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# *Ozone and climate change impacts on forest ecosystems*

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# SUMMARY

Tropospheric ozone ($O_3$) is a secondary pollutant not directly emitted from sources and it is formed, in presence of sunlight, by precursors like nitrogen oxide ($NO_x$) and volatile organic compounds (VOC). Tropospheric ozone is an important phytotoxic air pollutant and greenhouse gas and it is a serious concern for forest ecosystems. Since the industrialization, ambient ozone concentrations in the northern hemisphere are increasing at the annual rate of 0.5-2% affecting forest trees and crops. Ozone levels are damaging plants by causing leaf injuries, accelerating senescence, reducing leaf gas exchange and plants growth. There is also evidence that plants emit biogenic volatile organic compounds (BVOC) as a defense mechanism against biotic and abiotic stressors and have an important role in atmospheric chemistry.

Aim of the thesis was studying ozone and climate change impacts on forest ecosystems. The study focused on mechanisms of action of (1) ozone pollution on tree ecophysiology and (2) ozone and soil nutrients on BVOC emitted by vegetation. For the objective (1) two studies were conducted for investigating the impact of ozone exposure on stomatal dynamics, plant growth and wood traits. The experiments reported that ozone exposure slowed down the closure of stomata in trees and this was correlated to the ratio of stomatal ozone flux to net photosynthesis. The stomatal sluggishness depended on ozone flux and on the capacity for detoxification and repair. Long-term ambient ozone exposure reduced root biomass, stem diameter and changed biomass allocation but did not affect wood traits. Ozone effects on biomass partitioning to above ground organs depended on tree ontogenetic stage.

For the objective (2) one study was conducted for testing the effect of ozone and nitrogen on BVOC emission. The results pointed out that ozone exposure increased BVOC emissions and nitrogen had a compound-specific effect on BVOC. Both ozone and nitrogen changed BVOC emissions individually with no significant interactions.

This study demonstrated that the current ambient ozone levels and climatic factors are already having extensive impacts on vegetation. Therefore there is a need to coordinate effort for reducing the emissions of precursors of ozone pollution and find strategies for protecting forest ecosystems in a changing climate.

## RIASSUNTO

L’ozono ($O_3$) troposferico è un inquinante secondario che non viene emesso direttamente dalle fonti ma si forma, in presenza di luce solare, a partire da precursori, quali ossidi di azoto ($NO_x$) e composti organici volatili (COV). L’ozono troposferico è un importante inquinante fitotossico e gas serra che ha effetti negativi sugli ecosistemi forestali. Dall’inizio dell’industrializzazione le concentrazioni di ozono nell’emisfero settentrionale stanno aumentando di 0.5-2% l’anno causando danni alle foreste e alle colture. Le elevate concentrazioni di ozono causano lesioni alle foglie, accelerano la senescenza fogliare e riducono gli scambi gassosi e la crescita delle piante stesse. Inoltre è noto che le piante emettono composti organici volatili (COV) come meccanismo difensivo contro gli stress biotici e abiotici ed hanno un ruolo importante nella chimica atmosferica.

La tematica della tesi riguarda lo studio degli effetti dell’ozono e dei cambiamenti climatici sugli ecosistemi forestali. In particolar modo lo studio si è focalizzato sugli effetti e i meccanismi di azione (1) dell’ozono

sull’ecofisiologia delle piante; e (2) dell’ozono e dei nutrienti del suolo sull’emissione di composti organici volatili (COV) prodotti dalle piante. Per l’obiettivo (1), sono stati svolti due studi per verificare l’impatto dell’ozono sulla dinamica degli stomi, sulla biomassa delle piante e la qualità del legno. Gli studi hanno riportato che l’ozono rallenta le risposte degli stomi nelle piante e ciò dipende dal rapporto flusso stomatico di ozono / fotosintesi netta. L’inerzia stomatica dipende dal flusso di ozono e dalla capacità di disintossicazione e di riparazione da parte della pianta stessa. L’esposizione a lungo termine all’ozono riduce la biomassa radicale, il diametro del fusto e cambia l’allocazione della biomassa ma non influisce sulla qualità del legno. L’effetto dell’ozono sulla diversa ripartizione della biomassa in particolar modo delle parti aeree dipende dallo stadio di sviluppo della pianta. Per l’obiettivo (2), è stato condotto uno studio per esaminare l’effetto dell’ozono e dell’azoto sull’emissione di COV. I risultati hanno evidenziato che l’esposizione all’ozono aumenta l’emissione di COV mentre l’azoto ha un effetto composto specifico. Sia l’ozono che l’azoto singolarmente hanno un effetto proprio sulle emissioni di COV ma non hanno interazioni significative sulle emissioni.

Lo studio ha dimostrato che gli attuali livelli di ozono troposferico e i cambiamenti climatici hanno ampi effetti sulla vegetazione. Pertanto c’è sempre più bisogno di adottare nuove strategie per ridurre le emissioni dei precursori di ozono e per proteggere gli ecosistemi forestali in un clima che cambia.

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# LIST OF PUBLICATIONS

This thesis is based on data presented in the following articles, referred to the Roman numerals I-III.

I. Hoshika, Y., Carriero, G., Feng, Z., Zhang, Y., Paoletti, E., 2014. Determinants of stomatal sluggishness in ozone-exposed deciduous tree species. *Science of Total Environment* 481: 453-458.

II. Carriero, G., Emiliani, G., Giovannelli, A., Hoshika, Y., Manning, W.J., Traversi, M.L., Paoletti, E., 2015. Effects of long-term ambient ozone exposure on biomass and wood traits in poplar treated with ethylenediurea (EDU). *Environmental Pollution* 206: 575-581.

III. Carriero, G., Brunetti, C., Fares, S., Hayes, F., Hoshika, Y., Mills, G., Tattini, M., Paoletti, E. BVOC responses to nitrogen and ozone in silver birch. *Environmental Pollution* (Under revision).

# AUTHOR’S CONTRIBUTION

In paper **I** Giulia Carriero planned and made the experiment with her colleague Dr. Yasutomo Hoshika in collaboration with CAS Chinese Academy of Sciences in Beijing.

In paper **II** and **III** Giulia Carriero had the primary responsibility of the experiment, analyzing data and writing the paper. Paper **III** was done in collaboration with CEH Institute (Centre for Ecology and Hydrology) of Bangor (UK) thanks to a COST STSM Short-Term Scientific Mission FP1202 (Strengthening conservation: a key issue for adaptation of marginal/peripheral populations of forest trees to climate change in Europe MaP-FGR).

# 1. INTRODUCTION

## 1.1. Tropospheric ozone and climate change

Ozone (the triatomic oxygen molecule, $O_3$) occurs naturally in the atmosphere. Ozone is the main driver of the photochemical processes which lead to the recycling of most of the gases that are emitted into the atmosphere by natural processes and anthropogenic activities (Collins *et al.*, 2000). It is formed by oxygen ($O_2$) in the presence of ultraviolet radiation. In the stratosphere (at altitude of 15 to 35 km) the ozone layer acts as a natural filter absorbing most of the sun's damaging ultraviolet rays. This is called stratospheric ozone and is the upper layer of ozone. At ground level, ozone is a major component of photochemical smog and is a secondary air pollutant formed in the troposphere from photochemically-driven reactions. This is called tropospheric ozone. The majority of tropospheric ozone formation occurs when nitrogen oxides ($NO_x$), carbon monoxide (CO) and volatile organic compounds (VOC) react in the atmosphere in the presence of sunlight (Prinn, 2003; Monks, 2005). The chemical reactions involved in tropospheric ozone formation are a series of complex cycles in which CO and VOC are oxidized to water vapour and carbon dioxide. The oxidation begins with the reaction of CO with the hydroxyl radical ($^{\bullet}OH$) (Reaction 1). The radical intermediate formed by this, reacts rapidly with oxygen to give a peroxy radical $HO_2$ (Reaction 2).

$$^{\bullet}OH + CO \rightarrow CO_2 + {}^{\bullet}H \qquad (R1)$$

$$^{\bullet}H + O_2 \rightarrow HO_2 \qquad (R2)$$

It is the peroxyradical which initiates the majority of organic oxidations in the troposphere and thus is a controlling element in atmospheric chemistry (Atkinson 2000; Collins *et al.*, 2002). Peroxyl radicals react with NO to give $NO_2$ (Reaction 3) which is photolysed to give atomic oxygen (Reaction 4) and a molecule of ozone (Reaction 5).

$HO_2^{\bullet} + NO \rightarrow {}^{\bullet}OH + NO_2$ (R3)

$NO_2 + h\nu \rightarrow NO + O\cdot$

(R4)

$O_2 + O\cdot + M \rightarrow O_3 + M$ (R5)

Photo dissociation of $NO_2$ by sunlight is the only significant anthropogenic source of ozone in photochemical smog. The M in the Reaction 5 is any third molecule which is needed to stabilize the excited intermediated formed on the addition of $O\cdot$ to $O_2$. Then in the presence of ozone and nitrogen oxides, the nitrate radical ($NO_3$) is formed (Reaction 6).

