and makes it difficult to draw general conclusions. It is also critical that nutrient dynamics between different elements may change under elevated CO_2 (Li *et al*. 2015; Shi *et al*. 2016; Wang *et al*. 2016a). However, the responses are still not well understood since many factors such as soil temperature, light condition, nitrogen deposition, O_3, etc. may confound the nutrient allocation under elevated CO_2 (Cao *et al*. 2008; Danyagri and Dang 2014; Koike *et al*. 2015; Shi *et al*. 2016; Wang *et al*. 2016a,b).

It should be mentioned that increased microbial activity contributes to P and root-derived materials immobilization in rhizosphere under elevated CO_2 (Jin *et al*. 2014; Wang *et al*. 2016a). Considering fine root dynamics, the soil microbial activity (e.g. ectomycorrhizae) could also be a critical and determinant factor in the modulation of nutritional infertility and elevated CO_2 interaction. Significant species-specific differences in root biomasses and structure of fine roots were not found. In order to understand this, the seasonal trends of above- and belowground should be assessed (Sato 1995). Moreover, future studies should consider further investigations on soil microbial activity under the interaction of nutrient-poor soils and elevated CO_2.

Summarizing, in agreement with similar studies (e.g. de Oliveira *et al*. 2012), the present results show that soil type has the potential to modify the response of trees to eCO_2. However, in contrast to the initial expectations, the data support the hypothesis that the root growth of the community in BF soil was not altered by eCO_2 while in the infertile VA soil it was positively altered. Elevated CO_2 led to largely higher coarse root biomass, TDM and Fine:Area (effect magnitude > 3) when the community was grown in VA soil than when the community was grown in BF soil. This indicates the magnitude of response varies considerably between communities growing in different regions and that models dealing with the estimation of C

allocation and storage under elevated CO_2 scenarios may overestimate the potential in some regions and underestimate it in others.

2.4.2 Eleven-year CO_2 exposure on beech

To my knowledge, this is the first study evaluating the decadal independent or interactive effects between elevated levels of CO_2 and soil fertility on root production of a late-successional, deciduous, broad-leaved species in a transition zone between cool temperate and boreal forests and at the Asian boreo-nemoral ecotone (Matsuda *et al*. 2002). Saplings were subjected to the treatments for the entire active growth period, and during the 11-year exposure, they were progressing towards the mature phase of wood production and canopy closure.

Interestingly, eCO_2 did not alter the trunk area while VA affected it. Particularly, saplings grown under aCO_2×VA had smaller trunk area, of a large effect magnitude, than those grown under aCO_2×BF. Similarly, although root biomass was significantly increased by eCO_2, as a single factor, this was mainly due to the large negative effect of VA on saplings under ambient CO_2 environment (*cf*. BF). There were no statistically significant differences among the treatments a) aCO_2×BF, b) eCO_2×BF, and c) eCO_2×VA, however the data come from very wet years. The insignificant responses are in agreement with aboveground ecophysiological findings: The leaf area index (mean of the canopy of a community of ten species including beech) was higher in eCO_2 in 2nd growing season of CO_2 treatment, but not in the following growing seasons (Koike *et al*. 2015).

In addition, leaf mass per area, area-based and mass-based N content of leaf, chlorophyll fluorescence and most photosynthetic traits of beech saplings in the BF were not affected by eCO_2 (Watanabe *et al*. 2016). A 10-year experiment with wet and dry years also revealed that there were no sustained increases in the biomass of a community of perennial plants (Newingham *et al*. 2013). It is nevertheless practically noteworthy that eCO_2 led to a largely higher TDM in VA saplings, compared to aCO_2. In both variables (trunk area and TDM), eCO_2

mediated the negative impact of VA and as such the interaction of CO_2×soil was significant. The present findings (CO_2×VA), do not support the conclusion of Oren *et al*. (2001), based on light demanding pine stands, that "…fertility can restrain the response of wood carbon sequestration to increased atmospheric CO_2."

As to the Area:TDM, the only significant difference was that of aCO_2 *vs.* eCO_2; apparently, eCO_2 led to a higher TDM per trunk area, decreasing thus the Area:TDM. Area:TDM derives from the *Pipe Model* theory of tree form and can be used as an index for foliage mass against stem mass (Shinozaki *et al*. 1964a, b), indicating C allocation within plant body. According to meta-analyses, on average, eCO_2 does not change plant allometry (Poorter and Nagel 2000; Poorter *et al*. 2012). However, the results of this study indicate changed plant allometry, and this is in contrast to the previous findings (Tables 3 and 4) from the sapling community of three birches and an oak exposed to eCO_2 for 4 years in the same facilities. In the latter case, the saplings were more widely spaced (50 cm *vs*. 30 cm in the present study).

These and previous (Tables 3 and 4), at a wet region, are in agreement and differ to the general conclusion that soil infertility affects plant allometry (Poorter and Nagel 2000; Poorter *et al*. 2012) according to the *functional equilibrium* theory (Brouwer 1962; Poorter and Nagel 2000). It can only be postulated that the degree of soil infertility was not adequate to change the plant allometry during 11 growing seasons. In long-term experiments, in contrast to short-term experiments, there is an input of nutrients, through litterfall and decomposition, which may increase the soil fertility.

Regarding the results of the root classes, the large eCO_2-induced fine root biomass was certainly moderate and practically significant (Wolf 1986). This is in agreement with the previous results (Tables 2 and 3, Agathokleous *et al*. 2016c), but does not coincide with the findings of Bader *et al*. (2009) where unchanged or reduced fine root biomass of trees occurred

at a mature deciduous forest exposed to seven years FACE. An increased length of the small class of roots was optically observed (Fig 3). Essentially, there was no individual or interactive soil effect, even though VA was expected to be a critical factor altering fine roots production through a force to seek nutrients. This is another indication that future research should consider more nutrient-starving soil. The significant independent and interactive effects of CO_2 and soil are again attributed to an effect of VA under eCO_2, which caused a reduction of the intermediate root biomass and thus an increase of the Fine/Intermediate. It cannot confidently be explained why the intermediate root biomass was reduced by VA only under eCO_2, but it could be explained by the large increase of fine root biomass – even higher than in eCO_2×BF. Through this morphogenesis of expanding fine roots at the expense of intermediate roots, saplings under eCO_2 succeed to compensate the negative effects of VA. It is also possible that root turnover was faster under eCO_2 (Wang *et al*. 2016a), and fewer roots grew older which might decreased intermediate roots.

Saplings underwent extensive foraging strategy of fine roots, as indicated by higher Fine:Area, under eCO_2 (Ostonen *et al*. 2011; Leppalammi-Kujansuu *et al*. 2014) so as to increase fine root mass and length in order to achieve greater absorbing area (Ostonen *et al*. 2011). This effect was as moderate as it was in the previous study (Tables 3 and 4), however VA had no significant effects which is inconsistent with the previous findings (Tables 3 and 4) where VA had a large effect. In the latter case, an initial effect might be caused by wider spacing and as such propagated in following years.

Short-term exposure of very young or small seedlings to CO_2 and artifacts usually reveal high responses of tree species to elevated CO_2 (Pregitzer *et al*. 1995; Tissue *et al*. 1997; Kgope *et al*. 2009; Lavola *et al*. 1995; Duan *et al*. 2014), which can be even higher than those of herbaceous species (Körner 2006). Artifacts are caused by inappropriate growth conditions such as wide spacing and fertile artificial substrates. The results of the present study do not

correspond with some of those short-term experiments where a high, and likely overestimated, total root biomass response to elevated CO_2 was found: There was an increase in biomass when saplings had grown in VA, however, there was an insignificant response when grown in BF. On the other hand, the fine root biomass was not only high, but often even higher than in some short-term experiments, for saplings grown in both soils.

With reference to short-term experiments, long-term CO_2 experiments with saplings usually provide contradictory evidence (e.g. Bader and Körner 2010; Norby *et al*. 2010; Bader *et al*. 2013; Li *et al*. 2014; Warren *et al*. 2015). For instance, Li *et al*. (2014) found that eleven years of FACE treatment with 475 μmol mol^{-1} of CO_2 led to widely-ranged (-6 to +28%) annual plant production of grazing pasture and as little as 3 % higher (or even lower) final pasture production for the elevated CO_2 treatment, *cf*. ambient. Kostiainen *et al*. (2014) exposed four clones of *Populus tremuloides* and *Betula papyrifera* saplings to 560 μmol mol^{-1} of CO_2 for eleven years and found that most saplings responses to treatments were observed in the early phase of the experiment. Similarly, Dawes *et al*. (2015) reported that nine years of FACE treatment (+200 μmol mol^{-1}) did not significantly change the coarse root biomass or total biomass of either *Larix decidua* or *Pinus uncinata*, approximately 40-year-old trees. Interestingly, Norby *et al*. (2004, 2010) found 24 % higher NPP –the prime contributor being a more than doubled annual fine root production- in plants of a more widely spaced, deciduous community during the 4^{th} to 6^{th} growing season after exposure to 550 μmol $mol^{-1}$of CO_2 began. However, the NPP enhancement declined to just 9 % after eleven growing seasons. In contrast, Pregitzer *et al*. (2008) exposed a community of *Populus tremuloides* to 560 μmol mol^{-1} of CO_2 for ten years and found that elevated CO_2 led to ≈ 20 % greater fine and total root mass.

It is obvious that there is a wide range of responses in short-term and long-term research. Nonetheless, in most of the long-term cases the elevated CO_2-induced differences were insignificant (e.g. Norby *et al*. 2010; Newingham *et al*. 2013; Kostiainen *et al*. 2014; Li *et al*.

2014; Dawes *et al*. 2015) and this is consistent with our findings. The total root growth simulation in mature Siebold's beech stands with closed canopies was much smaller or neutral for the present two common types of soil when compared to the control treatment of aCO_2×BF. However, if the large simulation caused by eCO_2 under VA, compared to aCO_2×VA, is taken into account, it will be very important for relevant regions.

The key issue is that CO_2 enrichment must be applied to closed canopy stands to avoid the compound interest effect artifact (including wide spacing, fertile artificial substrates, etc.). What is needed is intact undisturbed natural soil *in situ* such as Sigurdsson at al. (2013) used for experiments with mature Norway spruce (*Picea abies* (L.) Karst) trees exposed to elevated CO_2 for three years, where limited nutrient availability in soil restricted the tree response to elevated CO_2 (Sigurdsson *at al*. 2013). Spinnler *et al*. (2002, 2003) found that beech (*Fagus sylvatica* L.) responded negatively to 4-year CO_2 enrichment when grown in acidic soil, but responded positively when grown in calcareous soil, where growth stimulation was observed –due to compound interest effect- during the first two to three growing seasons. In agreement with the findings of Spinnler *et al*. (2002, 2003), the present findings show that false conclusions would be drawn if only one soil type (brown forest soil) was chosen to experiment with.

Increased below-ground allocation in poor soil caused by elevated CO_2 levels, as observed in our study, may have wider consequences in the long term. Saxe *et al*. (2001) noted "climatic adaptation seems to be the most important component in the evolutionary process of temperate and boreal tree species." The root response of trees may affect the performance of the whole trees and the interactions and distributions of populations and species (De Kroon 2007). Fine roots contribute significantly to NPP (DeLucia *et al*. 1999), however, the NPP impact by CO_2 is species specific and depends on other factors such as nitrogen deposition (Norby *et al*. 2010;

Yan *et al*. 2014). The variant NPP responses may affect species' composition of forests under future climate change (Yan *et al*. 2014).

Overall, Siebold's beech saplings may not experience significant belowground effects in regions with fertile soils, but may experience significant positive effects in regions with infertile soils with porous pumice. The former case can be translated to insignificant effects on NPP, while in the latter case to a higher NPP and quicker reach of high storage age, so called *buying time*, (Körner 2006) in such regions. Regarding the large increase in biomass production caused by eCO_2 under VA, there is no benefit if we compare it with $aCO_2 \times BF$, but the net (real) surplus will be large to areas with VA. Natural forests at mountainous and remote regions often have infertile soils and are not easily accessible to humans, while urban forests and trees at plains usually have fertile soils. At remote infertile areas eCO_2 impacts could be higher than at nearby areas and this should be taken into account when planning relevant experiments. There is, however, still way to go to comprehensibly understand the fate of C due to the complexity of multi-factorial dependency (Fig 4).

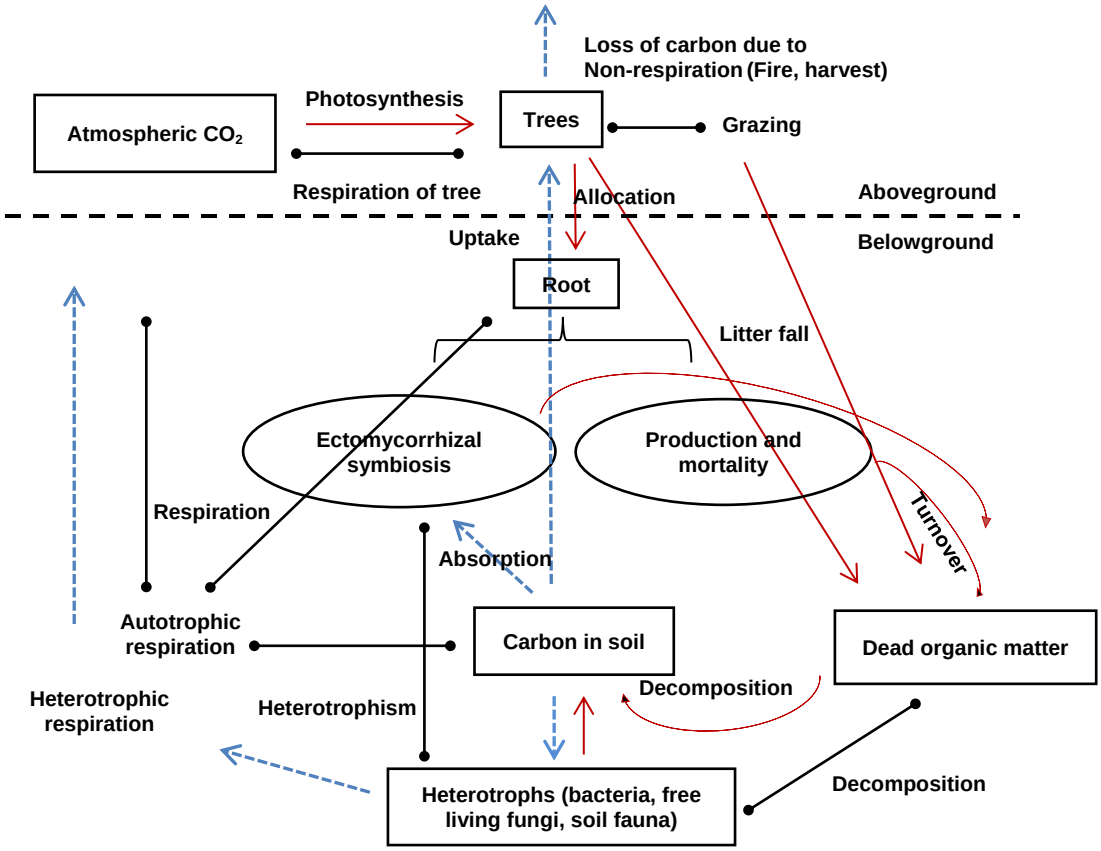

Fig 4 Schematic overview of the important role of ectomycorrhizal symbiosis and root dynamics in forests C cycling. Rectangles or circles indicate C pools, arrows indicate C fluxes (solid and dashed lines indicate increase and decrease in soil carbon, respectively, and round edges lines indicate that the processes do not always simply decrease the C pool in soil) and single text indicates processes (illustrated partly based on the idea of Fransson 2012). Source: Wang, Agathokleous *et al*. (2016a).

Chapter 3

EFFECTS OF ELEVATED O_3 LEVELS ON NATIVE TREE SPECIES AND THE ROLE OF SOIL

4.1 Study 1: the role of soil (larches as model trees)

4.1.1 Introduction

It is known that soil influences the tree responses to other applied treatments (Körner 2011; Sigurdsson *et al*. 2013), as it is shown in Chapter 2. However, the interaction of O_3 with soil

type is underexplored, with the majority of investigations conducted using potted seedlings (see references in Agathokleous *et al*. (2015c, 2016a)).

Larches are deciduous trees within the genus *Larix* (Pinaceae) with high economic and ecological value as they are primary components of Siberian and Canadian boreal forests, and have a wide range across the Northern Hemisphere (Gower and Richard, 1990; Farjon, 1990; Abaimov *et al*. 2000; Qu 2016). Larches hold an important role in Eurasia as maintainers of the CO_2 balance (Osawa *et al*. 2010) and important afforestation species in northeast China (Zhang *et al.* 2000). Japanese larch (*Larix kaempferi* (Lamb.) Carr.) is a light-demanding pioneer species. As a major plantation species in Japan, significant efforts in optimum breeding have been conducted (Kurinobu 2005). Consequently, the hybrid larch F_1 was developed by crossing Dahurian larch (*Larix gmelinii* var. *japonica* (Maxim. ex Regel) Pilg.) with Japanese larch (Miyaki 1990; Ryu *et al*. 2009). Properties of high growth, stem straightness and resistance to bark gnawing by voles contribute to the viability of hybrid larch as a plantation taxon. In Hokkaido, the northernmost island of Japan, 400 ha of hybrid plantations are established annually (Kurinobu 2005; Kita *et al*. 2014).

However, the breeding of hybrid larch has thus far occurred without taking into consideration elevated O_3 levels. This hybrid has been found sensitive to elevated O_3 levels (Ryu *et al*. 2009; Koike *et al*. 2012; Kam *et al*. 2015; Wang *et al*. 2015). Additionally, under combined conditions of elevated O_3 and CO_2 levels, the negative consequences of O_3 have been shown to dominate (Koike *et al*. 2012). These studies used: a) open-top chambers with plants directly planted in the ground (Wang *et al*. 2015); b) open-top chambers with plants in pots (Koike *et al*. 2012); or c) free-air O_3-concentration-enrichment (FACE) systems with plants grown in pots (Kam *et al*. 2015). The effect of O_3 on larch exposed to the natural environment (i.e. without chamber effects), and without root limitation (i.e. planted directly in the ground) remains unknown. Given the economic and ecological importance of larches in Eurasia, studies

on the future challenges of this hybrid larch are important to inform future afforestation practices.

The aims of this study were to investigate how two years of free-air O_3 enrichment influences a) the growth and photosynthetic pigmentation of Japanese larch in comparison to its hybrid when grown pot-free, and b) how O_3 impacts differ between two types of soil; fertile brown forest soil (BF: Dystric Cambisols) and BF mixed with infertile, immature volcanic ash plus pumice soil (VA: Vitric Andosols). The taxa were selected because of the prior scientific evidence explained above. A further aim was to quantify the magnitude of difference among experimental groups to provide practical insights for these ecologically and economically important taxa. It was hypothesized that in these fast-growing taxa, O_3 effects on growth would differ from previous findings derived from OTC experiments or pot-grown plants in FACE systems. This prediction is based on the assumption that epigeous growth depends on hypogeous growth and root expansion was restricted in potted experiments. Plants grown in infertile soils often allocate resources to root symbionts at the expense of photosynthetic products to overcome nutrient deficiencies (Hermans *et al*. 2006; Smith and Read 2008). It was therefore hypothesized that larch responses to O_3 would be affected by soil fertility through shifts in photosynthesis and nutritional demands.

4.1.2 Materials and Methods

3.1.2.1 Experimental site

The experiments were conducted at Sapporo Experimental Forest of Hokkaido University, Japan (43º.04’ N, 141º.20’ E, 15 m a.s.l.). The means of air temperature, wind speed, and relative humidity and the monthly totals of sunshine duration and precipitation can be found in Table 1 (Chapter 2). In addition, the PPFD was recorded by a HOBO Pendant data logger (UA-002-64, Onset Computer, Co., MA, USA) placed at 2 m height, in the center of each

experimental plot. An observation was recorded every 5 min, during the period June-October (2014 and 2015).

For both years, the PPFD in the experimental plots exceeded 70 μmol m^{-2} s^{-1} (*i.e*. light compensation point of photosynthesis, Koike (1988)) from 07:00 to 17:00. The daily 10-hr mean (07:00-17:00) for the experimental plots (n=6) was 362.9 ±14.0 μmol m^{-2} s^{-1} in 2014 and 354.3 ±25.8 μmol m^{-2} s^{-1} in 2015).