$NO + O_3 \rightarrow NO_2 + O_2$ (R6)

High presence of atmospheric NO favors the rate of removal of ozone, but the presence of VOC can increase the ratio of $NO_2$/NO by reacting with hydrossilic radicals ($HO^{\bullet}$) and forming other radical species (alchiperoxyradical, $RO^{\bullet}$, and hydroperoxyradical, $HOO^{\bullet}$).

$NO + ROO^{\bullet} \rightarrow RO^{\bullet} + NO_2$ (R7)

$NO + HOO^{\bullet} \rightarrow HO^{\bullet} + NO_2$ (R8)

This cycle involving $HO_x$ and $NO_x$ is terminated by the reaction of OH with $NO_2$ to form nitric acid or by the reaction of peroxy radicals with each other to form peroxides. The chemistry involving VOC is much more complex but the same reaction of peroxy radicals oxidizing NO to $NO_2$ is the critical step leading to ozone formation.

Increased global tropospheric ozone levels leads to increase OH production thus influencing the retention time of many gases in the atmosphere with implications on a more global scale (Prinn 2003). Ozone is also involved in nucleation events when reacting with VOC leading to aerosol and particle formation (Bonn and Moortgat 2003). The increasing emissions of precursors substances coupled with a rise in air temperature, results in an elevation of the ground-level ozone concentrations all over the world (The Royal Society, 2008) which are exceeding limits set to protect human health and vegetation (Sicard *et al.*, 2013; Paoletti *et al*., 2014). Ground levels ozone concentrations have increased continuously over the three decades before 2000, at a rate of 0.5-2% per year (Vingarzan 2004). Although a stagnation or slight decrease over Europe and North America was observed since 2000, ozone concentration is still increasing in Asia and other world parts (Oltmans *et al.*, 2013). In Europe, exposure of urban population to ozone has been increasing in the last decade (EEA 2013). Ozone pollution is pronounced in regions with strong photochemical activity, such as the Mediterranean basin (Paoletti 2006). The climate of the Mediterranean is characterized by seasonal changes between hot summers with high pressure, low winds and strong solar radiation. These conditions favor massive photochemical production of ozone with development of mesoscale processes and recirculation within air masses (Millàn *et al.*, 2000). Due to its central position in the Mediterranean, Italy may be considered as an hot-spot for ozone and representative of ozone impacts on Mediterranean vegetation.

## 1.2. Ozone effects on forest functionality

In addition to health impact (described in WHO 2010), ozone has the most widespread negative impact on vegetation than any other air pollutant (Ashmore, 2005). Forests are one of the most important global sinks for carbon (Houghton, 2003; Sitch *et al.*, 2007). Without this sink, which offsets a significant proportion of current global $CO_2$ emissions, the rate of increase in atmospheric $CO_2$ would be greater (Canadell *et al.*, 2007). Much has been written about the effects of ambient ozone on crops and forest trees (Fumagalli *et al.*, 2001; Matyssek and Sandermann, 2003; Manning 2005; Felzer *et al.*, 2007; Wittig *et al.*, 2009; Mills *et al.*, 2011) but knowledge is still imperfect. The phytotoxic nature of ozone may cause adverse effects on physiological and biochemical processes in tree species (Karnosky *et al.*, 2003; Matyssek and Sandermann, 2003; Ashmore, 2005; Paoletti, 2007; Bytnerowicz *et al.*, 2007; Serengil *et al.*, 2011). Ozone induces morphological and chemical changes in the leaves which adversely affect plant growth (Karnosky *et al.*, 2007; Matyssek *et al.*, 2007). Ozone may cause growth reduction, shift in biomass allocation (Hoshika *et al.*, 2013), impairment of physiological traits such as leaf gas exchange (Fares *et al.*, 2013), and visible foliar injury, i.e., interveinal dark stippling (Paoletti, 2007). Physiological studies suggest that chronic elevation of ozone decreases productivity primarily by lowering photosynthesis (Ainsworth *et al.*, 2012). Indeed ozone is a strong oxidant, and significant damage to photosynthesis occurs when $O_3$ enters the leaf through the stomata. The deposition of ozone into vegetation through stomata is an important sink for tropospheric ozone (Fowler *et al.*, 2001). Stomata are the primary route by which ozone enters leaves and this generates other reactive oxygen species and causes oxidative stress, which in turn decreases photosynthesis, plant growth, and biomass accumulation (Wittig *et al.*, 2007, 2009). This leads to a progressive loss of ribulose 1.5-bisphosphate carboxylase/oxygenase (Rubisco) activity and in a decline in light-saturated rate of leaf $CO_2$ uptake (Wittig *et al.*, 2007). The stomatal ozone sink is highly variable due to its dependence on environmental variables such as light, temperature and water availability in the soil-plant-atmosphere system, and is considered to be the main responsible for ozone injuries to plants (UNECE, 2010). If ozone causes a large-scale decrease in stomatal conductance in forests, there are major implications for regional hydrology, surface temperatures and the global climate system (Sellers *et al.* 1996). In general, short-term exposure to ozone stimulates a rapid reduction in stomatal aperture, while longer-term exposure causes stomatal responses to become sluggish (Hoshika *et al.*, 2014). These sluggish stomatal responses are also typical of leaves undergoing ageing processes and losing their ability to regulate water losses (Paoletti, 2005). Chronic exposures to ozone can damage the cuticle of plants, negatively modifying the protective action of cuticle. For instance, wax denaturation by ozone may increase cuticular water loss, increasing leaf permeability (Zhang *et al.*, 2011) and thus negatively affecting the water balance (Shreuder *et al.* 2001). Ozone indirectly affects the denaturation of membrane lipids (Pell *et al.* 1997) rapidly reacting with all compounds in the apoplast and in the gas phase, and generating reactive oxygen species (ROS) such as $O_2$, OH, and $HO_2$ (Apel and Hirt, 2004). The large amount of reactive oxygen species formed by ozone, rapidly leads to photo inhibition (Guidi *et al.*, 2002). ROS are strong oxidants and act as signal molecules in plants. ROS start hypersensitive responses, including the activation of ethylene, salicylic acid and jasmonate, and lead to programmed cell death in response to ozone (Pellinen *et al*., 1999; Kangasjarvi *et al*., 2005). Exposure to acute tropospheric ozone levels leads to visible injuries (Innes *et al.*, 2001; Paoletti *et al.*, 2009). Visible foliar $O_3$ injury reduces photosynthetic capacity and stomatal conductance in leaves (Paoletti *et al*., 2004; Hoshika *et al.*, 2012). Visible foliar $O_3$ injury has been investigated in many trees and herbaceous species, and partly validated under controlled conditions (e.g. Zhang *et al.*, 2000; Novak *et al*., 2005; Paoletti *et al.*, 2009). The most common method of assessing ozone injury involves visual estimation of the percent leaf surface area that is necrotic and chlorotic (Innes *et al*., 2001). Indeed visible symptoms induced by $O_3$ are generally recognized as visible alterations of leaf surface, including chlorosis (yellowing due to the chlorophyll breakdown, often distributed in spots over the leaf), bronzing (red-brown pigmentation caused by phenylpropanoid accumulation), bleaching (small unpigmented necrotic spots), flecking (small brown necrotic areas fading to grey or white), stippling (small punctuate spots, white, black or red in colour) and tipburn (dying tips, first reddish, later turning brown) (Innes *et al*., 2001).

Ozone is a major secondary pollutant and greenhouse gas which has an adverse effect on human health, crop yields, forests and food production (Ashmore, 2005; Paoletti, 2007; EPA, 2013). The surface ozone concentration has risen from less than 10 ppb prior to the industrial revolution to a day-time mean concentration of approximately 40 ppb over much of the northern temperate zone (Vingarzan, 2004). If current global emission trends continue, surface ozone is projected to rise a further 50% over this century with larger increases in many locations including Northern hemisphere forests (Vingarzan, 2004; EEA, 2013). Increasing atmospheric ozone will negatively impact plant production, reducing the ability of ecosystems to sequester carbon, and thus indirectly feedback on atmospheric $CO_2$ enhancing climate change (Sitch *et al.*, 2007; Collins *et al.*, 2010). In response to this problem, several working groups belonging to UNECE (United Nations Economic Commission for Europe) and CLRTAP (Convention on Long Range Transboundary Air Pollution) were set since 1983, for finding solutions for air pollution and implementing air quality. The International Cooperative Programme (ICP) was established in 1987 with the aim to assess the impacts of air pollutants on crops, in particularly ozone, but in later year also on (semi-) natural vegetation. An intensive monitoring activity of ozone damage was started, based on many other parameters such as the micrometeorology of the experimental sites, and the deposition of the pollutants on leaves. In Italy, the European Commission decisions within the ICP programme were adopted through different national programmes. In particular, in 1995 the CONECOFOR (Integrated National Programme for the Control of Forest Ecosystems) was started, with 31 permanent monitoring sites representing the most representative ecosystems where the directives of the ICP are applied. Regarding ozone pollution, an important aim of the ICP vegetation is to develop and validate ozone-flux effect relationship for vegetation. Different exposure indices have been developed (Kärenlampy and Skarby 1996; Fuhrer *et al.*, 1997) such as AOT40 expressed as the accumulated ozone over a threshold of 40 ppb, during daylight hours in which the global radiation is equal or above 50 $Wm^{-2}$. This index is widely employed in Europe and is based on the time-integrated atmospheric concentration of the pollutant (Equation 1).

$$\mathrm{AOT40} = \sum_{\substack{[O_3] > 40 \\ \mathrm{Rad} > 50\ Wm^{-2}}} \left[ [O_3] - 40 \right] \Delta t \qquad (1)$$

The AOT40 index assumes that the harmful ozone concentrations for vegetation are only those exceeding a threshold of 40 ppb. However plants absorb ozone almost exclusively through the stomata, while other uptake pathways are negligible (Kerstiens *et al.*, 1992). For this reason, the AOT40 index is calculated only for daylight hours, when stomata are open, and precisely when the solar radiation is above $50 W\mathrm{m}^{-2}$ (Kärenlampi and Skärbi, 1996). However AOT40 is not fully reliable for impact predictions since it does not take into account the amount of ozone directly absorbed by vegetation. Ozone damage to vegetation occurs through ozone uptake by plant stomata and is not necessarily related to ozone concentrations (Emberson *et al.*, 2000). Therefore, the scientific community has suggested a critical level of accumulated stomatal ozone flux (Wieser and Tausz, 2006; Karlsson *et al.*, 2007) which better indicates the real amount of ozone taken up by plants through stomata. This accumulated stomatal ozone flux index ($AF_{\mathrm{st}}$ Y), where $F_{\mathrm{st}}$ represents the stomatal flux of ozone above a flux threshold (Y), derives as the sum over time of the differences between hourly mean values of $F_{\mathrm{st}}$ and Y for the period when $F_{\mathrm{st}}$ exceeds Y. Also the climatic conditions strongly affect stomatal conductance, ozone uptake and the biological response of vegetation to the pollutant (Paoletti, 2006). The ability of stomata to serve as a sink for atmospheric gases, quantified by stomatal conductance, is, in turn, mainly controlled by water availability and by other environmental factors like air water vapor pressure deficit, solar radiation, wind speed, intensity of turbulence (Emberson *et al.*, 2000). Biomonitoring provides an approach to document direct foliar injury irrespective of direct measure of ozone uptake. Ozone concentrations are predicted to continue to rise across most of the world over the coming decades (Royal Society, 2008) and the ozone impact on vegetation will worsen. There is an urgent global need to reduce the emissions of ozone pollution precursors for favoring food security, improving human health and reducing global warming.

## 1.3. Biogenic Volatile Organic Compound emission as a defensive mechanism against biotic and abiotic stressors

The term Volatile Organic Compound (VOC) encompasses all organics (e.g. hydrocarbons, aldehydes, alcohols, nitrogen and sulfur-containing organics etc.) emitted from anthropogenic and biogenic sources. Plants, in particular trees, emit a considerable amount of different compounds known as biogenic volatile organic compound (BVOC) (Lerdau *et al.,* 1997; Guenther *et al*., 2012). Most of BVOC emitted by plants belong to the chemical class of isoprenoids or terpenes. The main volatile isoprenoids are monoterpenes, sesquiterpenes, and homoterpenes (Kesselmeier and Staudt, 1999). Isoprene and monoterpenes belong to the biochemical class of isoprenoids whose carbon skeletons are composed of characteristic $C_5$ units (McGarvey and Croteau, 1995). According to the number of $C_5$ units, they are subdivided into hemiterpenes ($C_5$ e.g. isoprene), monoterpenes ($C_{10}$ e.g. α-pinene, menthol, camphor), sesquiterpenes ($C_{15}$ e.g. β-caryophyllene, abscisic acid), diterpenes ($C_{20}$, e.g. gibberellins), triterpenes ($C_{30}$ e.g. sterols), tetraterpenes ($C_{40}$, e.g. carotenoids), prenols and polyterpenes ($>C_{45,}$ e.g. plastochinone, ubichinone). However, of importance for atmospheric chemistry are mainly the more volatile compounds isoprene, (2-methyl-1,3-butadiene, ($C_5H_8$)), the simplest and most volatile isoprenoid, and the backbone molecule of all isoprenoids, monoterpenes and sesquiterpenes. Isoprene is the most abundant compound emitted by plants; its annual emission is about half of the total BVOC emissions, and is comparable to the total emission of methane from all sources (Guenther *et al.*, 2006; Sharkey *et al.*, 2008). Monoterpenes are emitted especially by oak species (Loreto, 2002) in the regions with Mediterranean climate. The most abundant monoterpenes emitted are linalool, α- and β-pinene, limonene, and cis and trans β-ocimene. These compounds are emitted by a large class of tree species. Sesquiterpenes are very important in the chemistry of atmosphere but difficult to measure for their fast reaction in the atmosphere. Another class of BVOC is represented by the oxygenated volatile organic compound (OVOC). OVOC include carbonyls (aldehydes and ketones), alcohols and carboxylic acids. Methanol is one of the most abundant compounds among the emitted OVOC, especially in developing leaves, being an indicator of leaf expansion or emitted in response to injury (Nemecek-Marshall *et al.*, 1995). The pathway of isoprene and monoterpenes formation is strictly dependent on photosynthesis (Loreto *et al.*, 1996). All isoprenoids are synthesized via a common $C_5$ precursor, the isopentenyl pyrophosphate (IPP), called ‘active isoprene’. Isoprene is made through the methyl-erythritol 4-phosphate pathway (MEP pathway, also called the non-mevalonate pathway) in the chloroplast of plants. One of the two end products of MEP pathway, dimethylallyl pyrophosphate (DMAPP), is catalyzed by the enzyme isoprene synthase to form isoprene (Fig.**1**). Inhibitors that block the MEP pathway, such as fosmidomycin, block isoprene formation. Isoprene emission increases with temperature and this has led to the hypothesis that isoprene may protect plants against heat stress (Singsaas *et al.*, 1997; Sharkey *et al.*, 2005; Loreto *et al.*, 2006). Monoterpenes are a class of terpenes that consist of two isoprene units and have the molecular formula $C_{10}H_{16}$. By adding another IPP unit to DMAPP, the monoterpene geranylpyrophosphate (GPP) is formed, which is the starting unit for other monoterpenes, as well as the origin for a further addition of IPP units to produce sesqui- and diterpene species. The production of monoterpenes takes place within the plastids whereas sesquiterpenes are synthesized within the cytosol (Soler *et al*., 1992; McCaskill and Croteau, 1995) (Fig.**1**). OVOC are important products of the photo-oxidation of hydrocarbons and other organic species in the atmosphere. OVOC are formed through processes which are not as directly related to photosynthesis. Methanol is formed from the demethylation of cell wall pectins (Galbally and Kirstine, 2002). Large but transient releases of methanol in the atmosphere may be associated to wounding (Karl *et al.*, 2001), developing leaves (Nemecek-Marshall *et al*., 1995), senescing leaves because of irreversible decomposition of cell walls (Fall, 2003). A whole class of C-6 compounds, including ((Z)-3-hexenal, (E)-3-hexenal, (E)-2-hexenal, (Z)-3-hexenol and (E)-3-hexenol) are formed from the breakdown of membrane lipids (Hatanaka 1993; Matsui, 2006; Holopainen and Gershenzon, 2010). The emission of C-6 compounds is a consequence of insect feeding, high temperature (Loreto *et al.* 2006), or ozone exposure (Heiden *et al*., 2003). Beauchamp *et al.* (2005) also reported that C-6 compounds emission is related to the ozone damage to membranes.

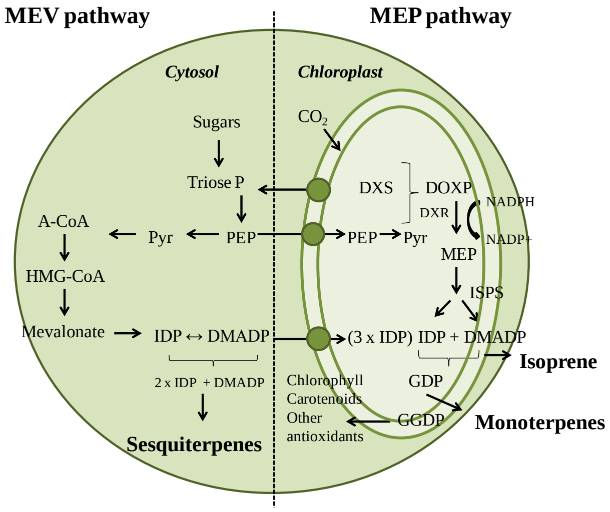

**Fig.1.** Scheme of the isoprenoids methabolic pathway in plants. On the left, the MEV (mevalonate) pathway in the cytosol describes the formation of sesquiterpenes; on the right, the MEP (non-mevalonate) pathway in the chloroplast describes isoprene and monoterpene formation (figure of *Giulia Carriero*).