3.1.2.2 Experimental Design

The O_3 treatments were ambient O_3 (AOZ) and elevated O_3 (EOZ), with three plot replicates for each treatment randomly scattered throughout the experimental forest. Each plot (diameter=6.5 m) contained three soil sub-plots from which BF and VA sub-plots were used in the present study. Each sub-plot covered an area of ≈42 % of the total plot area. For both BF and VA sub-plots, 30 cm of soil was removed and placed back. Brown forest soil is native soil this experimental forest. To create VA, volcanic ash soil from the Tomakomai Experimental Forest of Hokkaido University, Tomakomai, Japan, was mixed with the existing BF. Volcanic ash soil, which is poor in P and N content and has high porosity, is widely distributed throughout Hokkaido island, Japan. The pH of soil sub-plots was 5.69 ±0.11 for BF and 5.09 ±0.09 for VA. More information on the soils can be found in Chapter 2.

Two-year old seedlings of Japanese and hybrid larches were directly planted into the soils on May 15th, 2014. These seedlings were cultivated by the Forestry Research Institute of Hokkaido Research Organization in Bibai city, ≈60 km north from Sapporo. Agrochemicals or fertilizers were not used during the experiment.

The FACE system was calibrated to maintain EOZ approximately twice the ambient O_3 concentrations, with a maximum of 80 nmol mol^{-1}. Ozone generated from pure oxygen was diluted with ambient air in a pressurized tank and released into the rings by 2.5-m teflon tubes

suspended vertically 2.5 m above ground, and two horizontal Teflon tubes fixed around the plots elevated to 0.5 and 1.5 m. An SM70 Fixed Ozone Monitor (Aeroqual Ltd., Auckland, NZ) operated at the center of each EOZ plot, and a Hioki LR5042-20 (HIOKI E.E. Corporation, Nagano, JP) recorded O_3 data in two-minute intervals. Plants were exposed to EOZ from August 15th to October 26th in 2014 and from April 24th to October 26th in 2015, during daylight hours with a PPFD higher than 70 μmol m^{-2} s^{-1} (07:00-17:00). The mean 10-hr O_3 levels for EOZ plots were 60.1 nmol mol^{-1} in 2014 and 72.1 nmol mol^{-1} in 2015, which are translated to accumulated exposure over a threshold of 40 nmol mol^{-1} (AOT40) values of 8.4 and 49.1 μmol mol^{-1} h in 2014 and 2015. Although a critical AOT40 level of 8-15 μmol mol^{-1} h has been proposed for sensitive forest trees in Japan (Kohno *et al*. 2005), AOT40 values higher than 30 μmol mol^{-1} h occur in Japan (Hoshika *et al*. 2012). Background O_3 concentrations, for the same hours of the corresponding months, were 20.4 and 33.8 nmol mol^{-1} in 2014 and 2015, respectively, as continuously monitored by an O_3 monitor (Model 202, 2B Technologies, Boulder CO, USA).

3.1.2.3 Measurements and samplings

At the time of planting, initial measurements of plant height (cm) and stem basal diameter (mm) of the lignified stem at the base were taken from each plant. Plant height means were 16.68 (± 0.34 se) and 14.57 (± 0.32 se) cm for hybrid larch and Japanese larch, respectively. Basal diameter means were 2.26 (± 0.06 se), 2.16 (± 0.06 se) mm for hybrid larch and Japanese larch, respectively.

Late in the second growing season, O_3 visible foliar injury was assessed on a continuous scale of 0-100 % for the foliage of each plant. Each plant was observed from above and four cardinal directions and the total foliar injury was estimated as the percentage of injured needles per plant. Observations were done for both AOZ and EOZ plants.

Needles of similar maturity, randomized for O_3 symptom intensity, were destructively sampled from each plant to assess chlorophyll-related characteristics as potential indicators of physiological activity (Gottardini *et al*. 2014). Collected needles were stored in a deep freezer until pigments analysis. For each plant extract, needles were immersed in dimethyl sulfoxide (DMSO) solvent and incubated at 65 ^{o}C for 4 h under dark conditions, until shadowed thalli became visible. After incubation, each extract was covered with aluminum foil until its measurement. For the extracts, disposable 4.5 mL tubes were used (Nunc CryoTube Vials, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Measurements were taken at 415, 435, 470, 648, 665 nm (GeneSpec III; Hitachi Genetic Systems; MiraiBio, Alameda, CA). The ratio 435/415 (OD_{435}/OD_{415}) was calculated as a potential index of phaeophytinization, which shows the relative proportion of Chl_a and phaeopigment *a* (Ronen and Galun 1984). The total carotenoids content (*TCar*) was calculated according to Lichtenthaler (1987).

As we performed the extraction with dimethyl sulfoxide as opposed to acetone, *TCar* should be interpreted for relative differences among experimental groups rather than on absolute values which may slightly differ. The chlorophyll *a* content (Chl_a), chlorophyll *b* content (Chl_b) and total chlorophyll content (*TChl*) were estimated according to Barnes *et al*. (1992). The fractions Chl_a/Chl_b and *TChl*/*TCar* were also calculated.

Growth measurements (cm) were taken at the end of the active growth period in the second growing season (2015). Crown thickness (from the point to which the first lateral shoot is attached on the stem to the highest point of the crown), and mean crown spread (distance between the two farthest points of the crown, as observed from above) were measured alongside height, using measuring tape. The middle of the stem was marked following plant height measurements. Diameter of the lignified stem was measured at the base, middle and top using a digital a caliber, by taking the mean of two crosswise measurements at each stem level. Stem volume was also calculated for each plant as:

$$Volume = \pi \times \frac{\left(\left(\frac{diameter}{2}\right)^2\right)}{10} \times height \text{ cm}^3,$$

where diameter is the mean of two diameter measurements at the base of the stem and π is set at 3.14.

3.1.2.4 Statistics

The level of significance for all statistical analyses was set at α=0.05.

The data of pre-treatment plant height and diameter and post-treatment *Chl*$_a$, *Chl*$_b$, *TChl*, *Chl*$_a$/*Chl*$_b$, *TCar* OD_{435}/OD_{415}, *TChl*/*TCar*, plant height., crown thickness, mean crown spread and stem volume were transformed using the maximum likelihood approach of Box-Cox transformation to approximate the Gaussian fit (Box and Cox 1964), using maximum iterations = 40, minimum λ = -5, maximum λ = 5 and ε = 0.00001. The transformation was based on *maximum likelihood* approach, with an algorithm which uses Golden Search to find the λ (Agathokleous *et al*. 2016b). The likelihood formula, called criterion *E*, used was

$$E(\lambda) = -\frac{N}{2} log\left(\tilde{\sigma}^2(\lambda)\right) + (\lambda - 1)\sum_{i=1}^{N} log(\tilde{X}_i)$$

where λ is the ideal value which respects the normality assumption by minimizing the equation and $\tilde{\sigma}^2(\lambda)$ is the standard deviation of the transformed variable $\tilde{X}$ (Agathokleous *et al*. 2016b). Transformed data were used for the statistical analyses and untransformed data were used for presentation purposes.

General Linear Models (GLMs) were conducted on transformed data of each pigment-related response variable with Ozone, Soil and Taxa as fixed factors and randomized by plot (Kuehl 1999). Covariate-adjusted GLMs were performed on transformed data for growth-related response variables (Senturk and Muller 2009). The baseline data of plant height were used as a covariate for post-treatment plant height, crown thickness, mean crown spread and stem

volume, whereas the baseline data of basal diameter were used as a covariate for post-treatment mean crown spread, stem volume and diameters of stem. For testing diameter across the stem levels (low, middle, upper), baseline-basal-diameter-adjusted GLM was applied using stem level as the within effects dependent variable. Type 6 Sums of Squares, which uses the σ-restricted coding of effects in order to provide unique effect estimates even for lower-order effects (straightforward computing method), was employed. Post hoc Tukey's HSD test was performed when required.

For the variables that GLMs revealed Ozone as a significant factor, simple linear regression analysis was conducted between each pigment-related variable (predictor) and each growth-related variable (criterion). The gain in plant height and stem basal diameter was calculated by subtracting the pre-treatment score from the post-treatment score. Values of *r* within the arbitrary segments [0.00, 0.10), [0.10, 0.30), [0.30-0.50), [0.50-0.70), [0.70-0.90) and 0.90+, indicate trivial, low, moderate, large, very large and nearly perfect correlation magnitude, respectively (Hopkins 2000).

Visible injury data were subjected to arcsine transformation before performing a Generalized Linear Mixed Model (GLMM) with identity link function; random and main, two-way and three-way fixed factors specified as in GLMs (Bolker *et al*. 2008). The variance component was selected for random effect covariance type. An *a priori* decision was made to exclude the two-way effects from the full factorial terms (full: Ozone + Soil + Taxa + Ozone × Soil + Ozone × Taxa + Soil × Taxa + Ozone × Soil × Taxa + (Plot)), despite their statistical significance, and include only effects with biological importance. Plot was not significant (Estimate = 17.8, se = 18.3, Z = 0.975, p=0.330, 95% CI [2.4, 133.0]) and excluded to simplify the model; the corrected Akaike Information Criterion (AIC_c) was smaller without Plot. The final model was Ozone + Soil + Taxa + Ozone × Soil × Taxa.

The unbiased Cohen's δ was estimated based on balanced real replicates (Hedges and Olkin 1985) so as to quantify the magnitude of difference when the predictor was statistically significant. For covariate-adjusted response variables, δ was estimated by subtracting the baseline effect from the post-treatment effect (Agathokleous *et al*. 2016b) using the formula

$$\delta_{unbiased(covariate)} = \delta_{unbiased(posttreatment)} - \delta_{unbiased(pretreatment)}$$

This process corrects the δ value against underestimates or overestimates by taking into account any effect that is present from baseline (Agathokleous *et al*. 2016b).

Furthermore, δ was converted to OVL, a potential measure of bioequivalence under the name proportion of similar response (Reiser and Faraggi 1999). This coefficient represents the common area under two probability density curves and indicates the agreement between two distributions (Reiser and Faraggi 1999). Estimations of δ and OVL were calculated as described in Chapter 2.

Log-transformed height of the plant at which stem diameter was measured was used to plot height against stem diameter (n=3 for each taxon in each soil, O_3 treatment and stem level). The data of each Ozone group were pooled from taxa and soils, for each stem level, and the slopes of AOZ and EOZ were tested (α=0.05) using comparison of regression lines slopes from two independent samples based on pooled variance (Howell 2010).

For data presentation purposes, the untransformed values are presented. Data processing and statistical analyses were conducted using MS EXCEL 2010 (Microsoft ©), PASW Statistics 18 (formerly SPSS Statistics, IBM ©) and STATISTICA v.10 (StatSoft Inc. ©) software.

4.1.3 Results

3.1.3.1 Visible injury

The average values of needle areas affected by O_3 visible injury per plant (%) in EOZ plants were 93 (± 3), 83 (± 7), 73 (± 3) and 84 (±5) for hybrid larch×BF, hybrid larch×VA, Japanese

larch×BF and Japanese larch×VA, respectively. The value of the corrected AIC_c was 118.548. In the corrected model (F=81.5, P<0.001), only the predictor Ozone had a significant (F=564.2, P<0.001) fixed effect. Soil (F=0.02, P=0.895), Taxa (F=1.5, P=0.239), and Ozone×Soi×Taxa (F=1.3, P=0.318) were not significant. The coefficient of AOZ was -67.42 ± 5.68 se, Wald t statistic (t) = -11.87, P<0.001.

3.1.3.2 Photosynthetic pigments and plant size

Ozone was not a significant factor for OD_{435}/OD_{415} (Table 7, Fig 5). However, EOZ had a small negative effect on Chl_a/Chl_b (δ=-1.20, OVL=0.550) and a moderate negative effect on plant height (δ=-1.80, OVL=0.362), crown thickness (δ=-2.26, OVL=0.259), mean crown spread (δ=-2.28, OVL=0.255) and stem volume (δ=-2.89, OVL=0.148) (Fig 5-6). Furthermore, EOZ had a large negative effect on Chl_a (δ=-6.58, OVL=0.001), Chl_b (δ=-5.86, OVL=0.002), *TChl* (δ=-6.54, OVL=0.001), *TCar* (δ=-3.49, OVL=0.081) and *TChl/TCar* (δ=-3.46, OVL=0.084). For Chl_a, Chl_b, *TChl*, *TCar* and *TChl/TCar*, 10 % (maximum) of the groups (AOZ and EOZ) overlap and there is a >99 % chance that a random observation from EOZ would reveal a lower score than a random observation from AOZ (area under the receiver operating characteristics (AUC); Ruscio and Mullen 2012).

The effect of soil type was significant only for Chl_a/Chl_b (Table 7, Fig 5) to which VA caused a small positive effect (δ=0.83, OVL=0.678).

Taxa differed in all the variables except the three pigmentation ratios (Chl_a/Chl_b, OD_{435}/OD_{415}, *TChl/TCar*), with hybrid larch having superior performance to Japanese larch in all the measured characteristics (Table 7, Fig 5-6). In AOZ, hybrid larch and Japanese larch had a difference of small magnitude in *TCar* (δ=1.19, OVL=0.550). A moderate difference between species existed for Chl_b (δ=1.48, OVL=0.458), mean crown spread (δ=2.15, OVL=0.283), Chl_a (δ=1.55, OVL=0.438) and *TChl* (δ=1.58, OVL=0.431). Similarly, in AOZ, they had a difference of large magnitude in plant height (δ=3.53, OVL=0.078), crown thickness (δ=3.70,

OVL=0.064) and stem volume (δ=4.08, OVL=0.041). In EOZ, hybrid larch and Japanese larch had a difference of small magnitude in Chl_b (δ=1.13, OVL=0.573) and *TChl* (δ=1.43, OVL=0.474) and a difference of moderate magnitude in all the other characteristics including plant height (δ=1.72, OVL=0.391), crown thickness (δ=2.05, OVL=0.304), mean crown spread (δ=1.61, OVL=0.420), stem volume (δ=1.67, OVL=0.404), Chl_a (δ=1.64, OVL=0.412) and *TCar* (δ=1.66, OVL=0.406).

Ozone×Soil was significant only for Chl_b and Chl_a/Chl_b, whereas only for Chl_a/Chl_b there was a difference of interest in multiple comparisons. A moderately higher Chl_a/Chl_b was observed in EOZ×VA compared with EOZ×BF (δ=-1.65, OVL=0.410).

The interactions Soil×Taxa and Ozone×Taxa were insignificant, for all the pigment and plant size-related variables.

Ozone×Soil×Taxa was significant only for stem volume. Hybrid larch had a smaller stem volume in EOZ×BF compared with AOZ×BF (δ=-7.53, OVL<0.001) and in EOZ×VA compared with AOZ×VA (δ=-6.80, OVL=0.001) with large differences in magnitude. Similar large differences were observed for Japanese larch, which had a smaller stem volume in EOZ×BF compared with AOZ×BF (δ=-3.03, OVL=0.130) and in EOZ×VA compared with AOZ×VA (δ=-6.83, OVL=0.001). Finally, hybrid larch had a greater stem volume than Japanese larch in AOZ×BF (δ=5.45, OVL=0.006), AOZ×VA (δ=3.04, OVL=0.129) and EOZ×VA (δ=2.41, OVL=0.229), of large, large and moderate magnitude, respectively. Hybrid larch stem volume was 29% larger in EOZ×BF, however high RSD (35.5% and 28.4%) masked the difference.

GLM analysis of diameters across the stem (Fig 7) revealed Ozone (F= 102.13, P<0.001), Taxa (F=5.30, P<0.05) and Stem level (F=9.02, P<0.001) but not Soil (F=0.01, P=0.924) as significant predictors. Stem diameter was higher in AOZ than in EOZ and higher in hybrid

larch compared with Japanese larch. Stem diameter was also higher at the lower part compared with the middle part of the stem, and at the middle part compared with the upper part of the stem. From second order interaction, only Ozone×Stem level (F=46.38, P<0.001) and Taxa×Stem level (F=9.70, P<0.001) were significant. Stem diameter was decreased in EOZ treatments at each level. Only the lower part of the stem was different between taxa, where Japanese larch had lower stem diameter than hybrid larch. Ozone×Soil (F=1.84, P=0.195), Ozone×Taxa (F=0.75, P=0.401), Soil×Taxa (F=0.27, P=0.611), Soil×Stem level (F=0.10, P=0.905) were insignificant.

There were no significant effects for third and fourth order interactions: Ozone×Soil×Taxa (F=2.33, P=0.148), Ozone×Soil×Stem level (F=2.52, P=0.098), Ozone×Taxa×Stem level (F=1.72, P=0.196), Soil×Taxa×Stem level (F=0.48, P=0.621) and Ozone×Soil×Taxa×Stem level (F=1.43, P=0.256). The slope between the height of the stem, at which diameter was measured, and the stem diameter was lower in EOZ compared to AOZ (T=-3.61, P<0.001).

The simple linear regressions (Fig 8-9) showed that all the examined pigment-related variables can effectively predict scores on all the growth-related variables (P < 0.05, adjusted R^2 < R^2). *Chl_a/Chl_b* had a moderate correlation with all the growth-related variables except stem basal diameter with which it had a large correlation, and *TChl/TCar* had a large correlation with plant height and mean crown spread (Fig 9). All the other correlations of *TChl/TCar* and the other pigment-related variables with growth-related variables were very large or near-perfect.

Table 7 Results of the GLM statistical analysis. All the pigment-related data subjected to general linear models randomized by plot. Data obtained from Japanese larch and a hybrid larch F_1 seedlings grown in brown forest soil (BF) or BF mixed with volcanic ash soil (VA) and exposed to ambient air (AOZ) or O_3-enriched air (EOZ) (O_3≈66 nmol mol^{-1}) for two growing seasons. Each treatment condition is consisted of three replicates. Reprinted from Springer, Environmental Science and Pollution Research, Stem and crown growth of Japanese larch and its hybrid F_1 grown in two soils and exposed to two free-air O_3 regimes, 2017, Agathokleous E, Vanderstock A., Kita K, Koike T, "With permission of Springer"

	Ozone	**Soil**	**Taxa**	**Ozone×Soil**	**Ozone×Taxa**	**Soil×Taxa**	**Ozone×Soil×Taxa**
Plant height	*F*=67, ***P<0.001***	*F*=0.06, *P*=0.807	*F*=32, ***P<0.001***	*F*=0.38, *P*=0.547	*F*=0.15, *P*=0.703	*F*=0.38, *P*=0.546	*F*=0.04, *P*=0.844

Crown thickness	*F*=89, ***P*<0.001**	*F*=0.30, *P*=0.591	*F*=42, ***P*<0.001**	*F*=0.62, *P*=0.443	*F*=0.02, *P*=0.894	*F*=0.40, *P*=0.536	*F*=0.30, *P*=0.592
Mean crown spread	*F*=54, ***P*<0.001**	*F*=0.01, *P*=0.907	*F*=19, ***P*<0.001**	*F*=0.14, *P*=0.711	*F*=0.01, *P*=0.938	*F*=0.19, *P*=0.669	*F*=0.01, *P*=0.938
Stem volume	*F*=221, ***P*<0.001**	*F*<0.01, *P*=0.975	*F*=42, ***P*<0.001**	*F*=1.8, *P*=0.200	*F*=0.53, *P*=0.477	*F*<0.00, *P*=0.958	***F*=4.79, *P*<0.050**
Chl$_a$	*F*=633, ***P*<0.001**	*F*=1.13, *P*=0.304	*F*=17, ***P*<0.001**	*F*=1.33, *P*=0.266	*F*=0.04, *P*=0.855	*F*=0.22, *P*=0.649	*F*=0.11, *P*=0.750
Chl$_b$	*F*=333, ***P*<0.001**	*F*=0.21, *P*=0.655	*F*=11, ***P*<0.010**	*F*=5.2, ***P*<0.050**	*F*<0.01, *P*=0.957	*F*=2.94, *P*=0.106	*F*=0.153, *P*=0.701
TChl	*F*=557, ***P*<0.001**	*F*=0.38, *P*=0.544	*F*=15, ***P*<0.010**	*F*=2.2, *P*=0.156	*F*=0.03, *P*=0.867	*F*=0.7, *P*=0.422	*F*=0.01, *P*=0.940
Chl$_a$*/*Chl$_b$	*F*=13, ***P*<0.010**	*F*=7.8, ***P*<0.050**	*F*=0.23 *P*=0.636	*F*=6.6, ***P*<0.050**	*F*=1.18, *P*=0.182	*F*=3.3, *P*=0.087	*F*=0.66, *P*=0.430
TCar	*F*=161, ***P*<0.001**	*F*=4.3, *P*=0.055	*F*=16, ***P*<0.001**	*F*=0.19, *P*=0.669	*F*=0.07, *P*=0.794	*F*=1.2, *P*=0.282	*F*=1.55, *P*=0.230
OD$_{435}$/ OD$_{415}$	*F*=0.32, *P*=0.582	*F*=0.85, *P*=0.370	*F*=1.91, *P*=0.186	*F*=0.24, *P*=0.631	*F*=0.15, *P*=0.700	*F*=3.39, *P*=0.084	*F*=1.23, *P*=0.285
TChl/ TCar	*F*=82, ***P*<0.001**	*F*=3.25, *P*=0.090	*F*=0.56, *P*=0.464	*F*=0.47, *P*=0.503	*F*=0.17, *P*=0.690	*F*=2.95, *P*=0.106	*F*=0.23, *P*=0.635