A great amount of BVOC release in the atmosphere has a strong relevance for plant growth, reproduction and defense (Kesselmeier and Staudt, 1999) and atmospheric chemistry such as ozone and aerosol formation (Peñuelas and Staudt, 2010). BVOC play an important role in the oxidative chemistry of the atmosphere, consuming hydroxyl radicals and consequently the lifetime of radiatively active trace gases like methane (Brasseur and Chatfield, 1991). BVOC react in the troposphere and constitutes a significant input of precursors of photochemical oxidants (Guenther *et al.*, 1995), reactive carbon into the atmosphere (Peñuelas and Llusià, 2003) and aerosol formation (Peñuelas and Staudt, 2010). The importance of BVOC emission into the atmosphere is related to their reactivity with some of the compounds released from anthropogenic sources, especially nitrogen oxides ($NO_x$) which may contribute to the formation of ozone (Guenther *et al.*, 2006). A number of hypotheses exist regarding the role of constitutive BVOC and many of these highlight the role of these compounds in enhancing plant tolerance to certain abiotic stresses. A large number of stress factors affect the emission of BVOC. Heat stress (Velikova *et al.*, 2005b), drought (Sharkey and Loreto, 1993; Peñuelas *et al.*, 2009), salinity (Teuber *et al.*, 2008) and ozone (Loreto *et al.*, 2004) lead to an increase of BVOC emission. All studies on the emission of isoprene and monoterpenes show clear temperature dependence. Additionally under severe drought, emissions decrease and increase after re-watering during stress recovery (Kesselmeier and Staudt, 1999; Wu *et al.*, 2015). The effect of nitrogen availability on isoprenoid emission is not well understood: a positive correlation between emission capacity and nitrogen was found (Litvak *et al.*, 1996) although not always (Lerdau *et al.*, 1995). General studies reported effects of ozone and other air pollutants on the plant contents of secondary compounds (Heller *et al.*, 1990; Kainulainen *et al.*, 1995; 2000; Loreto *et al.*, 2006). Has been reported that BVOC emission change under ozone fumigation depending on plant species (Heiden *et al.*, 1999), season (Peñuelas *et al.*, 1999) and BVOC species (Hewitt *et al.*, 1994). Sustained stress reduces constitutive BVOC and only the prolonged fumigation with ozone can lead to an increase of emissions (Llusià *et al.*, 2002; Loreto *et al.*, 2004b; Velikova *et al.*, 2005a). These changes in emission can lead to a consequence for the biosphere structure and functioning and can disturb biosphere feedback on atmospheric chemistry and climate. Research in the role of BVOC in stress tolerance is required for assessing plant potential in maintaining fitness and survival. This research will lead to improve BVOC emission models for understanding BVOC impact on atmospheric chemistry and climate.

## 1.4. Objective of the study and overview of the experiments

Numerous studies concerning the impacts of ozone concentrations on plants have been conducted but knowledge about ozone effects on gas exchange dynamics, carbon allocation and BVOC emission both in the field and in controlled conditions, is still incomplete. Also there is a need to detect real plant damages in the field induced by ozone and other climatic variables associated to climate change. Aim of my research was studying the effect of tropospheric ozone and climate change on trees. My study focused on effects and mechanisms of action (1) of ozone pollution on forest ecophysiology and ecology; and (2) of soil nitrogen and ozone on BVOC emissions.

This thesis includes three chapters referred by Roman numerals (I-III) related to the experiments conducted during my PhD study. I summarized below the rationale, methods and main results for each of the original publications. A general discussion of the results related to the two aims of this PhD is then presented. The original publications are also appended to this thesis.

### *1.4.1. Effects and mechanisms of action of ozone pollution on forest ecophysiology*

The primary experiment of the first aim of the thesis (paper **I**) was studying the dynamic variation of stomatal conductance in response to different ozone conditions. The experiment took place in an open field site in China where we examined chronic ozone impacts on stomatal dynamics in common tree Chinese species (*Ailanthus altissima* Mill., *Fraxinus chinensis* Roxb. and *Platanus orientalis* L.). Seedlings of these species were grown in open-top chambers and exposed to three levels of ozone (NF: non-filtered ambient air, NF+40: NF supplied with 40 nmol mol$^{-1}$ ozone, NF+80: NF supplied with 80 nmol mol$^{-1}$ ozone) during daytime. We measured leaf gas exchange with a portable infra-red gas-analyzer (Li-cor Instruments, Model 6400). When the stomatal conductance reached the steady state, the leaf petiole was cut just outside the leaf cuvette to assess dynamic variations of stomatal conductance. Ozone exposure slowed the closing of stomata after leaf cutting. The ozone impairment on stomatal response was better explained by on index incorporating the amount of ozone entering a leaf and the capacity of biochemical detoxification and repair. These results were very helpful for understanding the stomatal response to ozone pollution of different Asian plant species.

A second experiment (paper **II**) was carried out for determining the effects of long-term ambient ozone exposure on tree biomass. The study was carried out in a Mediterranean field site located in central Italy (Antella: 43°44′ N, 11°16′ E, 50 m a.s.l., 15.8°C as mean annual temperature). Cuttings of an ozone-sensitive clone (Oxford, *Populus maximoviczii* Henry × *berolinensis* Dippel) were treated differently with and without ethylenediurea (EDU), an antioxidant used for protecting leaves from ozone injury. EDU was used as a ‘control’ for determining ambient ozone effects on field grown plants. Ozone concentrations were recorded at canopy height by an annually-calibrated ozone monitor (Mod. 202, 2B Technologies, Boulder CO, USA). The plants were exposed to ambient ozone and treated with EDU or water for six consecutive years (from 2008 to 2013). After six years, plants were harvested for determining ambient ozone effect on biomass and wood traits of poplar trees. The results showed that ozone reduced below ground biomass, decreased stem diameter and increased moisture content of the wood along the stem. Long term ambient ozone exposure influenced biomass allocation that was related to tree ontogenetic stage. This was the first experiment that highlighted the long-term effect of ambient ozone exposure on a tree species.

### *1.4.2. Effects and mechanisms of action of ozone and soil nutrients on BVOC emitted by plants*

The experiment of the second aim of the thesis (paper **III**) was carried out in a rural site in Bangor (UK) in collaboration with CEH Institute (Centre for Ecology and Hydrology). The site was provided with solardomes, hemispherical glasshouses, in which three years old saplings of silver birch were exposed to ozone and potted with soil nitrogen. The main purpose of this research was exploring the effects of realistic exposure to ozone and soil nitrogen, single and in combination, on BVOC emitted by silver birch. Mean daily ozone levels during the sampling period were: low, 36 ± 0.2 ppb; medium, 49 ± 1.1 ppb; high, 70 ± 1.8 ppb. The levels of nitrogen applied in the potting soil were: low, 10 kg $ha^{-1}$ $yr^{-1}$; medium, 30 kg $ha^{-1}$ $yr^{-1}$; high, 70 kg $ha^{-1}$ $yr^{-1}$. BVOC sampling was carried out from 19 – 26 July 2013. A modified portable gas exchange system (LI6400, Li-Cor, Lincoln, USA) was used to trap BVOC in silico-steel cartridge packed with 200 mg of tenax (Superlco, PA, USA). Ozone exposure increased BVOC emission while nitrogen fertilization showed compound-specific effect on BVOC. There was no interaction between ozone and nitrogen on BVOC emission. The results showed that ozone and nitrogen had the potential to affect BVOC emission and helped understanding their effects on atmospheric chemistry and plant ecology.

# *I. Determinants of stomatal sluggishness in ozone-exposed deciduous tree species*

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# Determinants of stomatal sluggishness in ozone-exposed deciduous tree species

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## HIGHLIGHTS

- Our knowledge of ozone ($O_3$) effects on dynamic stomatal response is still limited.
- Determinants of $O_3$-induced stomatal sluggishness were examined in deciduous tree species in open-top chambers.
- Ozone exposure slowed closing of stomata after leaf cutting.
- Stomatal sluggishness was well explained by stomatal $O_3$ flux per net photosynthesis.
- Stomatal sluggishness depended both on ozone flux and on the capacity for detoxification or repair.

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## ABSTRACT

Our knowledge of ozone effects on dynamic stomatal response is still limited, especially in Asian tree species. We thus examined ozone effects on steady-state leaf gas exchange and stomatal dynamics in three common tree species of China (*Ailanthus altissima*, *Fraxinus chinensis* and *Platanus orientalis*). Seedlings were grown and were exposed to three levels of ozone in open-top chambers (42, 69, 100 nmol mol$^{-1}$ daylight average, from 09:00 to 18:00). At steady-state, ozone exposure induced an uncoupling of photosynthesis and stomatal conductance, as the former decreased while the latter did not. Dynamic stomatal response was investigated by cutting the leaf petiole after a steady-state stomatal conductance was reached. Ozone exposure increased stomatal sluggishness, i.e., slowed stomatal response after leaf cutting, in the following order of sensitivity, *F. chinensis* > *A. altissima* > *P. orientalis*. A restriction of stomatal ozone flux reduced the ozone-induced sluggishness in *P. orientalis*. The ozone-induced impairment of stomatal control was better explained by stomatal ozone flux per net photosynthesis rather than by stomatal ozone flux only. This suggests that ozone injury to stomatal control depends both on the amount of ozone entering a leaf and on the capacity for biochemical detoxification or repair. Leaf mass per area and the density of stomata did not affect stomatal sluggishness.

## 1. Introduction

Tropospheric ozone ($O_3$) is recognized as a significant phytotoxic air pollutant and greenhouse gas (Bytnerowicz et al., 2007; Serengil et al., 2011). Ozone concentrations have been increasing in the northern hemisphere since the pre-industrial age (Akimoto, 2003; Vingarzan, 2004). Especially in East Asian countries, further increases in $O_3$ concentrations are predicted throughout this century because of rapid economic growth (Ohara et al., 2007; Yamaji et al., 2008).

The phytotoxic nature of $O_3$ may cause adverse effects on physiological and biochemical processes in tree species (Karnosky et al., 2003; Matyssek and Sandermann, 2003; Ashmore, 2005; Paoletti, 2007). There is still little information on effects of $O_3$ on native plant species of Asia (Royal Society, 2008). Many studies on European and North American species reported that $O_3$ may reduce carbon assimilation, and limit the growth of trees (e.g., Wittig et al., 2007, 2009). On the other hand, the effect of $O_3$ on stomatal conductance is not straightforward (Mansfield, 1998; Paoletti and Grulke, 2005). Ozone has been reported to induce stomatal closure (Wittig et al., 2007). However, slower or less efficient stomatal control may occur, especially a weaker ability to close stomata, referred to as "$O_3$-induced stomatal sluggishness" (Paoletti, 2005; Mills et al., 2009; Paoletti et al., 2009; Paoletti and Grulke, 2010; Hoshika et al., 2012a, 2013a,b; Dumont et al., 2013). Sluggishness may occur because $O_3$ reduces stomatal sensitivity to abscisic acid (ABA) (Mills et al., 2009). This loss of stomatal response to ABA is related to $O_3$-induced ethylene emission (Wilkinson and Davies, 2010). Although our knowledge of the mechanism is still

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limited, such a reduced stomatal control may impair an efficient water use for plants (Sun et al., 2012).

Stomatal $O_3$ flux is a crucial factor for the assessment of $O_3$ effects, because stomata are the principal interface for entry of $O_3$ into a leaf (Omasa et al., 2002; Karlsson et al., 2007; Mills et al., 2010). Stomatal response to environmental stimuli may be related to leaf anatomical traits such as stomatal density (e.g., Drake et al., 2013). Small stomata and their general association with high density of stomata provide the capacity for rapid response in the stomatal conductance of a leaf (Aasamaa et al., 2002; Hetherington and Woodward, 2003; Drake et al., 2013). This implies that fast response of stomata in leaves with high stomatal density and/or lower stomatal conductance during $O_3$ exposure may result in less diffusion of $O_3$ into a leaf, and may lead to a decrease in $O_3$-induced injury (Pääkkönen et al., 1995).

Foliar $O_3$ injury may also depend on the available resource for repair or biochemical $O_3$ detoxification of a leaf (e.g., Tausz et al., 2007; Paoletti et al., 2008). Wieser et al. (2002) suggested that antioxidative capacity increased with increasing leaf mass per area (LMA). Inherent LMA may thus be roughly related to sensitivity to $O_3$ stress (Bussotti, 2008; Zhang et al., 2012). Also Musselman and Minnick (2000) suggested that plant tolerance to $O_3$ stress may depend on its photosynthetic capacity because detoxification and repair require energy (Noctor and Foyer, 1998). The sensitivity to foliar $O_3$ injury may thus be explained by the ratio of stomatal $O_3$ flux to net photosynthesis (Fredericksen et al., 1996; Kolb and Matyssek, 2001), indicating a balance between $O_3$ exposure of mesophyll cells and availability of photosynthates for repair or detoxification.

In this study, we examined $O_3$ effects on steady-state leaf gas exchange and dynamic stomatal response under severe water stress imposed by cutting a leaf in three tree species that are common in China. The objective of the study was to test whether the degree of $O_3$-induced injury to stomatal control was related to stomatal density, LMA, stomatal $O_3$ flux or the ratio of $O_3$ flux to net photosynthesis.

## 2. Materials and methods

### 2.1. Plant materials

We used one-year-old seedlings of *Ailanthus altissima* (Mill.) Swingle, *Fraxinus chinensis* Roxb. and *Platanus orientalis* L. Before bud burst, bare rooted seedlings were planted in 20 L circular plastic pots on 31 March, 2013 and grown at ambient field condition (outdoors). Pots were filled with native light loamy soil. Seedlings with similar height and basal diameter were selected for this study and pre-adapted to open-top chamber conditions for 10 days before $O_3$ fumigation. All plants were watered at field capacity at 1–3 day intervals to avoid water stress.

### 2.2. $O_3$ treatments

The experiment was carried out in three open-top chambers (OTC, octagonal base, 12.5 $m^2$ of growth space and 3.0 m of height) in Changping (40°19′ N, 116°13′ E), Northwest Beijing with warm temperate and semi-humid continental climate. The annual mean temperature is 11.8 °C, and total precipitation is 550 mm. All seedlings were exposed to the following treatments for three months with a daily maximum of 9 h (from 09:00 to 18:00) when there was no rain, fog, mist or dew: nonfiltered ambient air (NF, averaged $O_3$ concentration of 42 nmol $mol^{-1}$ from 09:00 to 18:00), NF supplied with 40 nmol $mol^{-1}$ of $O_3$ (NF + 40, averaged $O_3$ concentration of 69 nmol $mol^{-1}$ from 09:00 to 18:00), and NF supplied with 80 nmol $mol^{-1}$ of $O_3$ (NF + 80, averaged $O_3$ concentration of 100 nmol $mol^{-1}$ from 09:00 to 18:00). Ozone was generated from pure oxygen by an $O_3$ generator (HY003, Chuangcheng Co., Jinan, China). The concentrations of $O_3$ in the OTCs were continuously monitored at approximately 10 cm above the plant canopy using a UV absorption $O_3$ analyzer (Model 49*i*-Thermo, USA). The monitors were calibrated by a 49iPS calibrator (Thermo Scientific, USA) before the experiment and once per month during the experiment. The chambers were turned off in the evening and the door was opened to allow for dew formation. Four or five potted plants per each tree species were set in each OTC. There was no replication of $O_3$ treatments. In order to eliminate the positional and chamber effects (Potvin and Tardif, 1988), the plant positions were changed every week within each OTC, and all seedlings were switched between chambers every month, according to Feng et al. (2008, 2011).

### 2.3. Measurements of leaf traits and gas exchange

Measurements of leaf gas exchange were carried out with a portable infra-red gas-analyzer (Model 6400, Li-Cor Instruments, Lincoln, NE, USA), at controlled value of leaf temperature (30 °C), leaf-to-air vapor pressure deficit (1.5 kPa), saturating light (1500 μmol $m^{-2}$ $s^{-1}$ of PPFD, photosynthetic photon flux density) and ambient $CO_2$ concentration (400 μmol $mol^{-1}$) from 8 to 14 August, 2013. Four or five plants per each $O_3$ treatment were used. A fully expanded sun leaf (leaf order: 4th to 6th in a shoot) was selected as a target. When stomatal conductance reached the equilibrium under constant light at 1500 μmol $m^{-2}$ $s^{-1}$, the methodology described by Paoletti (2005) was applied to assess dynamic variations of stomatal conductance after cutting the leaf petiole (Fig. 1). Data were logged at 1 min intervals for 30 min after cutting the petiole. Two phases of stomatal response were observed (Fig. 1). At first, stomatal conductance showed an increase called as the transient "wrong-way response" (WWR) (Powles et al., 2006). This transient increase is due to a difference in turgor pressure between guard cell and epidermal cells. Subsequently, stomatal conductance decreased with increasing leaf water stress. In the present study, the magnitude of WWR and time for 50% decrease of stomatal conductance were recorded. All gas exchange measurements were performed from 9:00 to 12:00 to avoid the midday depression of stomatal conductance (cf. Zhang and Gao, 1999).