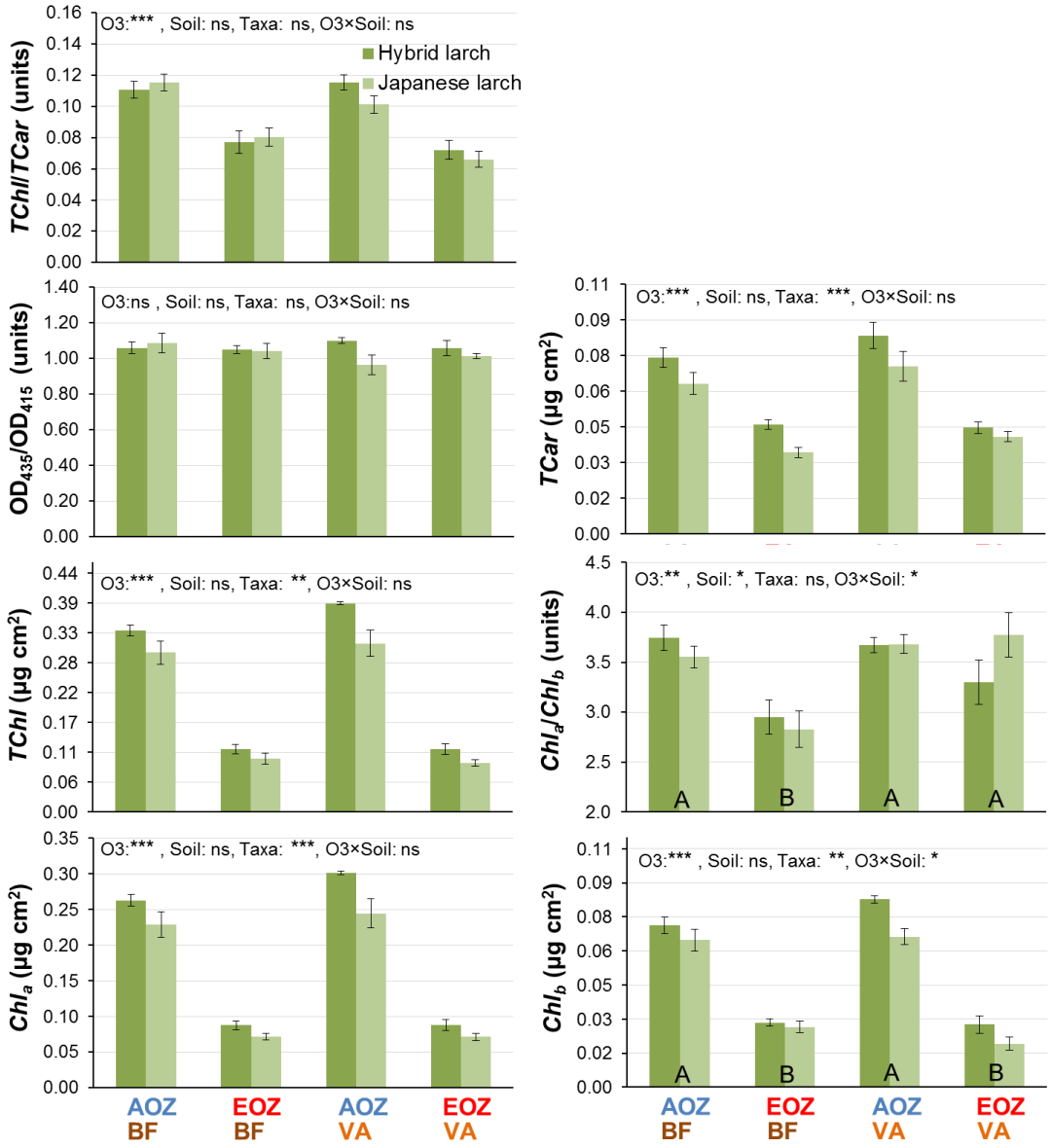

Fig 5 Means (± se) of chlorophyll *a* content (Chl_a), chlorophyll *b* content (Chl_b), total chlorophyll content (*TChl*), Chl_a/Chl_b ratio, ratio of optical densities 435 to 415 (OD_{435}/OD_{415}), total carotenoid content (*TCar*) and *TChl*/*TCar* ratio. Data obtained from Japanese larch (light shading) and a hybrid larch F_1 (dark shading) seedlings grown in brown forest soil (BF) or BF mixed with volcanic ash soil (VA) and exposed to ambient air (AOZ) or O_3-enriched air (EOZ) (O_3≈66 nmol mol^{-1}) for two growing seasons. Different uppercase letters show statistically significant differences within O3×Soil interaction (Taxa pooled), according to Tukey's HSD posthoc test. The significance of the main factors and O3×Soil interaction is indicated by ns (non-significant, P>0.05), * (P<0.05) ** (P<0.01) or *** (P<0.001). The interactions O3×Taxa, Soil×Taxa and O3×Soil×Taxa were insignificant for all the response variables. Each value of the eight means resulted from three replicates. Reprinted from Springer, Environmental Science and Pollution Research, Stem and crown growth of Japanese larch and its hybrid F_1 grown in two soils and exposed to two free-air O_3 regimes, 2017, Agathokleous E, Vanderstock A., Kita K, Koike T, "With permission of Springer"

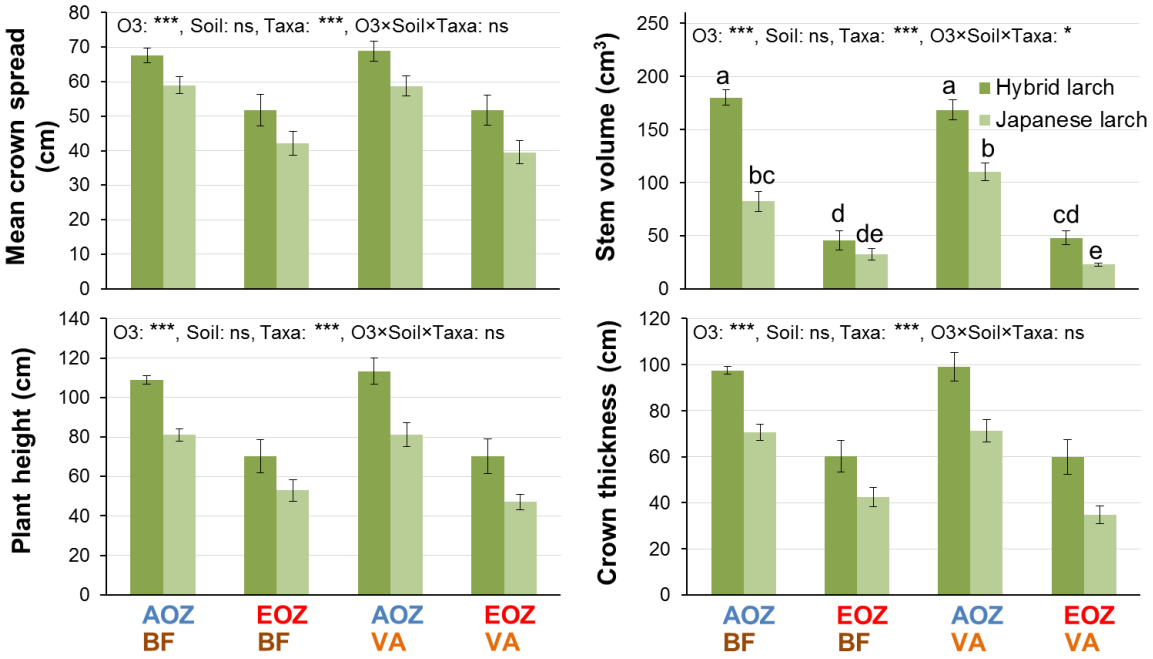

Fig 6 Means (± se) of plant height, crown thickness, mean crown spread and stem volume. Data obtained from Japanese larch (light shading) and a hybrid larch F_1 (dark shading) seedlings grown in brown forest soil (BF) or BF mixed with volcanic ash soil (VA) and exposed to ambient air (AOZ) or O_3-enriched air (EOZ) (O_3≈66 nmol mol^{-1}) for two growing seasons. Different lowercase letters indicate statistically significant differences among the means, when O3×Soil×Taxa was significant, and different uppercase letters show statistically significant differences within O3×Soil (Taxa pooled), according to Tukey's HSD posthoc test. The significance of the main factors is indicated by ns (non-significant, P>0.05), * (P<0.05) ** (P<0.01) or *** (P<0.001). The interactions O3xSoil, O3×Taxa and Soil×Taxa were insignificant for all the response variables. Each value of the eight means resulted from three replicates. Reprinted from Springer, Environmental Science and Pollution Research, Stem and crown growth of Japanese larch and its hybrid F_1 grown in two soils and exposed to two free-air O_3 regimes, 2017, Agathokleous E, Vanderstock A., Kita K, Koike T, "With permission of Springer"

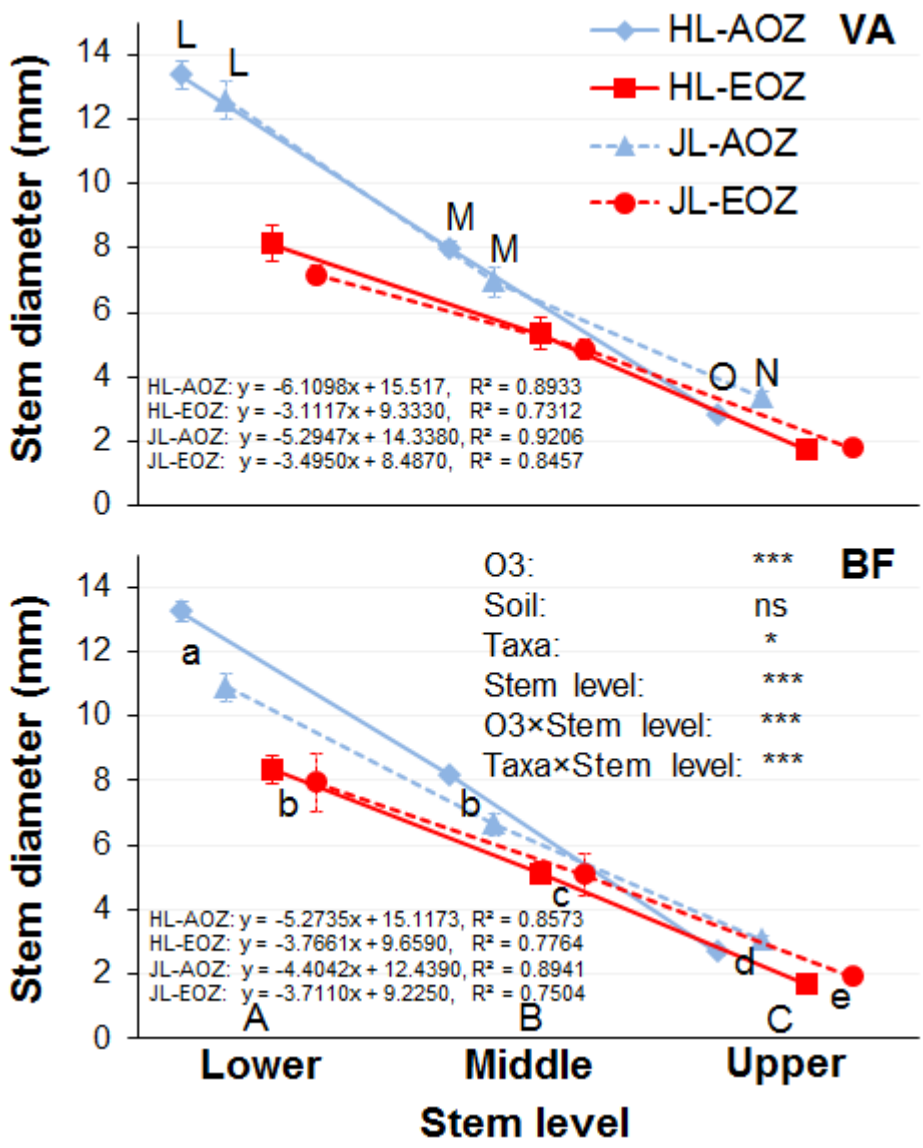

Fig 7 Means (± se) of diameter across stem as measured at the base (Lower), middle (Middle) and top (Upper) of the stem. Data obtained from Japanese larch (JL, dashed lines) and a hybrid larch F_1 (HL, solid lines) seedlings grown in brown forest soil (BF, lower figure) or BF mixed with volcanic ash soil (VA, upper figure) and exposed to ambient air (AOZ) or O_3-enriched air (EOZ) (O_3≈66 nmol mol^{-1}) for two growing seasons. Different uppercase letters A, B and C show statistically significant differences among stem levels (other treatments pooled). Different lowercase letters between means of taxa in each stem level show statistically significant differences within the interaction O3×Stem level (Soil and Taxa pooled). Different uppercase letters L, M, N and O above means of taxa in each stem level show statistically significant differences within the interaction Taxa×Stem level (O3 and Soil pooled). Differences are marked according to Tukey's HSD posthoc test. The significance of the main factors and the interactions O3×Stem level and Taxa×Stem level is indicated by ns (non-significant, P>0.05), * (P<0.05) ** (P<0.01) or *** (P<0.001). The remaining second order interactions and the third and fourth order interactions were insignificant. Each mean is the product of three replicates. Reprinted from Springer, Environmental Science and Pollution Research, Stem and crown growth of Japanese larch and its hybrid F_1 grown in two soils and exposed to two free-air O_3 regimes, 2017, Agathokleous E, Vanderstock A., Kita K, Koike T, "With permission of Springer"

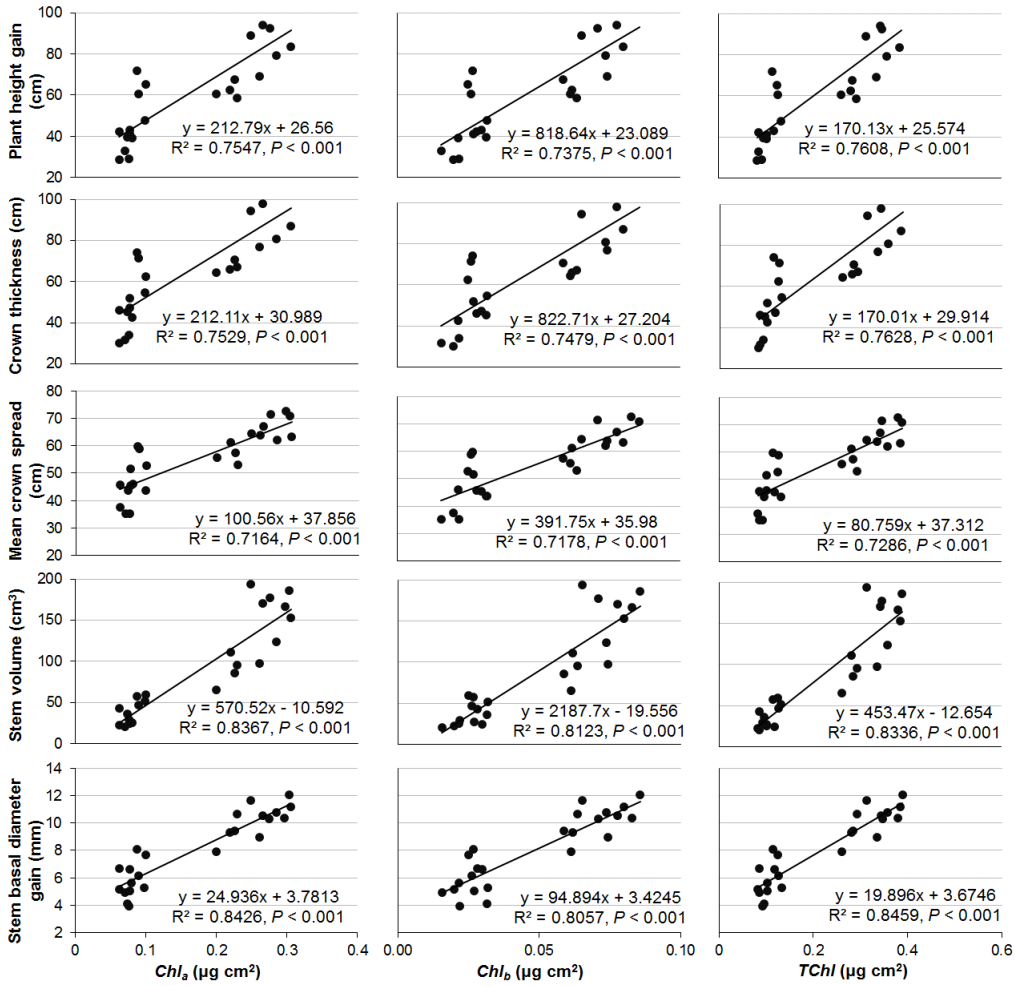

Fig 8 Regression analysis of chlorophyll *a* content (*Chl*$_a$), chlorophyll *b* content (*Chl*$_b$) and total chlorophyll content (*TChl*) with each growth-related variable. Each data point is the average of the plants in each research condition (N=24). The regressions were tested at an *α* level of 0.05. Reprinted from Springer, Environmental Science and Pollution Research, Stem and crown growth of Japanese larch and its hybrid F_1 grown in two soils and exposed to two free-air O_3 regimes, 2017, Agathokleous E, Vanderstock A., Kita K, Koike T, "With permission of Springer"

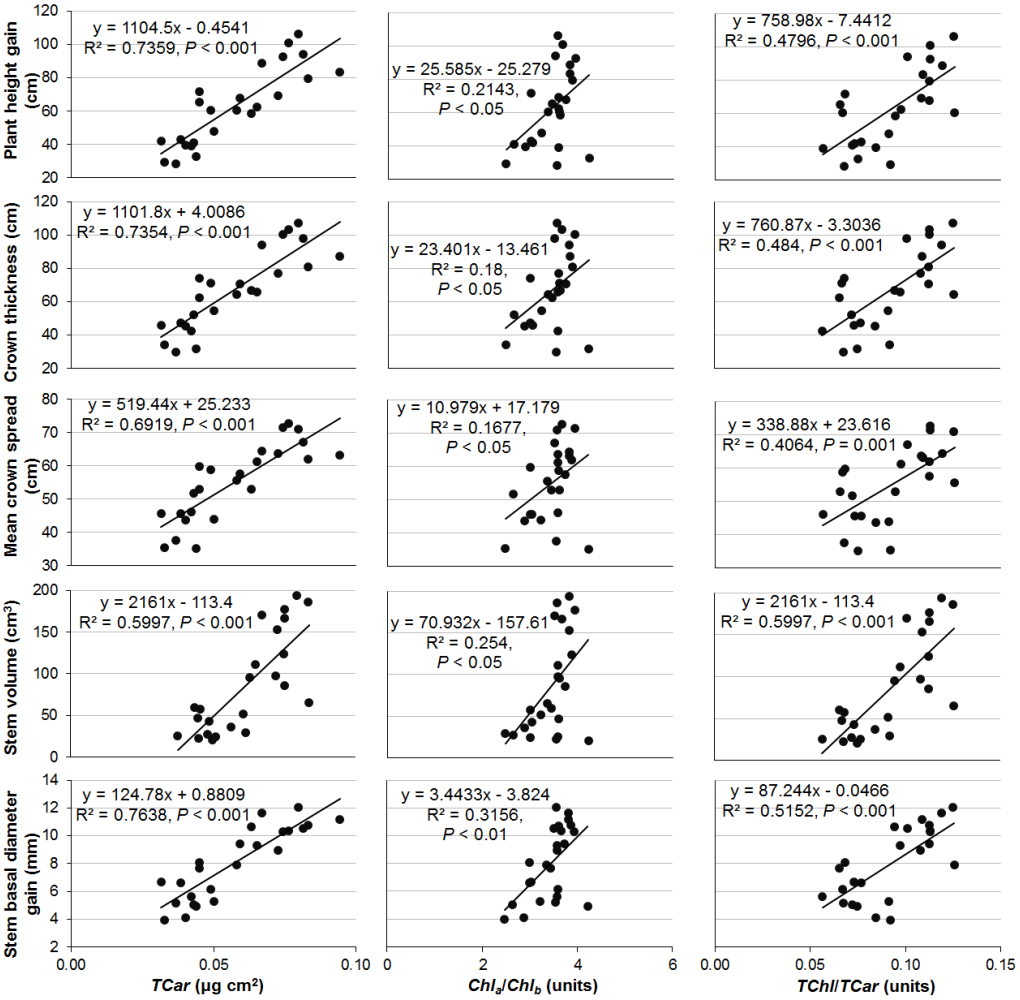

Fig 9 Regression analysis of total carotenoid content (*TCar*), Chl_a/Chl_b ratio and *TChl/TCar* ratio with each growth-related variable. Each data point is the average of the plants in each research condition (N=24). The regressions were tested at an α level of 0.05. Reprinted from Springer, Environmental Science and Pollution Research, Stem and crown growth of Japanese larch and its hybrid F_1 grown in two soils and exposed to two free-air O_3 regimes, 2017, Agathokleous E, Vanderstock A., Kita K, Koike T, "With permission of Springer"

4.1.4 Discussion

The O_3-induced visual injury was high in both soil types, and of similar magnitude for both larch taxa. This is consistent with the lower *Chl$_a$*, *Chl$_b$*, *TChl*, *TCar* and *TChl/TCar* of both taxa in EOZ than in AOZ which was of large magnitude. Importantly, despite the large differences in chlorophyll contents, the difference of hybrid larch and Japanese larch in terms of *Chl$_a$/Chl$_b$* between EOZ and AOZ was small, with 55% of the groups overlapping and an 80% chance that a haphazard observation from EOZ will reveal a lower score than a haphazard observation from AOZ. Chlorophyll production indicates nutritional, photosynthetic and productive efficiency of plants and relates to a unique plant defensive system against insect herbivory through the catalysis of chlorophyll by chlorophyll chlorophyllidohydrolase to produce chlorophyllide and phytol (Curran *et al*. 1990; Hu *et al*. 2015).

Therefore chlorophyll molecules are of utmost importance for plant health. Loss of chlorophyll may relate to chlorophyll oxidation or altered regulation of pigment biosynthesis (Caregnato *et al*. 2013), where the synthesis of new chlorophyll *a* is inhibited or the synthesis of chlorophyll *b* is increased as compared with uninjured needles (Knudson *et al*. 1977). The present *Chl$_a$/Chl$_b$* findings are consistent with higher sensitivity of chlorophyll *a* than chlorophyll *b* to O_3 in C3 plants reported in previous studies (Knudson *et al*. 1977; Saitanis *et al*. 2001; Neufeld *et al*. 2006; Feng *et al*. 2008b). However, they are inconsistent with other studies where a higher sensitivity of chlorophyll *b* than chlorophyll *a* to O_3 was found in C3 plants in the framework of a rearrangement of the pigments ratio as an adaptive defense mechanism within the chloroplasts to protect PSII reaction centers from photoinhibition (Fernandez *et al*. 1993; Döring *et al*. 2013; Pellegrini 2014). The structure of a chlorophyll molecule is characterized by a porphyrin head and a phytol tail with O_3-sensitive double bonds, however, while chlorophyll *a* has a $–CH_3$ group, chlorophyll *b* has an –CHO group at carbon atom 3 of pyrrole ring II.

Despite their similar structure, chlorophylls *a* and *b* have different locations, light absorption spectra and tasks within the photosystems and these dissimilarities may define their difference in sensitivity. The present and previous findings (Manninen *et al*. 1999; Saitanis *et al*. 2001; Feng *et al*. 2008b; Agathokleous *et al*. 2016b; Gao *et al*. 2016) indicate that Chl_a/Chl_b is a more efficient indicator of O_3 stress when O_3 injury is high than when O_3 injury is low, since a reduction of Chl_a associates with an increase in foliar injury (Knudson *et al*. 1977; Neufeld *et al*. 2006; Caregnato *et al*. 2013; Gottardini *et al*. 2014).

Contrastingly, O_3 sensitivity of *TChl/TCar* was 1.9 times higher (δ) than that of Chl_a/Chl_b. Such observation is consistent with higher sensitivity of *TChl* than *TCar* found in C3 plants (Agathokleous *et al*. 2014, 2016b; Pellegrini 2014). Alterations in *TChl/TCar* indicate malfunctions in plant physiological and phenological status caused by stress-induced photo-oxidation through breakdown of chlorophylls and a need for photo-protection (Peñuelas *et al*. 1995; Pellegrini 2014). In addition to the higher sensitivity of *TChl/TCar* compared with Chl_a/Chl_b, the correlations of *TChl/TCar* with the growth-related variables were large or very large whereas those of Chl_a/Chl_b were moderate in four out of five cases.

It is thus postulated that *TChl/TCar* may be a more sensitive O_3 biomarker than Chl_a/Chl_b and suggest further investigation of this index for O_3 pollution assessment studies of deciduous coniferous species. It is also worthwhile to test whether Chl_a/Chl_b increases under low stress in the framework of biological plasticity with the central player of Chl_b, and decreases under high oxidation damage in Chl_a (Calabrese and Blain 2009; Pellegrini 2014).

Physiological malfunctions indicated by pigmentation assessment reflected in plant growth through strong intercorrelations. Similarly to pigment-related traits, EOZ moderately suppressed plant height, crown thickness, mean crown spread and stem volume. However, heterosis was observed in the hybrid larch. Such a heterosis was previously observed in stem

and crown of the hybrid larch *Larix decidua* (Mill.) x *L. leptolepis* (Sieb and Zucc.) Gord. (Matyssek and Schulze 1987). Hybrid larch heterosis, observed in this study, was maintained in EOZ despite EOZ negatively affected hybrid growth too. Although vertical growth has been extensively studied, horizontal growth is poorly understood in open-grown tree species (Pretzsch *et al*. 2015).

Tree crown size is an important index both for forest and urban trees showing the epigeous space a tree occupies, which relates to the total leaf area and biomass (Binkley *et al*., 2013; Forrester 2014; Pretzsch *et al*. 2015). An EOZ-induced decrease in crown spread and thickness shows smaller crown size along with smaller epigeous space occupied by a tree. Such a decrease may further show decreased light use efficiency, as the latter may decrease with decreasing epigeous tree size (Gspaltl *et al*. 2013). Cachectic crown size in forest trees may have long term implications for forest ecosystems. Changes in epigeous competition between species of different O_3 sensitivity, undergrowth light intensity, crown faunal composition and carbon sequestration may be affected. In urban environments, suppressed crown size has implications of decreased particulate pollution absorption, reduced shading and a greater risk of branches breaking in high winds (Pretzsch *et al*. 2015).

With EOZ, stem diameter was reduced at each stem level across taxa and soils. However, plants in EOZ had a significantly lower drop of stem diameter over the run of stem height (from base to top) compared to plants in AOZ. This drop indicates a disproportional loss of stem diameter from the base to the top of the stem and may be attributed to a shorter exposure of the upper stem to EOZ than the middle and base. Disproportional inhibition of stem diameter growth across different heights indicates more warped growth and a possible susceptibility to winds. An O_3-induced reduction in stem diameter at the lower third of the stem was reported in a plantation of the O_3-sensitive Oxford clone of poplar (*Populus maximoviczii* Henry x *berolinensis* Dippel) in a 6- year open-field study (Carriero *et al*. 2015). A lower increment or

failure to increase in stem diameter also occurred mostly at the lower third of the stem of mature European beech trees (*Fagus sylvatica* L.) after a 7-year treatment with elevated O_3 levels (see Pretzsch *et al*. 2010 for discussion and references). No difference in the diameter at breast height but suppression in growth in the crown detected by a canopy crane. The findings of this study support conclusions by Pretzsch *et al*. (2010) that a decline in diameter increment at the lower third of the stem should not be interpreted as an overall growth decline due to overestimations.

The present findings of an EOZ-induced diameter suppression of both hybrid and Japanese larches contradict studies with potential experimental artifacts of O_3 facilities and root restrictions. For example, in a study using a FACE system with 60 nmol mol^{-1} daytime exposure over a growing season, no significant effects of O_3 were found on growth of the stem basal diameter and stem volume of potted hybrid and Japanese larch seedlings (Kam *et al*. 2015). Contrastingly, in an OTC study where potted seedlings were exposed to 60 nmol mol^{-1}, 7 h per day over a growing season, there was a significant O_3-induced reduction of diameter growth increment and height of potted seedlings of hybrid larch but not its parental species (Koike *et al*. 2012). However, the whole plant dry mass and the shoot/root ratio of all taxa were unaffected (Koike *et al*. 2012). Another OTC study on hybrid larch seedlings directly planted in the soil (i.e. no root limitations) with the same O_3 treatment as in Koike *et al*. (2012), found an O_3-induced reduction in final stem dry mass after two growing seasons of exposure to O_3 (Wang *et al*. 2015). However, O_3- induced reductions in plant height and stem diameter in the first growing season did not translate to early or late in the second (Wang *et al*. 2015). Thus, under experimental conditions with OTCs or potted plants in FACE systems, O_3 appears to influence plant growth differently with altered conditions. The present contradictory results in a system without containment artifacts and root restrictions reveal the importance of free air

studies in natural soils to understand how co-occurring environmental changes will impact forest tree species.

Soil, independently from Taxa and Ozone, was only a significant factor for Chl_a/Chl_b. In contrast to the EOZ-induced decrease in Chl_a/Chl_b, VA caused an increase in Chl_a/Chl_b, showing higher Chl_a than Chl_b. This increase was upon prevention of the EOZ-induced imbalance in Chl_a/Chl_b by VA, which was the only difference of interest in the Soil×Ozone interaction. Chl_a/Chl_b is an efficient indicator of the light environment of plants (Dale and Causton 1992; Lei *et al*. 1996), and its increase associates with acclimation to high light conditions and low N availability (Kitajima and Hogan 2003). Since the light environment was similar for both soils in this experiment, the increase in Chl_a/Chl_b in VA may be attributed to the lower N content in VA than in BF (Eguchi *et al*. 2008). It is also possible that Mg content differed between soil types. As Mg is a central atom in chlorophyll porphyrin ring, such a potential difference might have affected chlorophyll biosynthesis.

4.2 Study 2: The role of EDU (willow as a model tree)

4.2.1 Introduction

As mentioned in Chapter 1, the only currently known method for sufficiently protecting plants against O_3 injury is EDU. The complete molecule of EDU has a mass of 256 (Manning *et al*. 2011), it is consisted of phenylurea and urea by 53.2 and 23.5 %, and its total N content is estimated to be 21.9 % (Carnahan *et al*. 1978; Godzik and Manning 1998). The EDU concentrations required for a sufficient protection of plants against O_3 are usually quite small, e.g. 200-400 mg L^{-1} (Paoletti *et al*. 2008, 2009; Feng *et al*. 2010; Manning *et al*. 2011; Oksanen *et al*. 2013; Agathokleous *et al*. 2015a; Singh *et al*. 2015).

To depict the current state of art in EDU research, 50 peer-reviewed original articles published up to 2014 were reviewed (Agathokleous *et al*. 2015a, d). It seems there is a linear increase in EDU publications over time (Fig. 10).

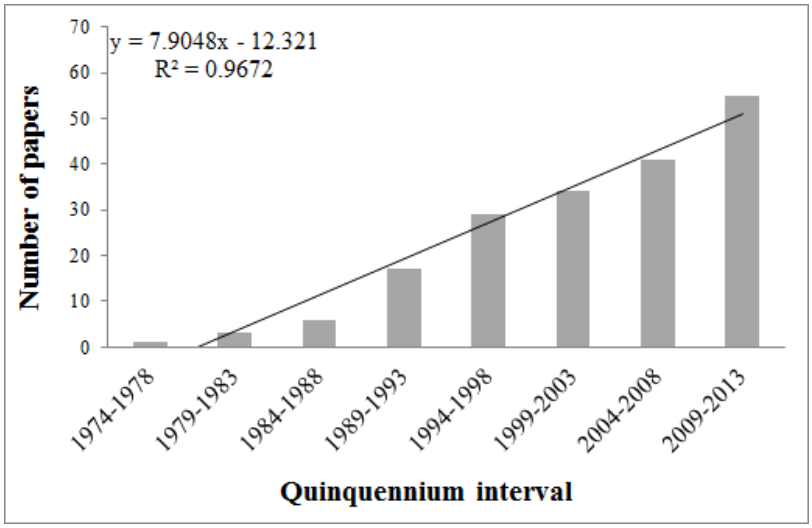

Fig 10. Cumulative number of peer-reviewed SCI original articles published on ozone-ethylenediurea during the last decades. On the x-axis the time interval (5-year) and on y-axis the number of published papers. This figure is based on the review work by Agathokleous *et al*. (2015a,d).

According to the review (Agathokleous *et al*. 2015a,d), the main EDU application methods are soil drench (>65 %), b) foliar spraying (20 %) and c) stem injection (10 %). In two papers EDU was dissolved in a nutrient solution and applied in either drip irrigation or hydroponic application; these applications were considered as soil drench (root absorbance). Approximately one hundred parameters have been studied to several plant species treated by EDU under O_3 exposure. For some parameters, the findings are complex or even contradictory. This may be the case due to several factors: EDU doses, plant developmental stage at EDU application, intervals of application, interval between the first application and the exposure to O_3, O_3 cumulative dose, treated species or cultivars, and the uncontrolled error especially in the open field studies.

EDU has been studied as a protectant of plants against O_3, as an O_3 biomonitoring tool or as a comparative tool for screening other chemicals as to their efficacy to protect plants against O_3 impact (Paoletti *et al*. 2009; Feng *et al*. 2010; Manning *et al*. 2011; Agathokleous *et al*. 2015a; Singh *et al*. 2015). EDU has been applied to plenty of agricultural crops. However, it has been applied only to few tree species: *Fagus sylvatica* L., *Fraxinus americana* L., *F. excelsior* L.

and *F. pennsylvanica* Marshall., *Liriodendron tulipifera* L., *Pinus taeda* L., *Prunus serotina* Ehrh, and different poplars (Paoletti *et al*. 2009; Agathokleous *et al*. 2015d; Xin *et al*. 2016). This is because such experimentations with trees are more difficult to be conducted (Manning *et al*. 2011).

As mentioned in the previous chapters, soil infertility, and particularly P scarcity, is one of the most critical issues nowadays as a large proportion of global soils are P deficient and acidic, phosphate rock reserves are decreasing, and P demands are increasing (von Uexkull and Mutert 1995; Van Vuuren *et al*. 2010; Cordell and Neset 2014; Ulrich and Frossard 2014). Notably, the effectiveness of EDU in plants grown in an infertile soil substrate has not been studied. Thus, the effectiveness of EDU against O_3 injury is unknown under such a scenario of soil infertility and when plant demands of nutrients are high.

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

The two main methods for applying EDU are soil drench and foliar spray (Paoletti *et al*. 2009; Agathokleous *et al*. 2015b), although stem injections were tested too (Ainsworth and Ashmore

1992; Paoletti *et al*. 2007). It was suggested that soil influences EDU effectiveness (Manning *et al*. 2011; Agathokleous *et al*. 2015a) while foliar applications of EDU are technically difficult in the case of big trees (Paoletti *et al*. 2011).

The present study aimed to assess the effectiveness of these two application methods of EDU, in the common range of 200-400 mg L^{-1} (Feng *et al*. 2010), to protect against O_3 damage in this fast-growing species. The study was designed to address five principal research questions. The first question (Q1) was "Does EDU applied at low doses affect *S. sachalinensis* plants grown in infertile and organic-matter-free soil under ambient conditions?" The second question (Q2) was "Does elevated O_3 alone affect *S. sachalinensis* plants?" In order to investigate EDU soil drench, the third question (Q3) was "Do EDU soil-drench applications at the dosage of 200 ml with the common concentrations of 200-400 mg L^{-1} every nine days protect against O_3 impact on *S. sachalinensis* plants grown in infertile and organic-matter-free soil?", where dosage means the rate of application of a dose. Similarly, to investigate EDU foliar spray, the next question (Q4) was "Do EDU spray applications at the common concentration range of 200-400 mg L^{-1} every nine days protect against O_3 impact on *S. sachalinensis* plants grown in infertile and organic-matter-free soil?" Finally, this study aimed to answer the question (Q5) "Which application method is more appropriate for protecting this fast growing species against O_3 phytotoxicity?" For this purpose, the amount of EDU needed for foliar spray applications was also recorded in order to estimate the consumption of EDU in relation to plant leaf area. This information would be important for designing future experiments. For these questions, it was further of interested to estimate the magnitude of the effect in case the alternative hypothesis (H_1) is accepted.

In order to answer the above questions of the second study, production-related response variables were selected rather than other ones, such as biochemical and physiological variables, because the O_3 impact on biomass production reflects the actual accumulated O_3 damage

(Larch 2003; Agathokleous *et al*. 2015a, 2016a) and is used in O_3 risk assessment (U.S. EPA 2014).

4.2.2 Materials and Methods

3.2.2.1 Experimental site

The experiments were conducted at Sapporo Experimental Forest of Hokkaido University, Japan (43º.04' N, 141º.20' E, 15 m a.s.l.). Description of the experimental site can be found in Chapter 2. Over the experimental period (August-October), data of temperature, wind speed, relative humidity, sunshine and precipitation were recorded by a nearby station at Sapporo (WMO, ID: 47412, 43º03.6'N 141º19.7'E), which is monitored by the Japan Meteorological Agency (2016). In addition, the photosynthetic photon flux density (PPFD) was recorded by a HOBO Pendant data logger (UA-002-64, Onset Computer, Co., MA, USA) located in the center of each experimental plot at a height of two meters.

3.2.2.2 Plant materials and experimental design

Willows can be propagated clonally from branch fragments (Newsholme, 1992) by rooting cuttings (Hayashi *et al*. 2005). One hundred fifty current-year cuttings of *S. sachalinensis* with height and basal diameter of 12.09±0.25 (mean ±s.e.) and 1.90±0.05 cm, respectively, were obtained from the Hokkaido Horti-Tree Planting Center, Co. Ltd; their origin was from the river basin of the Ebetsu city. The cuttings were stored at 0-4 ºC, in an incubator, for a month, in order to break the dormancy. Plant growth containers were filled with a mixture (1:1) of Akadama (well-weathered volcanic ash) and Kanuma (well-weathered pumice) soil – free from organic matter. Volcanic ash soils are phosphorus deficient and poor in N, and are commonly found in Hokkaido (Schmincke, 2004; Kam *et al*. 2015). Soils, originated from Kanuma town of Tochigi prefecture, were obtained (DCM Homac CO., LTD., Sapporo, JP) and opened just before the filling of the containers. Cuttings were planted for rooting on May 13th, in both 2014 and 2015, irrigated, and kept under field conditions. Irrigation was repeated two weeks later. On June 9th, when the cuttings were well rooted, 72 of them were selected for uniformity based

on total number of leaves per plant (39 ±2) and transplanted into 15 L pots filled with the same soil mixture, irrigated, and left in the field until establishment and full adaptation. The pH of this pot soil mixture was 5.90 ±0.01; details on sampling and composition of Akadama and Kanuma soils are in Agathokleous *et al*. (2015e). Irrigation was repeated two times, every seven days. On August 14th, the potted plants were randomly assigned and transferred to six different plots (12 pots per plot), of which three served as elevated O_3 and three as ambient O_3 treatment, and, further, four plants were randomly assigned to each of the three EDU treatments in each plot. All the pots within each plot were subjected to a fortnight rotation and the three plots of each O_3 treatment were interchanged three times over each growing season, during late evening hours. Irrigation was done using tap water (pH=6.57 ±0.04).

The plants were not fertilized. Plants were visually checked daily, and when insects were present they were manually removed. Visible injury by pests or pathogens was rarely observed, and thus plants were not treated by agrochemicals during the experiment.

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

3.2.2.3 Ozone treatments

For the O_3 treatments, a novel free-air O_3-enrichment system was established in the Sapporo Experimental Forest of Hokkaido University, Japan (see Chapter 2). The O_3 treatments were ambient O_3 (AOZ) and elevated O_3 (EOZ). Exposure of plants to EOZ lasted from August 15th to October 26th, in 2014 and 2015, during daytime, when the PPFD exceeded 70 μmol m^{-2} s^{-1} (*i.e*. light compensation point of photosynthesis of related plants as determined by Koike, 1988). The PPFD in the experimental plots exceeded 70 μmol m^{-2} s^{-1} during the hours 07:00 up to

17:00, for both experiments, and was not different between AOZ and EOZ plots (not shown). The AOZ and EOZ 10-h means were 22.3±3.3 and 60.1±2.2 nmol mol^{-1}, respectively, in 2014 and 34.3±5.5 and 71.5±1.3 nmol mol^{-1}, respectively, in 2015

3.2.2.4 Ethylenediurea treatments

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

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

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

needed for each plant. The 88 ml corresponded to 18±1 leaves or a total plant leaf area of 120.5±11.7 cm^2.