After measurement of leaf gas exchange, the same leaves were analyzed for determining the leaf mass per unit area (LMA). Three leaf disks (12 mm diameter) per measured leaf were punched out and dried in an oven at 70 °C for 1 week and then weighed. LMA was calculated as the ratio of dry mass to area of the leaf disks. The stomatal density was determined by the SUMP method (Koike et al., 1998), which involves making a replica of the abaxial leaf surface using a celluloid sheet (Universal Micro-printing, SUMP, Tokyo, Japan). Stomata were counted at 5–7 locations, randomly chosen from interveinal fields, of a total area of 0.4 $mm^2$, under a light microscope.

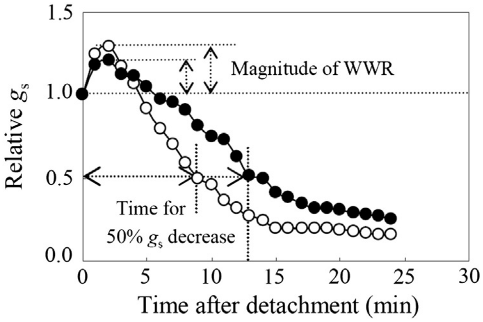

**Fig. 1.** Example of time course of stomatal conductance ($g_s$) after severing a *F. chinensis* leaf at time zero (open circle: NF, closed circle: NF + 80), with calculation of wrong way response (WWR) magnitude, and time for 50% decrease in stomatal conductance with increasing leaf water stress.

### 2.4. Stomatal ozone flux

Stomatal $O_3$ flux was estimated from stomatal conductance to water vapor ($g_s$), employing the water vapor surrogate method (Emberson et al., 2000; Hoshika et al., 2012b,c). This method assumes the $O_3$ concentration inside leaves to be close to zero (Laisk et al., 1989; Omasa et al., 2002). Stomatal conductance to $O_3$ can be then derived from $g_s$ and ambient $O_3$ concentrations multiplied by 0.613, i.e., the ratio of diffusivities between $O_3$ and water vapor (Wieser et al., 2002). In the present study, we used the mean $O_3$ concentration during daytime (09:00–18:00) in each treatment. Finally, stomatal $O_3$ flux per net photosynthetic rate was calculated at steady-state conditions.

### 2.5. Statistical analysis

The statistical unit was the single plant. Two-way analysis of variance (ANOVA) was used to assess the effects of species and $O_3$ treatments on leaf gas exchange parameters. Data were checked for normal distribution (Kolmogorov Smirnov D test) and homogeneity of variance (Levene's test). A simple linear correlation was used to test the relation of the parameters of $O_3$-induced stomatal sluggishness with leaf traits (i.e., LMA or stomatal density) or indices of $O_3$ flux (i.e., stomatal $O_3$ flux, or stomatal $O_3$ flux per net photosynthetic rate). Species-specific differences of the relationship of $O_3$-induced stomatal sluggishness were tested by analysis of covariance (ANCOVA) with indices of $O_3$ flux as covariate. Results were considered significant at $p < 0.05$. All statistical analyses were performed with SPSS software (20.0, SPSS, Chicago, USA).

## 3. Results

### 3.1. Leaf traits

Enhanced ozone concentrations did not affect stomatal density for any of the three species (data not shown). Average stomatal density was 211 $mm^{-2}$ in *A. altissima*, 116 $mm^{-2}$ in *F. chinensis*, and 118 $mm^{-2}$ in *P. orientalis*. There was no difference in LMA between $O_3$ treatments for any species (data not shown). Average LMA was 44.9 g $m^{-2}$ in *A. altissima*, 53.6 g $m^{-2}$ in *F. chinensis*, and 40.7 g $m^{-2}$ in *P. orientalis*.

### 3.2. Steady-state leaf gas exchange and dynamic stomatal response after leaf excision

Ozone exposure induced a decline of steady-state net photosynthetic rate (Fig. 2). Lower net photosynthesis was found in enhanced $O_3$ treatments compared to NF (−17% in *A. altissima*, −28% in *F. chinensis*, and −1% in *P. orientalis* in NF + 40; −29% in *A. altissima*, −43% in *F. chinensis*, and −35% in *P. orientalis* in NF + 80). Stomatal conductance did not show significant difference between $O_3$ treatments (Fig. 2).

Magnitude of WWR was not affected by $O_3$ (data not shown). Time for 50% decrease of stomatal conductance in NF was 5.5 ± 0.6 min in *A. altissima*, 7.5 ± 0.9 min in *F. chinensis*, and 6.0 ± 0.4 min in *P. orientalis* (Fig. 3). Increase in the time elapsed for 50% decrease of stomatal conductance was found especially in NF + 80 compared to NF (+50% in *A. altissima*, +57% in *F. chinensis*, and +17% in *P. orientalis*).

There was no significant relationship between time for 50% decrease of stomatal conductance after cutting a leaf and leaf traits (stomatal density or LMA: Fig. 4A,B). Time for 50% decrease of stomatal conductance increased with increasing stomatal $O_3$ flux (Fig. 4C), and with increasing ratio of stomatal $O_3$ flux to net photosynthesis (Fig. 4D) in *A. altissima* and *F. chinensis*. The relationships among $O_3$-induced stomatal sluggishness and indices of $O_3$ flux (stomatal $O_3$ flux or the ratio of stomatal $O_3$ flux to net photosynthesis) differed between these two species (Fig. 4C,D). Higher y-intercepts in those relationships were recorded in *F. chinensis* compared to *A. altissima* ($p < 0.01$).

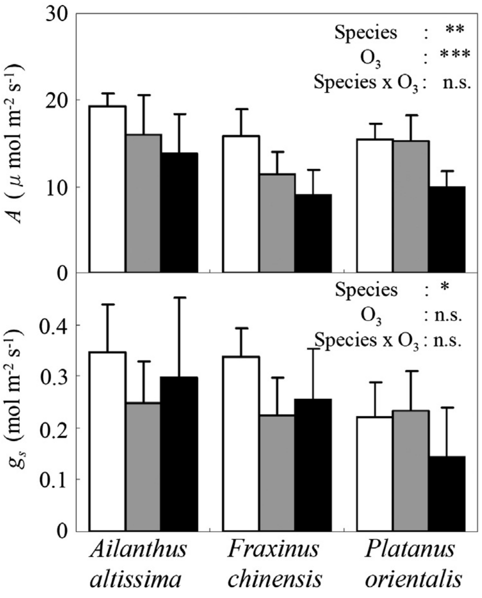

**Fig. 2.** Effect of chronic ozone exposure at three levels (NF: non-filtered ambient air, shown as white bar; NF + 40: NF supplied with 40 nmol $mol^{-1}$ of ozone, shown as gray bar; NF + 80: NF supplied with 80 nmol $mol^{-1}$ of ozone, shown as black bar) on steady-state net photosynthetic rate ($A$) and stomatal conductance ($g_s$) in 1 year old seedlings of three Chinese tree species. Data are means (N = 4–5 plants) + SD. Two-way ANOVA: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s. indicates no significance.

## 4. Discussion

Ozone exposure caused a decrease in the steady-state values of net photosynthesis in all three species, while no difference in stomatal conductance was found (Fig. 2). This response of stomatal conductance is in contrast with a meta-analysis of physiological responses to $O_3$ by trees (Wittig et al., 2007). Stomatal conductance is generally regulated so as to maintain the ratio of internal $CO_2$ concentration to ambient $CO_2$ concentration (Lambers et al., 2008). At a moderate level of chronic $O_3$ exposure, $O_3$-induced decline of photosynthetic capacity may therefore cause stomatal closure (Reich, 1987; Farage and Long, 1995; Heath and Taylor, 1997). However, free-air $O_3$ exposure experiments showed that such $O_3$-induced stomatal closure was diminished in European and Seibold's beech (*Fagus sylvatica* and *Fagus crenata*, respectively) during the late growing season, although $O_3$ decreased photosynthetic capacity (Löw et al., 2007; Hoshika et al., 2013b). The observation

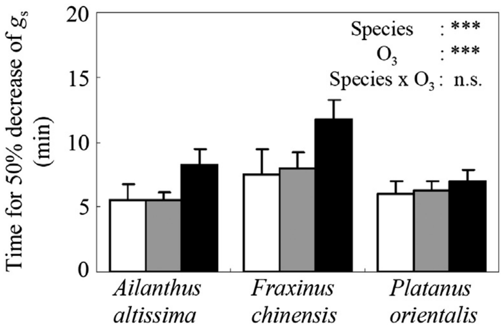

**Fig. 3.** Effect of chronic ozone exposure at three levels (NF: non-filtered ambient air, shown as white bar; NF + 40: NF supplied with 40 nmol $mol^{-1}$ of ozone, shown as gray bar; NF + 80: NF supplied with 80 nmol $mol^{-1}$ of ozone, shown as black bar) on time for 50% decrease of stomatal conductance ($g_s$) after severing a leaf in 1 year old seedlings of three Chinese tree species. Data are means (N = 4–5 plants) + SD. Two-way ANOVA: ***$p < 0.001$; n.s. indicates no significance.