3.2.2.5 Measurements and samplings

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

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

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

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

The basal diameter of each shoot was measured by a caliper (mm), and the average shoot diameter (shoot diameter) was calculated per plant. The length of each shoot was also measured and the average shoot length per plant was calculated. The number of buds of each shoot was counted and the buds of all the shoots were summed up to give the total number of buds per plant.

At the end of each experiment, each shoot and each leaf were harvested and put in a separate paper bag with an ID so as to know the position for the leaves on the shoots and the position

of the shoots on the stem and thus to group them into lower-level and upper-level compartments. Roots were also put into separate bags with an ID informing about the plant to which they belonged.

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

3.2.2.6 Statistics

Each comparison of interest derived from a particular hypothesis, requiring thus straightforward interpretation (Agathokleous *et al*. 2016e). Yet, the total number of possible pair-wise comparisons was quite huge (high number of independent variables with at least two levels each), the majority of which was meaningless, increasing thus the experimental error and further making the *a posteriori* comparisons inappropriate. Thus, based on prior theoretical knowledge and in order to answer only the most biologically meaningful questions (Ruxton and Beauchamp, 2008) the approach of contrasts was chosen and applied to *a priory* planned comparisons which offer a better trade-off between type I and type II errors than unplanned comparisons.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

To quantify the effect magnitude for Q2 and Q4a (plant DM) and of EOZ for each of the 18 plant response variables for each experiment (EDU0*EOZ vs. (EDU0*AOZ + EDU200*AOZ + EDU400*AOZ)), the unbiased Cohen δ was estimated as described in Chapter 2.

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

4.2.3 Results

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

Q1 tested if EDU affected the plants in the absence of O_3 exposure (AOZ). H_0 was accepted (α=0.0085) for all response variables in this species (Table 8, Fig 11-13) suggesting that EDU by itself did not affect *S. sachalinensis* plants when grown in infertile and organic-matter-free soil under ambient conditions. There was only a trend (P<0.05) towards increased shoot DM and lower number of shoots (Table 8, Fig 12).

Q2 tested if EOZ alone affected the plants in the absence of EDU exposure (0 mg EDU L^{-1}). H_0 was rejected (α=0.0085) for all leaf traits variables (Table 8, Fig 11), crown width, shoots

DM (total DM of shoots per plant), foliage DM, aboveground DM and plant DM (Table 8, Fig 13), suggesting a significant effect of EOZ on *S. sachalinensis* plants grown in infertile and organic-matter-free soil. EOZ did not affect the shoot traits (Table 8, Fig 12). EOZ led to decreased number of leaves, average leaf size, average leaf DM, plant leaf area, crown width and foliage DM (Table 8, Fig 11-13). It further led to reduced DM of shoot and aboveground DM. There was a trend for root DM reduction (P<0.05) by EOZ as well. As a result, there was a small effect of EOZ on plant DM (δ = -1.43, CI [-3.15, -0.28]); however, the biomasses of aboveground and belowground parts were equally suppressed by EOZ as indicated by the shoot:root ratio (S/R=1.18±0.16 for AOZ and 1.23±0.07 for EOZ). The effect magnitude of EOZ on plant DM was close to moderate and very close to the conservative margin for practical significance. Still, δ of the 18 plant response variables was -1.63±0.36 in 2014 and -1.39±0.35 in 2015, showing no difference in the effect magnitude of EOZ. The average δ of the two experiments across all the 18 plant response variables was -1.51, indicating an overall moderate effect of EOZ on plants which is of practical significance.

Q3a tested if EOZ plants treated with soil drench of 200 and 400 mg EDU L^{-1} had similar performance with those treated with 0 mg EDU L^{-1}. H_0 was rejected (α=0.0085) only for number of leaves (Table 8, Fig 11), evidencing that, for all the other response variables, the means of plants treated with 200 ml soil drench of 200 and 400 mg EDU L^{-1} were comparable to those treated with 0 mg EDU L^{-1} in EOZ. Thus, there was a trend for lower foliage DM (P<0.05) and plant leaf area (P=0.058) in plants treated with 0 mg EDU L^{-1} than those treated with 200 or 400 mg EDU L^{-1} (Table 8, Fig 11).

Q3b tested if the performance of EOZ plants treated with soil drench of 400 mg EDU L^{-1} differed from that of EOZ plants treated with 200 mg EDU L^{-1}. H_0 was accepted (α=0.0085) for all plant response variables (Table 8, Fig 11-13), evidencing that the means of plants treated with 200 ml soil drench of 400 mg EDU L^{-1} were comparable to those treated with 200 mg

EDU L^{-1} in EOZ. However, there was a trend for increased (P<0.05, Table 8) number of shoots (Fig 12) and crown width (Fig 13) in plants treated with 400 mg EDU L^{-1} than those treated with 200 mg EDU L^{-1}. In addition, there was an insignificant decrease (P=0.066) in shoot diameter (Table 8, Fig 12) in plants treated with 400 mg EDU L^{-1} than those treated with 200 mg EDU L^{-1}.

Q4a tested if EOZ plants treated with foliar spray of 200 and 400 mg EDU L^{-1} had similar performance with those treated with 0 mg EDU L^{-1}. H_0 was rejected (α=0.0085, Table 8) for number of leaves, plant leaf area, average leaf DM (Fig 11) and root DM (Fig 13). Furthermore, average leaf size (Fig 11) and DM of foliage and plant (Fig 13) showed a trend for higher (P<0.05, Table 8) means of plants treated with foliar spray of 200 or 400 mg EDU L^{-1} than those treated with 0 mg EDU L^{-1} in EOZ. Yet, there was an insignificantly higher crown width, shoots DM and aboveground DM of EOZ plants treated with 200 or 400 mg EDU L^{-1} than those treated with 0 mg EDU L^{-1} (Table 8, Fig 13). H_0 was accepted (α=0.0085) for all the response variables of shoot traits (Table 8, Fig 12). The effect magnitude of EDU on plant DM was close to moderate (δ = 1.41, CI [0.45, 2.59]) and very close to the conservative margin for practical significance.

Q4b tested if the performance of EOZ plants treated with foliar spray of 400 mg EDU L^{-1} differed from that of EOZ plants treated with 200 mg EDU L^{-1}. H_0 was accepted (α=0.0085) for all the plant response variables (Table 8, Fig 11-13), with the means being similar between the components, proving that the means of plants treated with foliar spray of 400 mg EDU L^{-1} were indifferent from those treated with 200 mg EDU L^{-1} in EOZ. Only a trend was observed towards lower shoot-stem angle (Table 8, Fig 12) of EOZ plants treated with 400 mg EDU L^{-1} than those treated with 200 mg EDU L^{-1}, which, however, was insignificant (P>0.05). Except the shoot-stem angle, there was no difference between plants treated with 200 mg EDU L^{-1} and those treated with 400 mg EDU L^{-1} in EOZ.

As to the meteorological conditions, average air temperature and maximum air temperature were 0.1 and 0.3 ºC higher in 2014 than in 2015 while minimum air temperature was 0.3 ºC lower in 2014 than in 2015 (Table 9). Wind speed was 0.1 m s^{-1} lower in 2014 compared to 2015 and relative humidity was indifferent between years. Sunshine duration was 17.2 h longer and precipitation 20 mm higher in 2014 than in 2015. Moreover, the average daily PPFD, as measured within the experimental plots, was 161.7 ±6.8 μmol m^{-2} s^{-1} (n=6) in 2014 and 141.6 ±13.9 μmol m^{-2} s^{-1} (n=6) in 2015. These variations in meteorological conditions were not biologically significant (both for O_3 and EDU effects) as the effect magnitude of EOZ was indifferent between 2014 and 2015. In addition, these variations were insignificant for comparison between the two EDU application methods due to the binomial effect of the methods ("failure" of soil drench and "success" for foliar spray).

Table 8 Typical difference of 10 cm root tips. Samples were obtained from Siebold's Beech (*Fagus crenata*) saplings which were grown in two different types of soil (BF: brown forest soil or VA: volcanic ash soil including pumice) and exposed either to ambient CO_2 (370–390 μmol mol^{-1}) or to elevated CO_2 (500 μmol mol^{-1}) for 11 growing seasons (2003-2013). Reprinted from Science of The Total Environment, 573, Agathokleous E, Paoletti E, Saitanis CJ, Manning WJ, Sugai T, Koike T, Impacts of ethylene diurea (EDU) soil drench and foliar spray in *Salix sachalinensis* protection against O_3-induced injury, 10, 2016, with permission from Elsevier.

	Q1	Q2	Q3a	Q3b	Q4a	Q4b
Leaf traits (leaf level)						
Number of leaves	*t*=2.112, *P*=0.074	*t*=14.418, *P*<0.001	*t*=14.235, *P*<0.001	*t*=0.866, *P*=0.420	*t*=4.092, *P*=0.006	*t*=1.376, *P*=0.218
Leaf size	*t*=1.707, *P*=0.101	*t*=6.328, *P*<0.001	*t*=0.404, *P*=0.700	*t*=1.100, *P*=0.314	*t*=3.337, *P*=0.016	*t*=0.355, *P*=0.735
Plant leaf area	*t*=1.293, *P*=0.208	*t*=7.059, *P*<0.001	*t*=2.338, *P*=0.058	*t*=0.619, *P*=0.559	*t*=4.339, *P*=0.005	*t*=0.057, *P*=0.956
Leaf DM	*t*=1.683, *P*=0.105	*t*=4.444, *P*<0.001	*t*=0.075, *P*=0.943	*t*=1.087, *P*=1.319	*t*=12.367, *P*=0.006	*t*=0.691, *P*=0.516
Shoot traits (shoot level)						
Number of shoots	*t*=2.181, *P*=0.039	*t*=0.402, *P*=0.700	*t*=0.333, *P*=0.750	*t*=2.887, *P*=0.028	*t*=0.007, *P*=0.995	*t*=0.105, *P*=0.920
Shoot DM	*t*=2.688, *P*=0.013	*t*=0.882, *P*=0.386	*t*=0.901, *P*=0.402	*t*=1.028, *P*=0.344	*t*=0.727, *P*=0.540	*t*=1.270, *P*=0.251
Shoot length	*t*=2.015, *P*=0.055	*t*=0.546, *P*=0.604	*t*=0.862, *P*=0.422	*t*=1.072, *P*=0.325	*t*=0.513, *P*=0.626	*t*=0.293, *P*=0.779
Shoot diameter	*t*=2.040, *P*=0.071	*t*=1.902, *P*=0.069	*t*=0.033, *P*=0.975	*t*=2.244, *P*=0.066	*t*=0.489, *P*=0.642	*t*=0.434, *P*=0.680

Shoot angle	*t*=0.612, *P*=0.546	*t*=1.064, *P*=0.298	*t*=0.087, *P*=0.933	*t*=0.930, *P*=0.388	*t*=0.245, *P*=0.815	*t*=1.834, *P*=0.116
Number of buds	*t*=0.792, *P*=0.436	*t*=0.428, *P*=0.673	*t*=0.345, *P*=0.742	*t*=0.679, *P*=0.522	*t*=0.069, *P*=0.947	*t*=0.894, *P*=0.406
Plant traits (plant level)						
Crown length	*t*=1.750, *P*=0.093	*t*=0.380, *P*=0.707	*t*=0.468, *P*=0.657	*t*=1.292, *P*=0.209	*t*=0.808, *P*=0.450	*t*=1.175, *P*=0.284
Crown width	*t*=1.395, *P*=0.176	*t*=5.287, *P*<0.001	*t*=0.881, *P*=0.412	*t*=2.895, *P*=0.028	*t*=2.392, *P*=0.054	*t*=0.719, *P*=0.499
Root DM	*t*=1.780, *P*=0.123	*t*=3.060, *P*=0.042	*t*=0.836, *P*=0.435	*t*=1.336, *P*=0.230	*t*=5.180, *P*=0.002	*t*=1.000, *P*=0.423
Stem DM	*t*=0.867, *P*=0.395	*t*=1.599, *P*=0.123	*t*=0.947, *P*=0.380	*t*=0.615, *P*=0.561	*t*=0.200, *P*=0.848	*t*=1.139, *P*=0.298
Shoots DM	*t*=1.331, *P*=0.196	*t*=3.145, *P*=0.004	*t*=0.389, *P*=0.711	*t*=1.189, *P*=0.279	*t*=1.884, *P*=0.109	*t*=0.507, *P*=0.630
Foliage DM	*t*=0.897, *P*=0.379	*t*=7.855, *P*<0.001	*t*=3.112, *P*=0.021	*t*=0.810, *P*=0.449	*t*=3.561, *P*=0.012	*t*=0.308, *P*=0.768
Aboveground DM	*t*=0.847, *P*=0.406	*t*=4.442, *P*<0.001	*t*=1.007, *P*=0.353	*t*=0.693, *P*=0.514	*t*=2.169, *P*=0.137	*t*=0.698, *P*=0.511
Plant DM	*t*=0.462, *P*=0.658	*t*=5.337, *P*<0.001	*t*=1.037, *P*=0.340	*t*=0.685, *P*=0.519	*t*=3.515, *P*=0.013	*t*=0.533, *P*=0.613

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

Table 9 Monthly and experimental-period means of the main meteorological conditions at Sapporo, Japan, for the months August-October, of the years 2014-2015. Reprinted from Science of The Total Environment, 573, Agathokleous E, Paoletti E, Saitanis CJ, Manning WJ, Sugai T, Koike T, Impacts of ethylene diurea (EDU) soil drench and foliar spray in *Salix sachalinensis* protection against O_3-induced injury, 10, 2016, with permission from Elsevier.

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

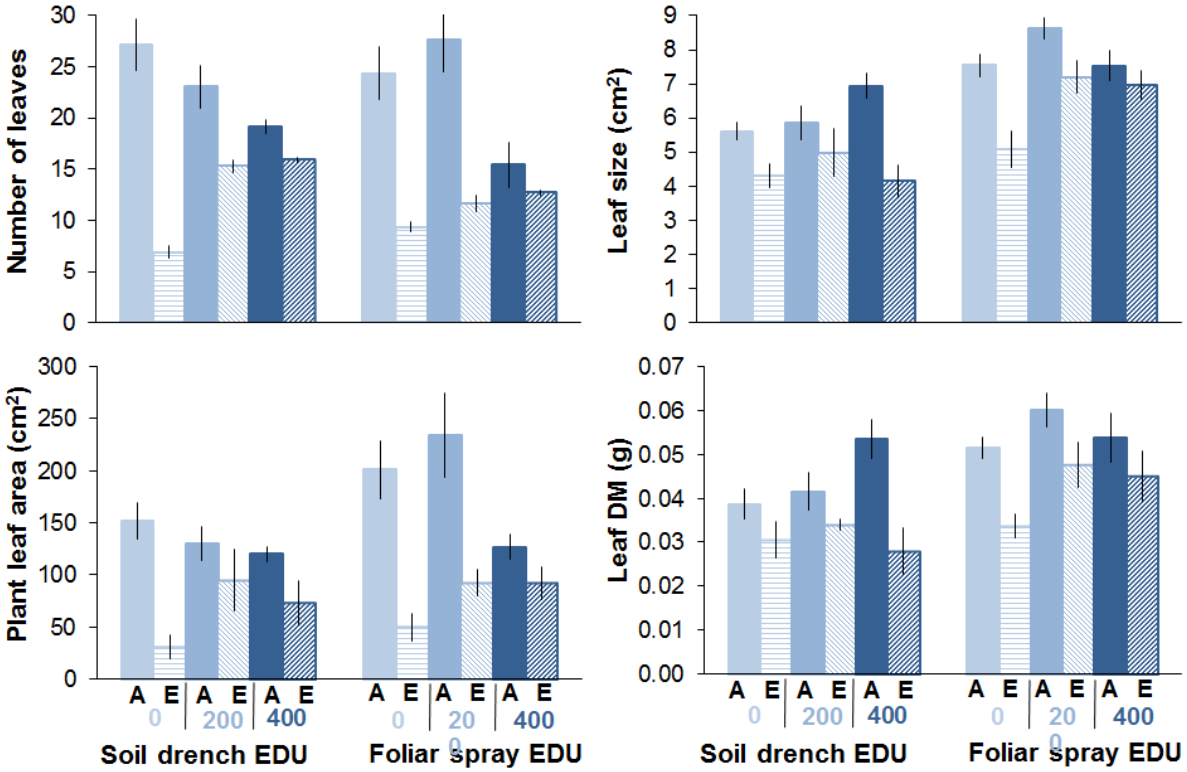

Fig 11 Arithmetic means (± s.e.) of leaf-level traits of *Salix sachalinensis* plants treated with 0, 200 or 400 mg EDU L^{-1} and exposed to ambient O_3 (A) or elevated O_3 (E) levels. In a growing season EDU was applied as soil drench and in the next growing season, following the same protocol, EDU was applied as foliar spray, to different plants. Reprinted from Science of The Total Environment, 573, Agathokleous E, Paoletti E, Saitanis CJ, Manning WJ, Sugai T, Koike T, Impacts of ethylene diurea (EDU) soil drench and foliar spray in *Salix sachalinensis* protection against O_3-induced injury, 10, 2016, with permission from Elsevier.

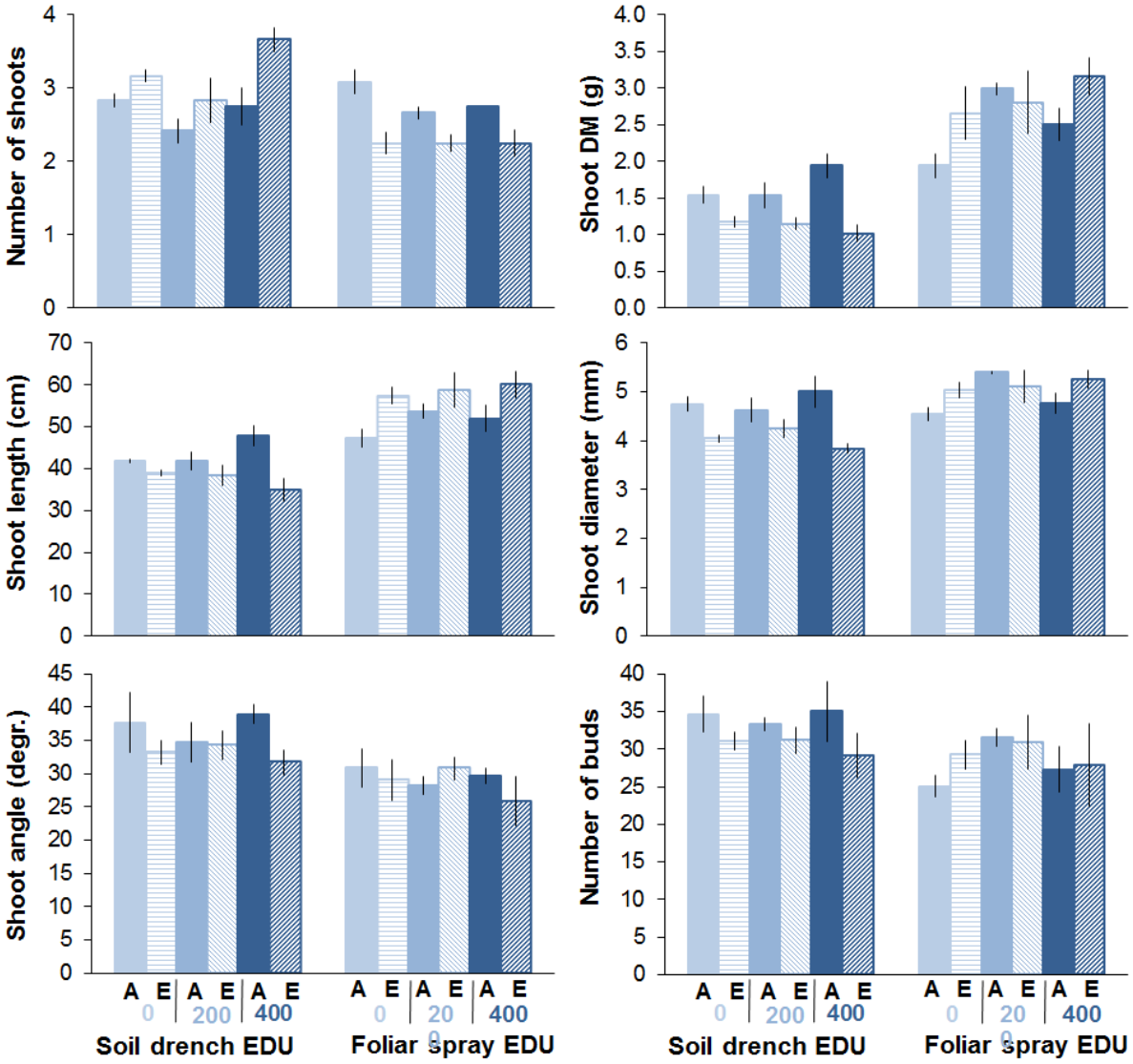

Fig 12 Arithmetic means (± s.e.) of shoot-level traits of *Salix sachalinensis* plants treated with 0, 200 or 400 mg EDU L^{-1} and exposed to ambient O_3 (A) or elevated O_3 (E) levels. In a growing season EDU was applied as soil drench and in the next growing season, following the same protocol, EDU was applied as foliar spray, to different plants. Reprinted from Science of The Total Environment, 573, Agathokleous E, Paoletti E, Saitanis CJ, Manning WJ, Sugai T, Koike T, Impacts of ethylene diurea (EDU) soil drench and foliar spray in *Salix sachalinensis* protection against O_3-induced injury, 10, 2016, with permission from Elsevier.