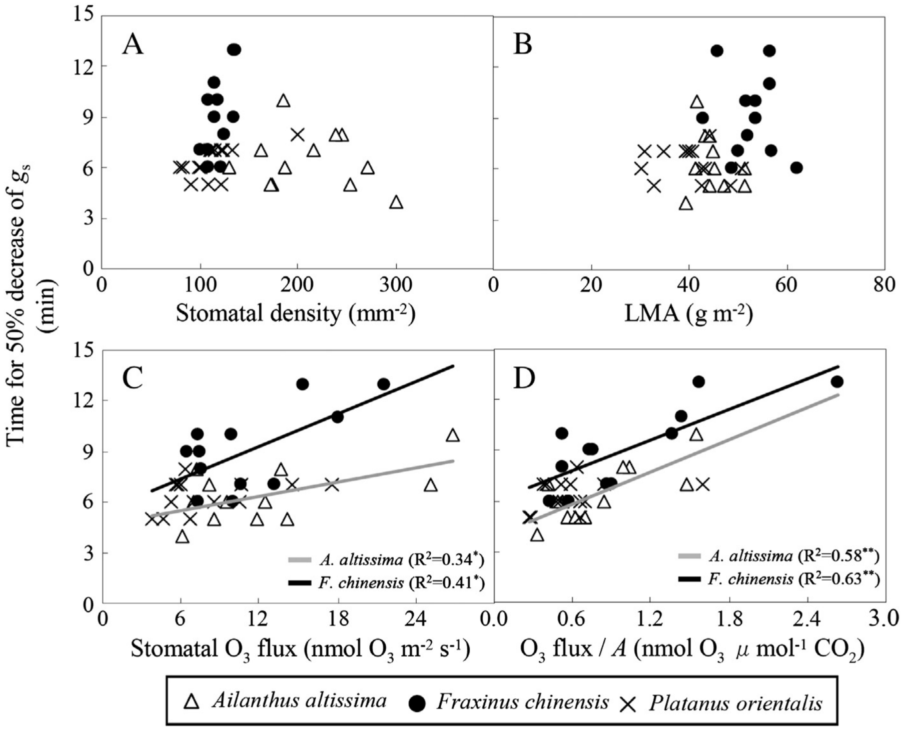

**Fig. 4.** Relationship between time for 50% decrease of stomatal conductance ($g_s$) after severing a leaf and (A) stomatal density, (B) leaf mass per area (LMA), (C) stomatal ozone (O$_3$) flux, and (D) stomatal O$_3$ flux per net photosynthetic rate in 1 year old seedlings of three Chinese tree species. $Y = 0.14X + 4.63$ (gray line, *A. altissima*) and $Y = 0.32X + 5.50$ (black line, *F. chinensis*) for stomatal O$_3$ flux vs time for 50% decrease of $g_s$. $Y = 3.21X + 3.87$ (gray line, *A. altissima*) and $Y = 3.04X + 5.96$ (black line, *F. chinensis*) for stomatal O$_3$ flux per net photosynthesis vs time for 50% decrease of $g_s$. Statistical significance for R$^2$ is denoted by $^*(p < 0.05)$ and $^{**}(p < 0.01)$. A significant difference of y-intercept in the regression lines was recorded between *A. altissima* and *F. chinensis* in panels C and D (ANCOVA, $p < 0.01$).

here implies a decoupling of stomatal conductance and photosynthesis after O$_3$ exposure.

Enhanced O$_3$ exposure caused slower closing response of stomata to the severe water stress imposed by excision of a leaf in all three tree species (Fig. 3). Similar findings were reported in previous studies (e.g., Paoletti, 2005; Mills et al., 2009; Paoletti and Grulke, 2010; Hoshika et al., 2012a). Maier-Maercker (1989) suggested that such slower stomatal response might be explained by delignification of the guard cell and subsidiary cells as recorded in ozonated *Picea abies*. No change in guard cell wall lignification, however, was observed in ozone-injured manna ash (*Fraxinus ornus*) leaves showing stomatal sluggishness (Paoletti et al., 2009). In manna ash, such slower stomatal responses were considered as accelerated senescence in the cell physiological processes (Paoletti et al., 2009). Mills et al. (2009) found that O$_3$ reduced stomatal sensitivity to ABA. Wilkinson and Davies (2010) suggested that the loss of stomatal response to ABA may be related to O$_3$-induced ethylene emissions. However, the mechanisms of the sluggish response of stomata caused by O$_3$ are still under investigation. We thus investigated whether leaf traits, i.e. stomatal density and LMA, can affect sluggishness of stomata.

There was no significant relationship between stomatal sluggishness and stomatal density (Fig. 4B). The higher stomatal density in *A. altissima* translated into a slightly faster response of stomata in NF than those in the other two species (Fig. 3). Our result however indicated that observed slightly faster stomatal responses associated with higher stomatal density in leaves were not enough to reduce O$_3$ injury to stomatal control.

The degree of O$_3$-induced impairment of stomatal control was greater in leaves with higher stomatal O$_3$ flux in *A. altissima* and *F. chinensis* (Fig. 4C). No significant relationship was found in *P. orientalis.* Interestingly, *P. orientalis* showed relatively lower stomatal O$_3$ flux than the other two species (Fig. 4C), and showed the lowest increase in time for 50% decrease in stomatal conductance after cutting a leaf even under elevated O$_3$ condition (Fig. 3). This may be interpreted as more efficient limitation of stomatal O$_3$ flux in *P. orientalis* than in the other two species. Such capacity for avoidance of O$_3$-induced stress by smaller O$_3$ influx in *P. orientalis* may contribute to reduced injury to stomatal control under elevated O$_3$. In addition, *F. chinensis* showed higher y-intercept in the relationship between stomatal sluggishness and indices of O$_3$ flux (i.e., stomatal O$_3$ flux, or stomatal O$_3$ flux per net photosynthetic rate) compared to that in *A. altissima*. In fact, the time for 50% decrease of stomatal conductance (NF + 80 vs. NF) was greater in *F. chinensis* (+57%) than in the other species (+50% in *A. altissima*, and +17% in *P. orientalis*). This indicates the following order of sensitivity of O$_3$-induced stomatal sluggishness, *F. chinensis* > *A. altissima* > *P. orientalis*.

The ratio of stomatal O$_3$ flux to net photosynthesis, which is an index of both O$_3$ exposure of mesophyll cells and availability of photosynthates for repair or detoxification, was the best parameter explaining the impairment of stomatal control induced by O$_3$ (Fig. 4D). Fredericksen et al. (1996) similarly reported that greater foliar O$_3$ flux per net photosynthesis in shade leaves was related to the degree of visible foliar injury in black cherry (*Prunus serotina*) trees. This suggests that the sensitivity to the O$_3$-induced impairment of stomatal control is related not only to diffusion of O$_3$ into leaves but also to biochemical detoxification capacity or repair. Biochemical detoxification capacity may also be related to LMA because greater LMA implies a higher density of mesophyll tissues, suggesting a higher capability of repair or detoxification (Wieser et al., 2002). However, O$_3$-induced sluggish response of stomata did not show any relation with LMA (data not shown). Watanabe et al. (2013) reported that Siebold's beech (*Fagus crenata*) showed much greater O$_3$ injury to photosynthetic traits than that in deciduous oak (*Quercus mongolica* var. *crispula*), even though both species had similar LMA. Our results suggest that physiological leaf traits affect $O_3$-induced stomatal sluggishness more than anatomical leaf traits, although a relatively small range of LMA among the three species was examined here.

In conclusion, the present study demonstrated that enhanced $O_3$ exposure slowed the stomatal dynamics in three tree species of China. This effect was species-specific, being greater in *F. chinensis* and smaller in *P. orientalis*. The degree of $O_3$-induced impairment of stomatal control was related to stomatal $O_3$ flux. A restriction of stomatal $O_3$ flux reduced $O_3$ injury to stomatal control in *P. orientalis*. Yamaji et al. (2008) predicted further increase in $O_3$ concentration in East Asian countries in 2020. Climate change brings about further risk of drought and flooding (Bytnerowicz et al., 2007). This $O_3$-induced loss of stomatal function may therefore enhance both leaf water loss and stomatal $O_3$ flux under predicted $O_3$ pollution (e.g., Hayes et al., 2012). The present study also found that the $O_3$-induced sluggish response of stomata was better explained by stomatal $O_3$ flux per net photosynthesis rather than by stomatal $O_3$ flux only, supporting the use of carbon assimilation as a surrogate for tolerance to $O_3$ stress including a capacity for repair and detoxification (Paoletti et al., 2008). The concept of "effective $O_3$ dose" reflecting plant sensitivity to unit of stomatal $O_3$ flux may contribute to further development of our knowledge into the mechanisms of plant response to $O_3$-induced stress (Wieser et al., 2002; Musselman et al., 2006; Matyssek et al., 2008).