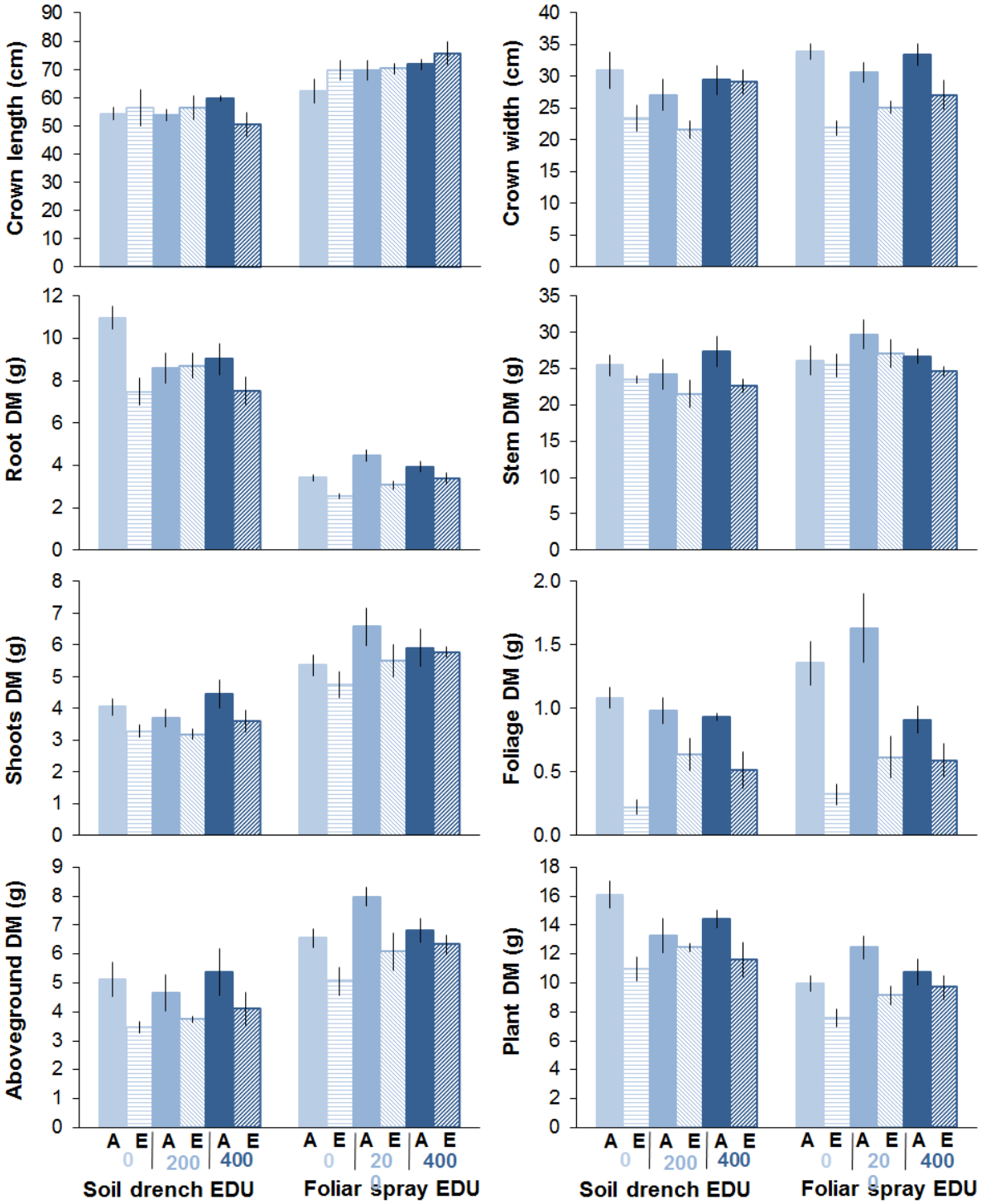

Fig 13 Arithmetic means (± s.e.) of plant-level dimensions and dry masses (DM) of *Salix sachalinensis* plants treated with 0, 200 or 400 mg EDU L^{-1} and exposed to ambient O_3 (A) or elevated O_3 (E) levels. In a growing season EDU was applied as soil drench and in the next growing season, following the same protocol, EDU was applied as foliar spray, to different plants. Reprinted from Science of The Total Environment, 573, Agathokleous E, Paoletti E, Saitanis CJ, Manning WJ, Sugai T, Koike T, Impacts of ethylene diurea (EDU) soil drench and foliar spray in *Salix sachalinensis* protection against O_3-induced injury, 10, 2016, with permission from Elsevier.

4.2.4 Discussion

At low ambient O_3 levels which are not expected to impact plants (AOZ), the present findings confirm suggestions made by Manning *et al*. (2011) and Agathokleous *et al*. (2015a) for absence of EDU-induced side effects on plants when EDU is applied in the appropriate range of doses (Q1). Regarding the trend of EDU-treated plants in AOZ towards increased shoot DM (DM per shoot) and decreased number of shoots, *i.e*. more biomass to be allocated to fewer shoots, it should be taken into account that shoots were formed before the exposure to the treatments. Thus, these observations are likely due to pre-treatment differences since plants were allocated to the treatments based on number of leaves. Further, these findings support evidence on the absence of EDU side effects in the range of 150-300 mg L^{-1} when hydrophyte communities (*Lemna minor* L.) were treated with EDU in an O_3-free atmosphere (Agathokleous *et al*. 2016g, see Chapter 4).

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

where S/R was significantly reduced and 27% where S/R was significantly increased (Agathokleous *et al*. 2016a). No effect of EOZ on shoot traits was due to the fact that the shoots were well-developed before the treatments started.

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

In contrast to previous experiments where woody plants were treated with EDU soil drench (Paoletti *et al*. 2009, 2011; Hoshika *et al*. 2013; Carriero *et al*. 2015), this experiment was conducted with current-year cuttings grown in infertile soil. The plant leaf area of these fast-growing plants was higher early in the treatments than it was at harvest when the autumn senescence was at the final stages, as it is indicated by the 63 leaves at first EDU application and the higher amount of EDU needed for the spray treatments in the second experiment. It is thus postulated that EDU as a soil drench was not enough for the high plant leaf area early in the treatments.

As observed for EDU applied as soil drench, EDU protected against EOZ-induced accelerated senescence in this species when applied as foliar spray at 200 and 400 mg L^{-1} (Q4a and Q4b),

as indicated by number of leaves, plant leaf area and foliage DM. A loss of leaves was more obvious around the middle of October, when the air temperature dropped suddenly to very low levels. This observation is supported by the more than two times higher amount of EDU needed to spray the plants at the semi-final EDU treatment, compared to the final one. The harvest was done at the end of the growing season when plants stopped producing new leaves and, therefore, cannot be proved if plants treated with spray of 200 and 400 mg EDU L^{-1} compensated the accelerated leaf senescence by producing more leaves during the growing season (Kolb and Matyssek 2001).

The reviews by Paoletti *et al*. (2009) and Singh *et al*. (2015) suggested that EDU delays the O_3-induced accelerated senescence and this coincides with the findings of the present study. However, the fact that EDU soil drench protected against EOZ-induced accelerated senescence while did not protect against EOZ damage to all the other response variables (which are not related to the leaf number) indicates that either the EDU mode of action in protecting against O_3 injury is not upon protecting against O_3-accelerated senescence –which is in agreement with suggestions by Eckardt and Pell (1996)- or EDU protection against EOZ injury was not complete as reported also by Paoletti *et al*. (2007). The higher biomass production of plants treated with foliar spray of 200 or 400 mg EDU L^{-1} than those treated with 0 mg EDU L^{-1} and the indifferent biomass production of plants treated with foliar spray of 200 mg EDU L^{-1} and those treated with 400 mg EDU L^{-1} in EOZ, suggest that EDU can reduce O_3-induced damage to plants of this species in the range of EDU doses 200-400 mg L^{-1}.

In the case of this study, the amount of EDU was the same when applied as spray and as soil drench and this evidences that no more EDU is needed when applied as foliar spray to current-year plants of fast growing species grown under similar experimental conditions (Q5). When the plant leaf area was relatively low, *i.e*. at the final EDU application, the amount of EDU needed for foliar spray was 2.3 times lower than that needed for soil drench, showing that EDU

foliar spray is more appropriate –in terms of financial cost- than EDU soil drench for plants with small leaf area.

Chapter 4

EDU toxic and fertilization effects

4.3 Introduction

As explained in Chapters 2 and 3, EDU is a substance which has been found to protect plants against O_3 impact (Carnahan *et al*. 1978) when appropriately applied in the usual range of doses,

i.e. 200-400 mg L^{-1} (Paoletti *et al*. 2009; Feng *et al*. 2010). However, the EDU mode of action in protecting plants against O_3 injury remains unclear (Paoletti *et al*. 2008, 2009; Feng *et al*. 2010; Manning *et al*. 2011; Oksanen *et al*. 2013; Agathokleous *et al*. 2015a; 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 O_3 (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, although EDU has been successfully used in numerous research studies (Paoletti *et al*. 2009; Feng *et al*. 2010; Manning *et al*. 2011; Agathokleous *et al*. 2015a), EDU application is limited by lack of toxicological studies and commercial unavailability (Agathokleous *et al*. 2015b). Despite the majority of research reports do not mention any toxicity effect of EDU to plants, there are a few publications which reported speculations about probable side effects (see the review by Agathokleous *et al*. (2015b)). Such speculations were based on experiments with growing substrates containing organic matter, which confounds the EDU applied doses (as reviewed by Manning *et al*. (2011) and Agathokleous *et al*. (2015a); see also Pasqualini *et al*. (2016). it remains hitherto unclear whether or not EDU *per se* is phytotoxic to plants when applied to roots.

On the other hand, as stated by Paracelsus (1525), "all substances are poisons, there is none which is not a poison. The right dose differentiates a poison and a remedy". Thus, since there is hitherto no clear evidence about side or toxic effects of EDU, the biological effects of its applications are unknown and relevant toxicological studies are urgently needed (Agathokleous *et al*. 2015a).

This study was carried out 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 in *S. sachalinensis* as an experimental plant. This species was selected as model plant because it is

a very fast growing species (Koike *et al*. 1995) and can grow on nutrient-poor substrates (Haruki and Tsuyuzaki 2001). EDU was applied 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).

4.4 Materials and Methods

4.4.1 Experimental site

The experiments were conducted at Sapporo Experimental Forest of Hokkaido University, Japan (43º.04' N, 141º.20' E, 15 m a.s.l.), in 2014. More details about the site can be found in Chapter 2. For the period May-October, the main meteorological conditions (mean ±s.e. in parenthesis) were: mean monthly average of air temperature = 17.83 (±1.83) ºC; daily maximum temperature = 22.52 (±1.75) ºC; daily minimum temperature = 14.07 (±2.02) ºC; wind speed = 3.52 (±0.17) m s^{-1}; relative humidity = 68.83 (±1.58) %; mean monthly total sunshine duration = 186.25 (±9.98) h; and mean monthly precipitation 120.50 (±23.19) mm, respectively. Ambient O_3 concentration at the experimental area was continuously monitored by an ultraviolet (UV) absorption O_3 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 O_3 concentration for the 80 days was 23.6 ± 0.7 nmol mol^{-1}. For the same period, the index of Accumulated exposure to O_3 over the Threshold of 40 nmol mol^{-1} (AOT40, Mills *et al*. 2007) was 104.9 nmol mol^{-1} h.

4.4.2 Plant material and 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 (±0.25)

and 1.90 (±0.05) cm, respectively. The cuttings were stored in an incubator, at 0-4 ºC, 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 ºC), 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).

4.4.3 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 ≈ 4.68 kg ha^{-1} for the 1600 mg EDU L^{-1} treatment which, based on Manning *et al*. (2011), equals to ≈ 1.02 kg N ha^{-1}; half of these amounts were applied for the 800 mg EDU L^{-1} treatment.

4.4.4 Measurements & Samplings

For pre-treatment assessing, 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 ($y = 0.5234x - 0.4106$, $R^2 = 0.5147$, $_{adj}R^2=0.504$, $p<0.001$) but not with chlorophyll *b* content ($y = 0.0221x + 4.3437$, $R^2 = 0.0034$, $_{adj}R^2=-0.019$, $p=0.697$) (data not shown). Therefore, SPAD indicated the chlorophyll *a* content in the present study.

For post-treatment assessing, A day 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. 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.

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 (HNO_3) and hydrogen peroxide (H_2O_2). 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.

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 ^{o}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 (OD_{435}/OD_{415}), according to Ronen and Galun (1984). The OD 470 was measured to check alterations in total carotenoid content (TCar) following the equation $TCar = (1000A_{470} - 1.90Chl_a - 63.14Chl_b)/214$ by Lichtenthaler (1987); however, it was given for acetone 100 % and therefore the actual values of carotenoid contents may differ.

The ODs 648 and 665 were measured to estimate the chlorophyll *a* content (Chl_a), chlorophyll *b* content (Chl_b) and chlorophylls *a+b* content (TChl), according to Barnes *et al*. (1992). The fractions Chl_a/Chl_b and *TChl*/*TCar* were calculated as well.

The root system of each plant was perfectly excavated as a whole, gently washed with tap water and dried in an air-dry oven at constant air temperature of 80 ^oC, until a constant dry mass. The dry masses of each leaf, shoot, root (Root DM) and stem (Stem DM) were measured. Apart from the mean leaf dry mass (Mean leaf DM) and mean shoot dry mass (Mean shoot DM) per plant, the total foliage dry mass (Foliage DM) and total shoot dry mass (Shoots DM) per plant were calculated. The sum of Foliage DM, Shoots DM and Root DM constituted the total plant dry mass (Plant DM). The Root DM/Foliage DM ratio was also calculated as a biomass partitioning indicator.

SPAD and N_{PPW} measurements were taken following the same methodology as described for pre-treatment measurements. For N/C analyses, soil, fine roots (diameter < 1 mm), shoots and buds were analyzed. For the soil, all the sampled soil from each pot (8 randomly selected pots per EDU treatment) was grounded. 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 metallic 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).

4.4.5 Data handling and 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 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); see Chapter 3 for details. Transformed data were used for the statistical analyses and untransformed data were used for presentation purposes.

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 (see explanations in Chapter 3.2). 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*. 2016g) 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 was estimated as explanation in Chapter 2.

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

Log-transformed concentrations of EDU were used to plot EDU against measured N content in mature leaves. From the data of the three EDU concentrations, data were estimated for 34

EDU concentrations using interpolation on a scale of 50 mg EDU L^{-1}. The interpolation was done based on a constrained version of the Bessel spline function that results to fewer overshoots and oscillations, giving a more constrained smooth fit to data. Two lines were distinguished, one for each of the two EDU intervals (*i.e*. 0-800 and 800-1600) of whose slopes were tested (α=0.05) using comparison of regression lines slopes from two independent samples based on pooled variance (Howell 2010).

For the analyses of all the response variables, the minimum *n* of each treatment of each variable was 6 and the maximum was 14. Data processing and statistical analyses were conducted using MS EXCEL 2010 (Microsoft ©), PASW Statistics 18 (formerly SPSS Statistics, IBM ©) and STATISTICA v.10 (StatSoft Inc. ©) software.

4.5 Results

Regarding the 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.

Regarding the post-treatment assessment, EDU800, *vs.* EDU0, induced a large increase in plant height gain (Fig 14C), mean leaf DM (Fig 14E), LAI (Fig 14H), mean shoot DM (Fig 15C), shoots angle (Fig 15D), shoots DM (Fig 16C), foliage DM (Fig 16D), plant DM (Fig 16E) and N_{PPW} of mature leaves (Fig 15F). Similarly, EDU800 led to higher Ni content of shed leaves

(Table 10, δ = 1.67, CI [1.67, 1.68]) and N content of mature leaves (Fig 17E), at a moderate effect magnitude. On the other hand, EDU800 caused a decrease of large effect magnitude in SLA (Fig 14G) and root DM/foliage DM (Fig 16F).

EDU1600, *vs.* EDU0, affected the plants in a similar way. It caused an increase of large effect magnitude in plant height gain (Fig 14C), mean leaf DM (Fig 14E), mean shoot DM (Fig 15C), SPAD (Fig 15E), N_{PPW} (Fig 15F) and N content of shed leaves (Fig 17F). Further, EDU1600 caused an increase of moderate effect magnitude on Ni content of shed leaves (Table 10, δ = 1.67, CI [1.67, 1.68]) and N content of mature leaves (Fig 17E). Finally, it lowered the SLA (Fig 14G) and N/C fraction of shed (Fig 17F) and mature (Fig 17E) leaves at a large magnitude and the shoot diameter gain at a small magnitude (Fig 15A).

The coefficients of variation of element concentrations in shed leaves were very high (Table 10).

The measured N content in leaves was well correlated (p<0.001) with the estimated N_{PPW} and with SPAD. 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 18); the two slopes were statistically different (t=-27.75, p<0.001).

Table 10 Mean ±se of post-treatment pigment characteristics of mature leaves and elemental composition of shed leaves 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). Chl_a is chlorophyll *a*, Chl_b is chlorophyll *b*, TChl is total chlorophyll, Tcar is total carotenoids and OD_{435}/OD_{415} 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 α=0.017. Reprinted from Science of The Total Environment, 566, Agathokleous E, Paoletti E, Saitanis CJ, Manning WJ, Koike T, High doses of ethylene diurea (EDU) are not toxic to willow and act as nitrogen fertilizer, 10, 2016, with permission from Elsevier.

	EDU0	**EDU800**	**EDU1600**	**EDU0 *vs.* EDU800**	**EDU0 *vs.* EDU1600**
Pigments of mature leaves (μg 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

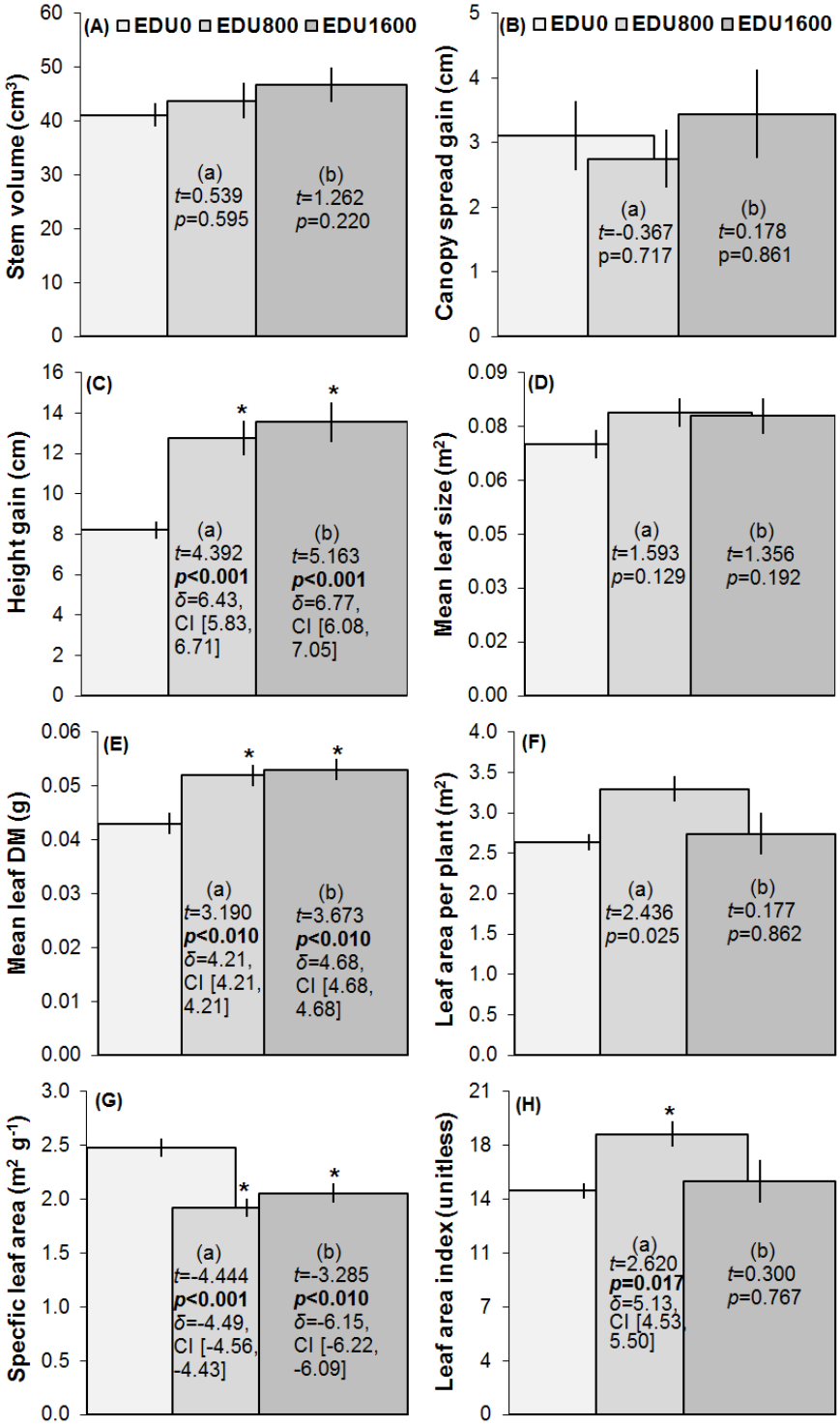

Fig 14 Post-treatment growth characteristics (A, B, C) and leaf traits (D, E, F, G, H) of willow (*Salix sachalinensis*) plants grown in ambient O_3 atmosphere and treated with 0 mg EDU L^{-1} (EDU0), 800 mg EDU L^{-1} (EDU800) or 1600 mg EDU L^{-1} (EDU1600). Asterisk above the error bar of an EDU800 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 α=0.017. The unbiased Cohen's δ (δ) and its confidence interval (CI) were estimated when there was a significant EDU effect. Reprinted from Science of The Total Environment, 566, Agathokleous E, Paoletti E, Saitanis CJ, Manning WJ, Koike T, High doses of ethylene diurea (EDU) are not toxic to willow and act as nitrogen fertilizer, 10, 2016, with permission from Elsevier.

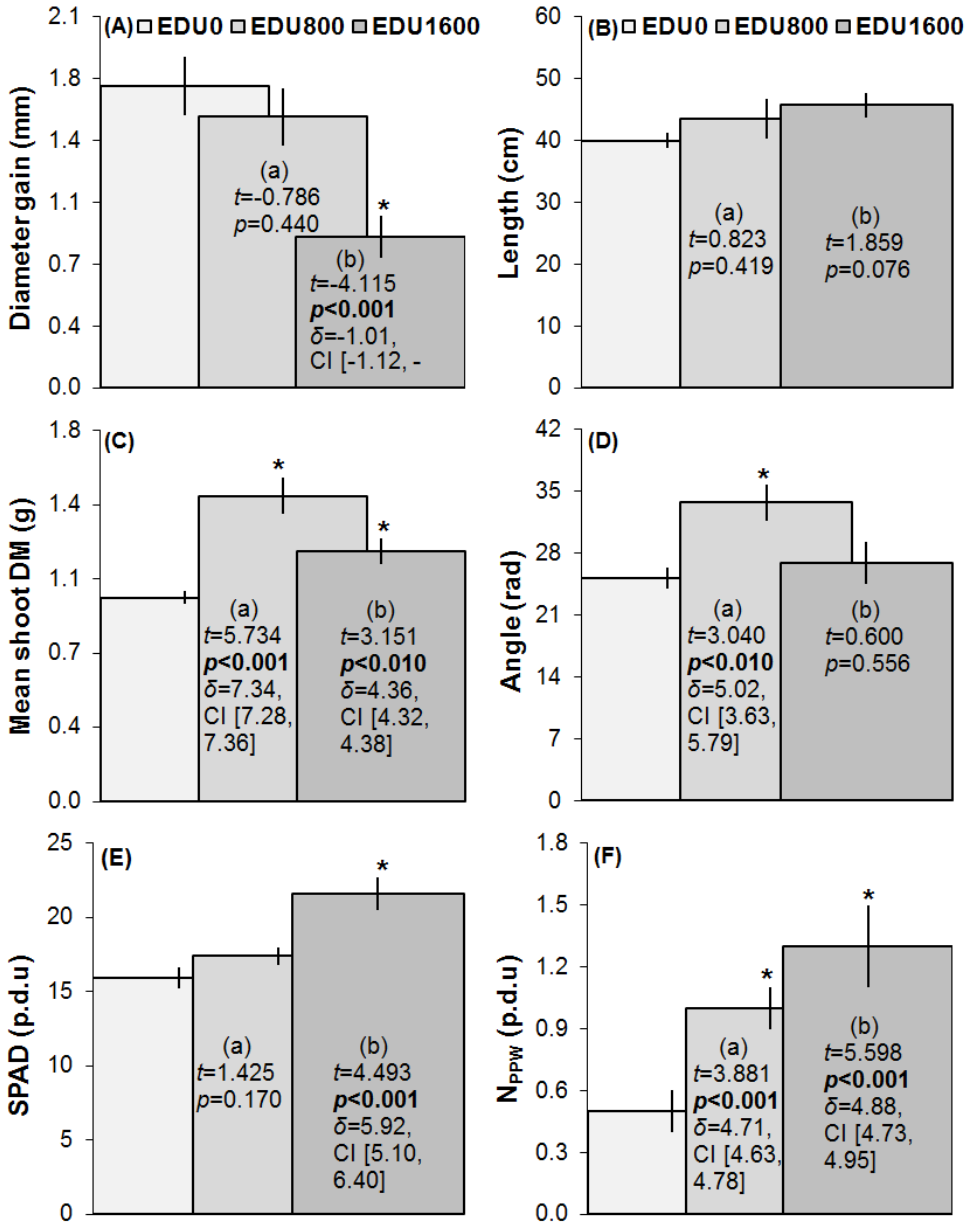

Fig 15 Post-treatment shoot traits (A, B, C, D), leaf greenness (E) and estimated leaf nitrogen content (F) of willow (*Salix sachalinensis*) plants grown in ambient O_3 atmosphere and treated with 0 mg EDU L^{-1} (EDU0), 800 mg EDU L^{-1} (EDU800) or 1600 mg EDU L^{-1} (EDU1600). Asterisk above the 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 α=0.017. The unbiased Cohen's δ (δ) and its confidence interval (CI) were estimated when there was a significant EDU effect. Reprinted from Science of The Total Environment, 566, Agathokleous E, Paoletti E, Saitanis CJ, Manning WJ, Koike T, High doses of ethylene diurea (EDU) are not toxic to willow and act as nitrogen fertilizer, 10, 2016, with permission from Elsevier.

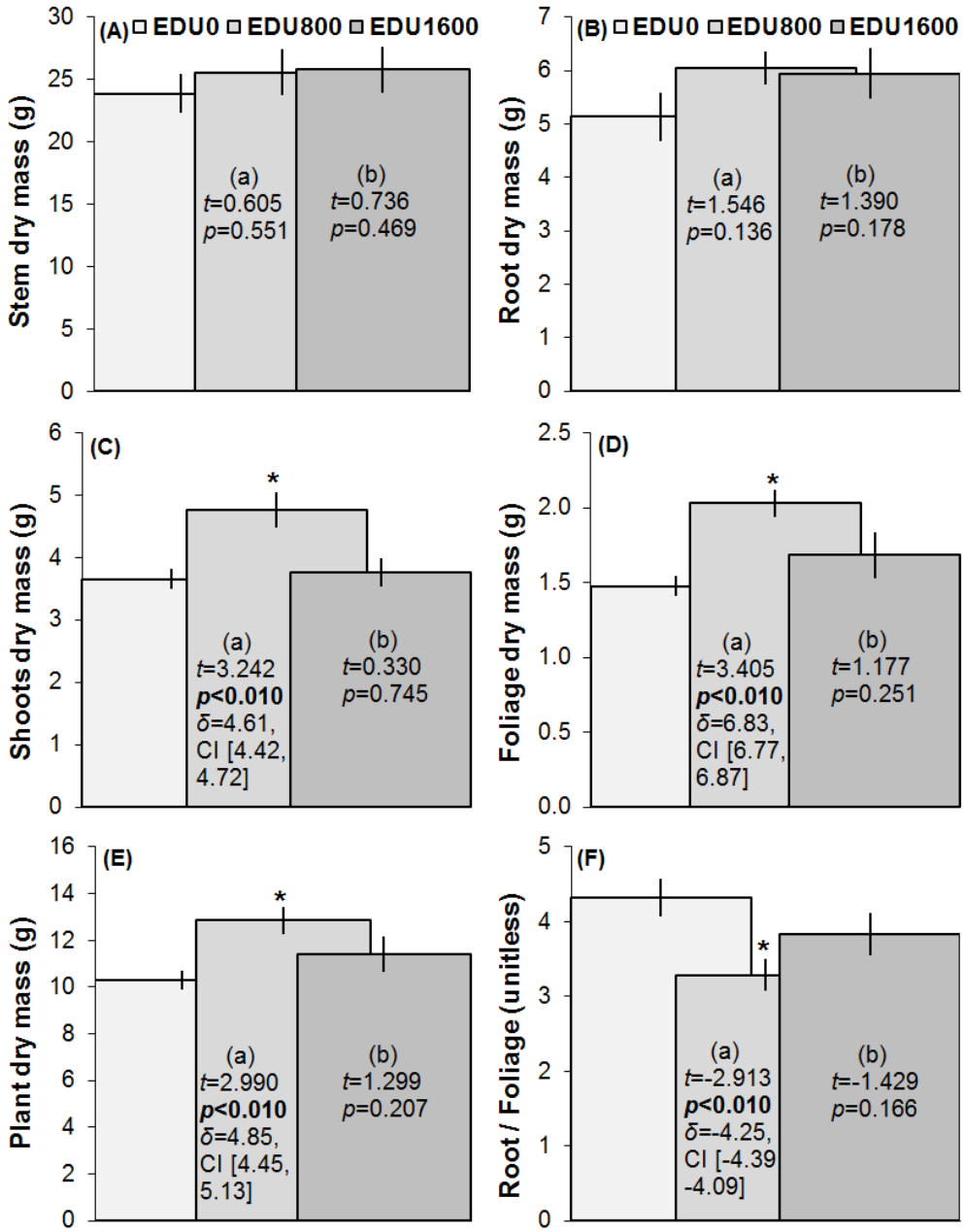

Fig 16 Post-treatment biomass production of willow (*Salix sachalinensis*) plants grown in ambient O_3 atmosphere and treated with 0 mg EDU L^{-1} (EDU0), 800 mg EDU L^{-1} (EDU800) or 1600 mg EDU L^{-1} (EDU1600). Asterisk above the error bar of an EDU800 or EDU1600 mean indicates statistical significance. 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 α=0.017. The unbiased Cohen's δ (δ) and its confidence interval (CI) were estimated when there was a significant EDU effect. Reprinted from Science of The Total Environment, 566, Agathokleous E, Paoletti E, Saitanis CJ, Manning WJ, Koike T, High doses of ethylene diurea (EDU) are not toxic to willow and act as nitrogen fertilizer, 10, 2016, with permission from Elsevier.

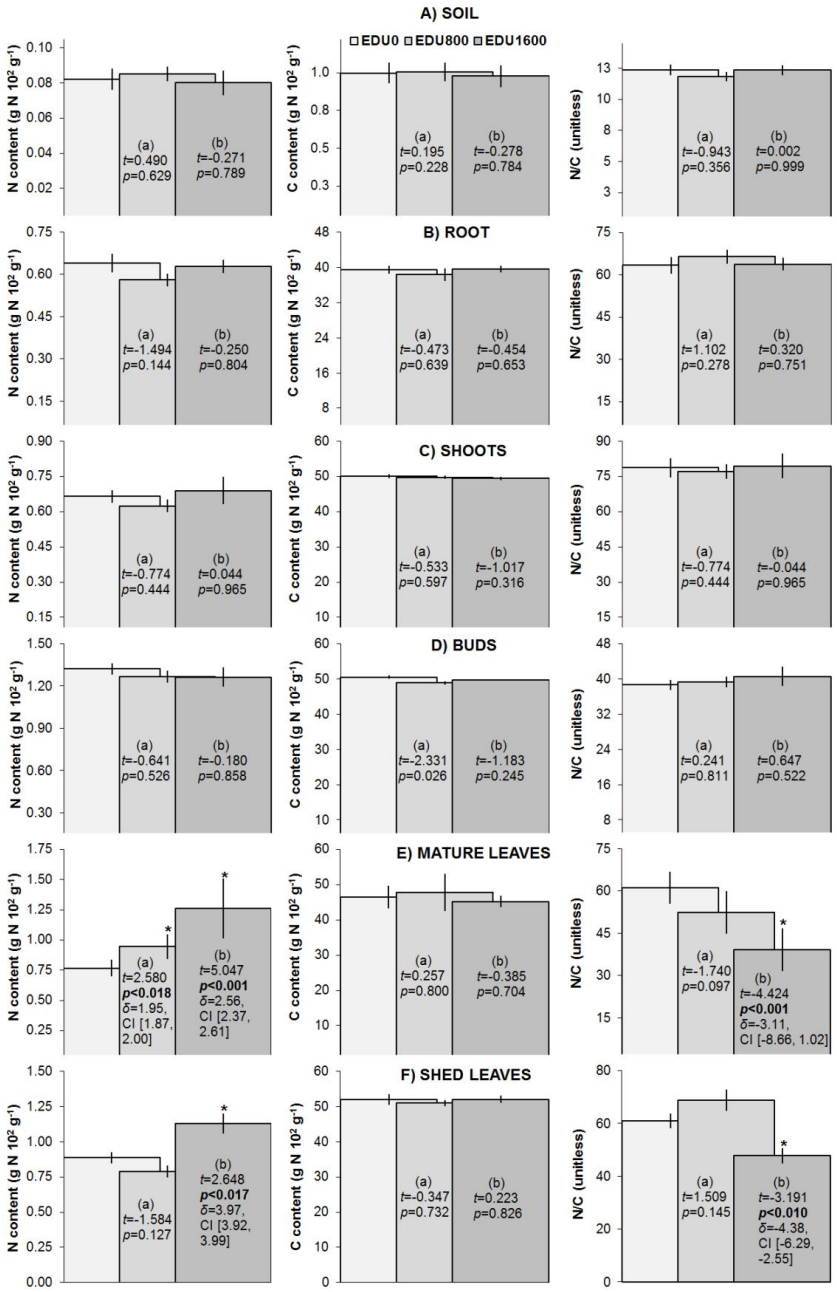

Fig 17 Post-treatment measured nitrogen (N) content, carbon (C) content, and N/C ratio in the soil (A) and different plant organs (B-F) of willow (*Salix sachalinensis*) plants grown in ambient O_3 atmosphere and treated with 0 mg EDU L^{-1} (EDU0), 800 mg EDU L^{-1} (EDU800) or 1600 mg EDU L^{-1} (EDU1600). 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 α=0.017. The unbiased Cohen's δ (δ) and its confidence interval (CI) were estimated when there was a significant EDU effect. Reprinted from Science of The Total Environment, 566, Agathokleous E, Paoletti E, Saitanis CJ, Manning WJ, Koike T, High doses of ethylene diurea (EDU) are not toxic to willow and act as nitrogen fertilizer, 10, 2016, with permission from Elsevier.

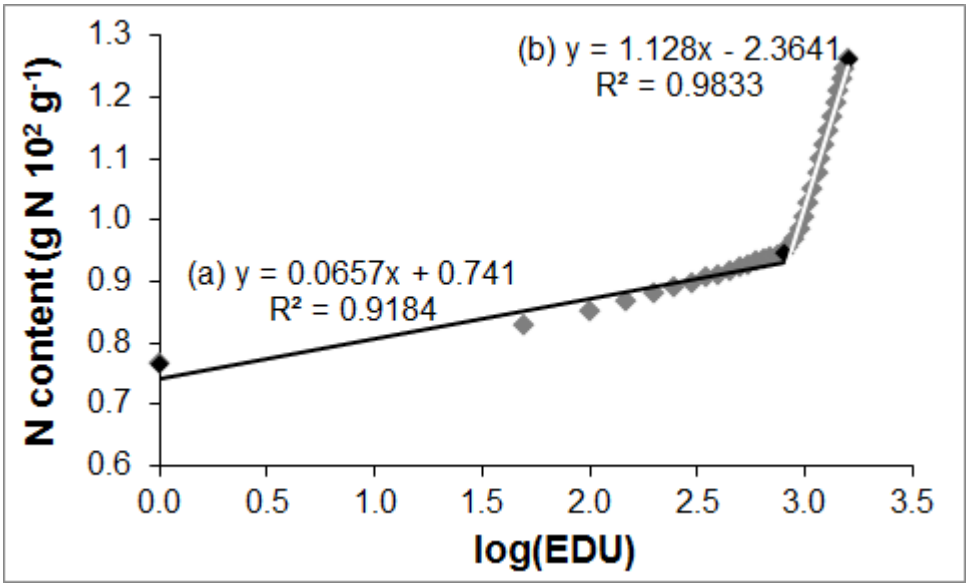

Fig 18 Interpolated nitrogen (N) content in mature leaves of willow (*Salix sachalinensis*) plants grown in ambient O_3 atmosphere *vs*. log-transformed concentrations of EDU which were repeatedly applied to the plants. The three original EDU treatments were 0, 800 and 1600 mg EDU L^{-1} (black data 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 interpolation was done based on a constrained version of the Bessel spline function. Two regression lines are shown; one for the EDU range [0, 800] (a) and one for the EDU range [800, 1600] (b). Reprinted from Science of The Total Environment, 566, Agathokleous E, Paoletti E, Saitanis CJ, Manning WJ, Koike T, High doses of ethylene diurea (EDU) are not toxic to willow and act as nitrogen fertilizer, 10, 2016, with permission from Elsevier.

4.6 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. It is thus concluded 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 used in this study) or with retention of bioavailable N which as may, for instance, happen in volcanic soils of temperate evergreen rainforests (Huygens *et al*. 2008).

Plants have different mechanisms to cope with O_3-induced stress according to the available nutrient supply (Utriainen and Holopainen, 2001). It is worthwhile to consider the potential impacts of elevated foliar N concentration on the leaf responses to O_3. Although some reports have shown no interactive effect of N availability with O_3 (Watanabe *et al*. 2007; Yamaguchi *et al*. 2007b), O_3-induced damage to aboveground physiological functioning or production of plants is possible to be higher when N availability or leaf N content is higher (Tjoelker and Luxmoore 1991; Lippert *et al*. 1996; Utriainen and Holopainen 2001; Izuta and Nakaji 2003; Yamaguchi *et al*. 2007a). In such a case, elevated leaf N content due to EDU would contrast with EDU prevention of O_3 damage.

Godzik and Manning (1998) applied 300 mg EDU L^{-1} and corresponding amounts of N as urea (70 mg L^{-1}) or phenylurea (159 mg L^{-1}) to leaves of Bel-W3 tobacco plants (*Nicotiana tabacum* L.), and suggested that N was not responsible for the EDU protection of plants: EDU and phenylurea were equally effective in preventing O_3 injury, whereas urea was not effective. Manning *et al*. (2011) also reported that a foliar application of 300 mg EDU L^{-1} to the O_3-sensitive bean line 156 (*Phaseolus vulgaris* L.) resulted to a slight increase of N and ^{15}N only one day after the application, followed by a decline. In contrast, three spray applications (biweekly) of 300 mg EDU (50 % a.i.) L^{-1} for one growing season increased the leaf N content of *Pinus taeda* L. seedlings late in the study but not early in the study (Kuehler and Flagler 1999). Paoletti *et al*. (2007, 2008) found that six gravitational trunk infusions (every 21 days) with 450 mg EDU L^{-1} did not contribute to leaf N content of adult ash (*Fraxinus excelsior* L.) trees; the 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 it is considered 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 mg EDU L^{-1} would be as little as 0.25 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 Chl_a/Chl_b 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 O_3 stress (Agathokleous *et al*. 2016a). EDU did not affect the carbon distribution within plant and between plant and soil (Table 10). Therefore, in EDU-O_3 studies, alteration in C accumulation or distribution within plant would be caused by O_3 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*. 2016g). 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 O_3 damage by EDU: No characteristic O_3 symptoms were observed on the EDU0 plants. Although such symptoms are not always present under O_3 stress (Agathokleous *et al*. 2015b), differences in C content or C distribution within plant which is a classical O_3 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*. 2016g), 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).

Chapter 5

General discussion

5.1 Effects of elevated CO_2 levels on root traits

5.1.1 Four-year CO_2 exposure on the community

I studied how elevated CO_2 and soil infertility could impact the belowground functioning, in terms of C allocation within plant body and productivity, of a community of trees of four deciduous broad-leaved species.

Elevated CO_2 did not cause an increase in trunk basal area or total root mass when the community had grown in fertile BF soil after canopy closure, but it did cause a large increase in both characteristics when the community had grown in infertile VA soil. Furthermore, eCO_2 caused an evident rhizo-morphogenesis through a large increase in fine root production in both soils. On the other hand, eCO_2 had no effect on C allocation to roots even in the case where eCO_2 increased Area and TDM in VA soil. Similarly, VA soil did not cause any significant disturbance to C allocation under ambient or elevated CO_2, albeit there was an insignificant trend for higher allocation to belowground when combined with eCO_2. Nevertheless, these results do not support the drawing of general conclusions because the adaptability of plants to VA soil as well as to eCO_2 may differ among species of different growth traits and soil physicochemical structure.

I conclude that eCO_2 treatment of 4 growing seasons has no potential to alter the total root production of a community of the 4 deciduous broad-leaved trees when it is growing in brown forest soil. Tree species growing in less fertile soils, such as VA with high porosity, may respond more vigorously to eCO_2 if they are well adapted to the single effects of such soils. New directions should also deal with more infertile soil than the one used in our study. Moreover, ectomycorrhizae research under similar conditions would be essential as their symbiosis may directly or indirectly help plants to mediate the VA- or CO_2-induced suppressing stressors.

5.1.2 Eleven-year CO_2 exposure on beech

I studied the response to eCO_2 and soil fertility of saplings of the late-successional, deciduous, broadleaved Siebold's beech (*Fagus crenata*) after 11 years of exposure, in a transition zone between cool temperate and boreal forests and at the Asian boreo-nemoral ecotone (Koike *et al*. 1998).

Elevated CO_2 led to a large enhancement of the total root production of saplings grown in VA, compensating the negative effect of VA under aCO_2, however, there was no significant effect of eCO_2 on saplings grown in BF. Since the effects of eCO_2×VA on total root production were not significantly different from aCO_2×BF, the eCO_2-enhancement is not quantitatively noteworthy compared to other soils, but this enhancement will be practically significant for regions with VA (or similar soil). In such regions, a higher NPP may be noticed, meaning that the projected elevated CO_2 concentrations may have a different impact in regions with different soil fertility.

A large eCO_2-induced fine root biomass (with higher biomass per basal trunk area) was observed, and it was certainly high, for both soils. Unexpectedly, there were no individual or interactive soil effects, something pointing out that future research should consider more nutrient-starving soil. A morphogenesis of roots was evident in saplings exposed to eCO_2 and VA, through which saplings succeed to compensate the negative effects of VA by expanding fine roots at the expense of intermediate roots.

5.2 Effects of elevated O_3 levels on trees

5.2.1 Investigating the role of soil (larches as model trees)

I tested how two years of free-air O_3 enrichment influences a) the growth and photosynthetic pigmentation of Japanese larch in comparison to its hybrid when grown pot-free, and b) how O_3 impacts differ between two types of soil; fertile brown forest soil (BF) and BF mixed with infertile, immature volcanic ash plus pumice soil (VA).

I conclude that hybrid larch is no more susceptible to elevated O_3 levels than Japanese larch for the growth and health parameters tested. The present findings provide evidence for sustained heterosis in hybrid larch under elevated O_3 levels. These results differ from previous studies conducted with the same taxa and at the same experimental area but with potential experimental artifacts. It seems there is a potential for success in tree breeding for O_3 tolerance, and, thus, long-term studies and breeding programs which account for improvement to O_3-tolerance capacity are required.

In contrast to the tested hypothesis of a soil-dependent response of larch to O_3, there was no relevant difference except in Chl_a/Chl_b. It is recommended including tests with more infertile soil in future studies.

The complexity of using Chl_a/Chl_b as a measure of the O_3 impact on plants in *in vitro* studies is demonstrated. Attention should be paid to the numerous factors influencing Chl_a/Chl_b, and particularly site-specific soil, which may lead to erroneous conclusions. OD_{435}/OD_{415} is an ineffective biomarker of O_3 injury in these taxa. *TChl/TCar*, may be more accurate in assessing O_3 effects on the studied taxa as it is more sensitive to EOZ than Chl_a/Chl_b and consistently related to O_3 alone.

Diameter across stem height could be utilized as a low-cost assessment tool of O_3 stress in large trees especially in remote areas where access to equipment and facilities is prohibitive. Still, diameter across stem height and crown size should be taken into account by urban plant pathologists and local authorities in risk analysis (Lorenzini and Nali 2014).

It is recommended to avoid interpretation of O_3 impact on overall growth based on a decline in diameter increment at the lower third of the stem and to consider stem shape as well as vertical and horizontal growth when making comparative viability assessments of rehabilitation and plantation species.

5.2.2 Investigating the role of EDU (willow as a model tree)

I investigated if chronic soil-drenched EDU exposure can cause phytotoxicity and if high doses of EDU can act as N fertilizer after chronic treatment in *S. sachalinensis* as an experimental plant.

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

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

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

When EDU is used as a research tool, it is recommended to be applied as foliar spray instead of soil drench to plants of small size (small plant leaf area as in our case at the final application) for economy and for minimizing the error that could be caused due to the influence of soil since EDU should cycle from soil up to the leaves. However, for adult trees of larger size and with more foliage while more EDU is expected to be needed when applied both as foliar spray and

soil drench (Paoletti *et al*. 2011), much more time would be needed for foliar spray application and it could be practically prohibitive to tall trees, unless motorized vehicles are available, which increases the financial cost in turn.

5.3 EDU toxic and fertilization effects

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

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 O_3 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 O_3 impact. It is however recommended to avoid applications of EDU concentrations $\geq$ 600 mg L^{-1} in uncontrolled environments because of potential EDU toxicity to sensitive organisms (Agathokleous *et al*. 2016g).

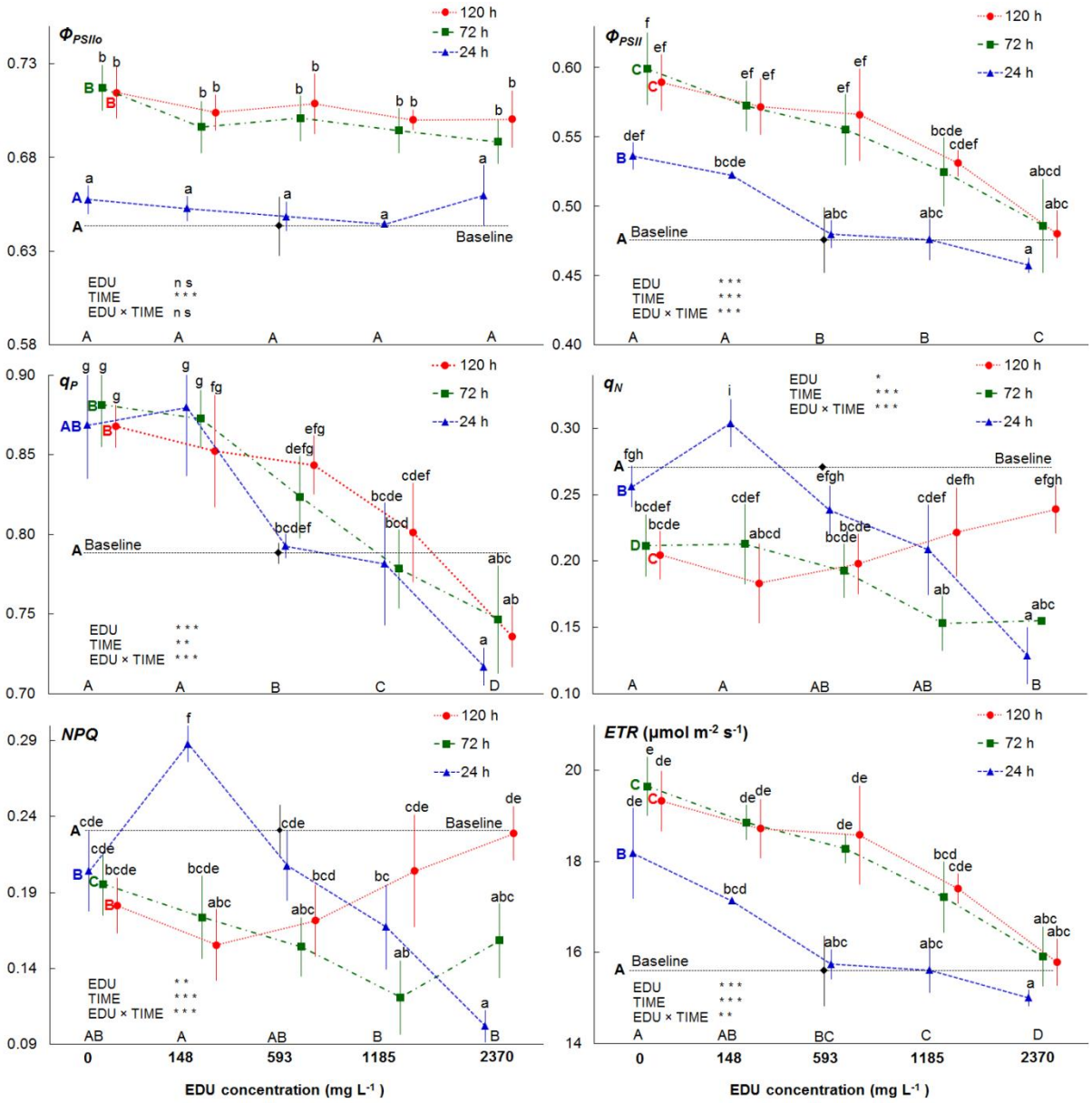

Fig 19 Effect of various EDU concentrations on fundamental photochemical ability of PSII, F_v/F_m, (Φ_{PSIIo}) (upper left), functional photochemical ability of PSII (Φ_{PSII}) (upper right), coefficients of photochemical quenching of photosystem II variable fluorescence (q_P) (middle left), non-photochemical quenching of fluorescence (q_N) (middle right), rate of non-photochemical quenching of fluorescence (*NPQ*) (lower left) and its apparent linear electron transport rate in photochemical chain (*ETR*) (lower right) of *Lemna minor* L. (duckweed) at 24, 72 and 120 h from tests initiation. One, two and three asterisks indicate statistically significant effect of GLMRs factors at $p < 0.050$, 0.010 and 0.001, respectively. Different lowercase letters above the confidence interval (CI) indicate statistically significant difference according to Tukey HSD posteriori tests. Uppercase letters at the left endpoint of the lines and above the EDU concentration show statistically significant difference among time and among EDU concentrations, respectively. Baseline value stands for the mean of each response variable 24 h before the initiation of the tests. Each data point (mean) is the product of three values ± 95% CI. Reprinted from Environmental Pollution, 213, Agathokleous E, Mouzaki-Paxinou A-C, Saitanis CJ, Paoletti E, Manning WJ, The first toxicological study of the antiozonant and research tool ethylene diurea (EDU) using a *Lemna minor* L. bioassay: Hints to its mode of action, 11, 2016, with permission from Elsevier.

At this point, it should be speculated that EDU protection of plants against O_3 injury may be upon a hormetic effect of EDU. A hormetic effect is characterized by stimulatory effect at low levels and inhibitory effects at high levels (Calabrese and Baldwin 2001; Calabrese *et al*. 2011) and describes biological plasticity (Calabrese 2014). Such stimulatory effects may hint to overcompensations in response to disruptions in homeostasis (Calabrese and Baldwin 2001; Calabrese *et al*. 2010). A toxicological bioassay of EDU along with the investigation of its mode of action was conducted by exposing *Lemna minor* L., as a model organism, to 0, 37, 74, 148, 296, 593, 1185, 1778, and 2370 mg EDU L^{-1} (Agathokleous *et al*. 2016g). This study provided evidence for the phenomenon of hormesis, early after exposing the colonies to EDU (Fig 19).

Hormesis observed in the rate of non-photochemical quenching of fluorescence (*NPQ*) which is is an indicator of heat dissipation by PSII antenna complexes (Muller *et al*. 2001; Calatayud *et al*. 2003; Calatayud *et al*. 2011); higher *NPQ* shows down-regulation of PSII (Moustaka *et al*. 2015). It was also observed in the non-photochemical quenching of fluorescence (q_N). The overcompensation that appeared in q_N and *NPQ* at 24 h in 148 mg L^{-1} EDU but not from 72 h and later may indicate a transient residence time (i.e. the duration of time that the xenobiotic-receptor complex persists) shorter than 72 h with conformational changes (i.e. lifetime of the xenobiotic-receptor complex is affected by dynamic processes) of the target - pharmacologic effect (Copeland 2011). That is, if the hormetic level is maintained for a long time, the effect can be negative. This stimulation coincides with the finding in the first gene expression experiment by Paoletti *et al*. (2014b) where EDU inhibited the O_3-induced production of reactive oxygen species (ROS) in French bean (*Phaseolus vulgaris* L.) plants within 24 h from the exposure with moderated H_2O_2 production, and protected gas exchange. It is suggested future EDU research to be directed towards the mechanism of EDU hormetic effects and its potential role in protecting plants against O_3 toxicity.

There has been an indication (Lee and Chen 1982) that EDU acted similarly to cytokinin hormone (e.g. phenylurea-type cytokinin). A cytokinin-like mode of action may retard the leaf senescence. EDU has been found to retard O_3-induced accelerated senescence, but not only upon EDU -i.e. under elevated O_3 (Paoletti *et al*. 2009; Singh *et al*. 2015; Agathokleous *et al*. 2016e). There is no further metabolism evidence supporting a cytokinin-like mode of action. Abscisic acid ($C_{15}H_{20}O_4$), a plant hormone which functions in several developmental processes of plants, is widely known for its potential to protect plants against stress (Cutler *et al*. 2010; Finkelstein 2013). There is currently no evidence for the metabolism of abscisic acid under EDU treatments. There may also be a potential relation of EDU with β-aminobutyricacid ($C_4H_9NO_2$) (BABA), possibly the most effective agent in priming plant defense system, which helps plants to cope with stress (Schwarzenbacher *et al*. 2014; Floryszak-Wieczorek *et al*. 2015; Thevenet *et al*. 2017). Similarly to abscisic acid, there is no study on BABA. It has been shown that EDU does not alter the emission of ethylene (C_2H_4) by plants and suggested that EDU protection of plants is not upon prevention of an interaction between ethylene and O_3 (Zilinskas *et al*. 1990); however further examinations are needed. Overall, future research should be directed towards elucidating potential role of cytokinin, abscicid acid, BABA and ethylene. EDU Dose-response experiments (with emphasis on low doses) in isolated environment, where the metabolism of EDU is assessed over time, has the perspectives for revealing the mode of action of EDU and O_3 in plants.

The most widely matter of concern in studies for EDU protection of plants against O_3 injury was the EDU concentration. However, present (Chapters 3 and 4) and prior (Paoletti *et al*. 2007) evidence suggests that EDU amount is important too. Thus, EDU should be applied at amounts calculated as a function of total plant leaf area, and, as EDU is applied repeatedly, should be varied over time, depending on the leaf area at a given time point. Regarding EDU

soil drench, the role of soil microorganisms in degrading EDU, which remains unknown, should be studied too, as EDU effectiveness may be affected.

5.4 Importance for the practice

Several morphological and physiological characteristics of representative trees native to northeast Asia, especially in Japan, have been revealed. The present findings may contribute to the silviculture techniques for breeding for abiotic stress tolerance, protecting against abiotic stress impacts, and developing tending methods in a future changing environment.

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ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude to my supervisor Prof. Takayoshi Koike, Hokkaido University, Japan, for sharing his helpful guidance, support and expertise throughout the course of my PhD work. His generous heart and hospitality made the difference. I appreciate all his contributions of time, ideas, and funding to make my Ph.D. experience productive and stimulating. The enthusiasm and passion he has for science could not leave me passive. He introduced me the "mysterious" world of Ecophysiology, in where I will select to walk "dark" pathways to enlighten shades.

I also appreciate Prof. William Manning of the Department of Plant, Soil and Insect Sciences, University of Massachusetts, Amherst, U.S.A., and Dr. Elena Paoletti of the Institute of Sustainable Plant Protection, National Council of Research, Florence, Italy. Their continuous guidance was exceptional. The inspiration by them was important at all the stages of my Ph.D. work.

The scientific world could be not so nice without the stimulation of Assoc. Prof. Costas Saitanis, Agricultural University of Athens. As a teacher, and later as a collaborator and friend, was continuously challenging me to get over myself day by day. Here, I should address him my many thanks for introducing me the Environmental science, Ecology, and Statistics, from early in my undergraduate days. Endless hours of challenge often kept us in the laboratory after midnight hours.

For this dissertation I would like also to thank my committee members of the School of Agriculture, Hokkaido University, Japan: Prof. Kazunori Iwabuchi, Prof. Ryusuke Hatano, Prof. Takashi Hirano, Prof. Yuzuo Sano, Assoc. Prof. Toshihiro Watanabe, Assoc. Prof. Masato Shibuya, lecturer Hideyuki Saito and Assis. Prof. Tomomichi Kato. Their time, interest, and helpful comments from a diverse scientific point of view are acknowledged.

I gratefully thank Prof. Edward J. Calabrese of the School of Public Health and Health Sciences, University of Massachusetts Amherst, USA, for warmly welcoming some proposed ideas of me and for critical comments on a new synthesis. I am also thankful to Assoc. Prof. Alexander Beaujean of the School of Education, Baylor University, TX, USA, for enlightenment on the bias correction which produces unbiased effect magnitude estimate, and to Dr. Yannis Georgalis of Faculty of Engineering, Hokkaido University, Japan, for discussions on problem-solving principles. Furthermore, I thank Dr. Evangelos Kontopantelis of the Centre for Health

Informatics, The University of Manchester, U.K., for feedback on some meta-statistical ideas whose development is in progress.

I appreciate the insights and feedback on some of my experiments by the following scientists (random order): (a) Prof. Rainer Matyssek, Ecophysiology of Plants, Technische Universität München, Germany, (b) Prof. Elina Oksanen, Department of Biology, University of Eastern Finland, Finland, (c) Prof. Kazuhiko Kobayashi, The University of Tokyo, Japan; (d) Prof. Heljä-Sisko Helmisaari, University of Helsinki, Finland; and (e) Prof. Zhaozhong Feng and Dr. Laiye Qu, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, P.R.China.

I also thank the following scientists for helpful discussions (random order): (a) Assoc. Prof. Makoto Watanabe (Environmental and Experimental Botany) of Tokyo University of Agriculture and Technology, Japan; (b) Dr. Matsumoto Naoyuki (Microbiology) of the Research Faculty of Agriculture, Hokkaido University, Japan; (c) Dr. Xiaona Wang (Environmental Soil Biology) of Agricultural University of Hebei, China; and (d) Dr. Yasutomo Hoshika (Gas Exchange Processes) of National Research Council, Italy.

I appreciate Mr. Tatsushiro Ueda of Dalton Co. (Hokkaido Branch) and Professsor Fuyuki Satoh (Hokkaido University) for continuous engineering assistance and financial support in the FACE system, respectively. Thanks are due to Mr. K. Ichikawa of Hokkaido University Forests for the operation of the bulldozer. Dr. Rhett Loban of the University of New South Wales, Australia, is acknowledged for proofreading some of my manuscripts, and Ms. Yuika Sugawara for making the drawing of some graphical abstracts of my articles.

My Ph.D. work could have never been carried out without the financial support by the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) and the Japan Society for the Promotion of Science (JSPS) (scholarship No: 140539). The studies included

in this Ph.D. work were funded by JSPS (Innovation research: 21114008, Grant-in-Aid Type B: 26292075, and Challenging Exploratory Research: 16K14932). The British Ecological Society is acknowledged for awarding me a Training & Travel Grant (reference No: TT16/1013). Furthermore, the Award of Sapporo Agriculture Alumni by the School of Agriculture, Hokkaido University, Japan, via the Japanese Society of Soil Science and Plant Nutrition, is acknowledged. Finally, financial support was provided by the Frank Laboratory of Neutron Physics, Joint Institute for Nuclear Research, Dubna, Russian Federation.

Evgenios Agathokleous
Hokkaido University
Sapporo, January 2017

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