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# *II. Effects of long-term ambient ozone exposure on biomass and wood traits in poplar treated with ethylenediurea (EDU)*

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# Effects of long-term ambient ozone exposure on biomass and wood traits in poplar treated with ethylenediurea (EDU)

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ABSTRACT

This is the longest continuous experiment where ethylenediurea (EDU) was used to protect plants from ozone ($O_3$). Effects of long-term ambient $O_3$ exposure (23 ppm h AOT40) on biomass of an $O_3$ sensitive poplar clone (Oxford) were examined after six years from in-ground planting. Trees were irrigated with either water or 450 ppm EDU. Above (−51%) and below-ground biomass (−47%) was reduced by $O_3$ although the effect was significant only for stem and coarse roots. Ambient $O_3$ decreased diameter of the lower stem, and increased moisture content along the stem of not-protected plants (+16%). No other change in the physical wood structure was observed. A comparison with a previous assessment in the same experiment suggested that $O_3$ effects on biomass partitioning to above-ground organs depend on the tree ontogenetic stage. The root/shoot ratios did not change, suggesting that previous short-term observations of reduced allocation to tree roots may be overestimated.

## 1. Introduction

Tropospheric ozone ($O_3$) is a harmful phytotoxic air pollutant and greenhouse gas which has become a serious concern for forest ecosystems (Matyssek et al., 2007; Paoletti, 2007). Ozone is recognized as a secondary pollutant not directly emitted from sources (Jenkin and Clemitshaw, 2000; Zeng et al., 2008). Its formation results from the interaction between precursors like nitrogen oxides ($NO_x$) and volatile organic compounds (VOCs) (Atkinson and Arey, 2003).

Ozone levels are exceeding limits set to protect human health and vegetation (Sicard et al., 2013; Paoletti et al., 2014). Although knowledge is still imperfect, much has been written about the effects of ambient $O_3$ on growth of forest trees (Matyssek and Sandermann, 2003; Manning, 2005; Felzer et al., 2007; Wittig et al., 2009). Ozone may cause growth reduction, shift in biomass allocation, impairment of physiological traits such as leaf gas exchange, and visible foliar injury, i.e., interveinal dark stippling (Paoletti, 2007).

Many scientists investigated $O_3$ effects on plants using the chemical ethylenediurea, N-[2-(2-oxo-1-imidazolidinyl)ethyl]-N′-phenylurea (abbreviated as EDU) to prevent foliar $O_3$ injury and to determine $O_3$ effects on plants (Szantoi et al., 2007; Paoletti et al., 2009; Feng et al., 2010). In the United Nations/Economic Commission for Europe International Co-operative Program on the effects of air pollution (primarily ozone) and other stresses on crops and non-woody plants (UN/ECE ICP-Vegetation), EDU has been used to investigate crop responses to ambient $O_3$ (Sanders et al., 1992). Many short-term experiments were performed especially on crops confirming that EDU is effective in protecting plants from $O_3$ injury (Lee et al., 1997; Paoletti et al., 2007a,b, 2008; Singh et al., 2010), but only a few studies about the long-term effect of EDU on trees are available. Manning et al. (2003) sprayed one-year-old in-ground seedlings of *Pinus taeda* with 450 ppm EDU for three growing seasons, and found that ambient $O_3$ (118 ppb as a peak) reduced stem biomass and height by 30% and 11%, respectively, while roots were not investigated. Long and Davis (1991) used foliar sprays of 1000 ppm EDU over a 4-year period to protect in-ground *Prunus serotina* seedlings and found that ambient $O_3$ (129 ppb as a peak)

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reduced tree height (−17%), basal diameter (−21%) and above-ground leafless biomass (−47%). Again, roots were not investigated. Two further multi-year EDU studies are part of the present experiment on poplar. Hoshika et al. (2013a) and Katanić et al. (2014) used 450 ppm EDU as soil drench for three and four growing seasons, respectively, and found that ambient $O_3$ (24 ppm h AOT40) reduced biomass of leaves (−64%), lateral branches (−46%), coarse (−40%) and fine roots (−52%), increased the ectomycorrhizal (ECM) colonization (+40%), and did not affect stem height and diameter.

Most of the studies included in Feng et al. (2010)'s meta-analysis investigated EDU effects on crops, due to the fact that crops are easier to be treated with EDU than trees. This meta-analysis reported that EDU significantly reduced $O_3$ visible injury, increased biomass production and yield of crops and grasses, without significant effects on trees with an exception of increased stem radial growth.

Previously, Wittig et al. (2009) reported that ambient $O_3$ exposure in controlled-conditions experiments significantly reduced total biomass of broadleaf trees, especially root biomass. Decreased carbon allocation below ground is often associated with decreased Root/Shoot ratio (Andersen, 2003). A few studies have reported long-term effect of $O_3$ on plants using Free-Air $O_3$ fumigation systems (Karnosky et al., 2007; Matyssek et al., 2010b; Watanabe et al., 2013). Because of changing climate, concern for wood productivity and quality is increasing (Corona, 2014). Knowledge on the effects of $O_3$ on wood properties, however, is imperfect, and usually limited to chemical and anatomical traits (Kaakinen et al., 2004; Kostiainen et al., 2006, 2008; Richet et al., 2011, 2012).

Wood characteristics are mainly determined by cambium activity and how derivative cambial cells differentiate up to become mature xylem passing through the phases of cell expansion, secondary wall apposition, cell death and lignification (Balducci et al., 2013). Basic density is considered one of the main indicators for wood quality and summarizes a suite of adaptive traits related to patterns of carbon gain, water transport, biomechanics, and growth (Cocozza et al., 2011). Basic wood density describes the proportion of the xylem conduit walls and the lumen xylem (Swenson and Enquist, 2007). In hardwoods, vessel area and cell wall thickness contribute to wood density in parallel by affecting the amount of lumen space in the wood. Short-term exposure to $O_3$ induced significant decrease of cell wall thickness in poplar (Richet et al., 2012) and *Betula pendula* juvenile wood (Matyssek et al., 2002). Three years of exposure to elevated $O_3$ induced significant changes in the secondary cell wall composition and wood structure in *Betula papyrifera* and *Acer saccharum* saplings whilst the effects on *Populus tremuloides* were genotype dependent (Kaakinen et al., 2004; Kostiainen et al., 2008). After 11 years of open-field $O_3$ exposure, mature wood of *P. tremuloides* showed an increased vessel density and a decreased cell diameter thus prioritizing hydraulic safety over efficiency (Kostiainen et al., 2014).

In this experiment, trees were exposed to ambient $O_3$ rather than to artificial $O_3$ fumigation because fumigating large trees throughout the entire canopy is difficult and costly. An $O_3$ sensitive poplar clone was irrigated for six years with EDU in a Mediterranean field site. We aimed at examining long-term effects of ambient $O_3$ exposure on poplar biomass production, allocation and wood physical traits, by investigating EDU-treated plants and non-treated ones, and comparing our results with those obtained after three years in the same experiment (Hoshika et al., 2013a; Katanić et al., 2014).

## 2. Materials and methods

### 2.1. Site and treatment description

The experiment was conducted at Antella (43°44′ N, 11°16′ E, 50 m a.s.l) in central Italy, in a poplar plantation ($O_3$ sensitive Oxford clone, *Populus maximoviczii* Henry *x berolinensis* Dippel) planted in two lines (20 root cuttings in each line) in autumn 2007 with a spacing of 1 m between trees along a line and of 3 m between trees of the two lines. Every week during the growing season (April to October) each tree was drip irrigated with 2 L of water (WAT or not-protected trees) as ambient $O_3$ treatment, or 450 ppm EDU solution (EDU or protected trees) as $O_3$ protection treatment. The application of EDU was repeated weekly because of degradation over time and reduced mobilization from old tissues to new sprouts (Carnhan et al., 1978; Weidensaul, 1980). Ozone concentrations were recorded at canopy height by an annually-calibrated $O_3$ monitor (Mod. 202, 2B Technologies, Boulder CO, USA). Ozone exposure was expressed as 24-h mean and AOT40 index, which represents the accumulated exposure over a threshold of 40 ppb during daylight hours (UNECE, 2010). This is the current legislative standard in the EU Air Quality Directive (2008/50/EC).

### 2.2. Tree harvesting and assessment of below and above ground biomass

In October 2010 and April 2014, after full development of leaves, five trees in each treatment (EDU or WAT) were selected, harvested and separated into each organ (stem, branches, leaves, fine and coarse roots). To avoid damages to the roots of nearby trees, alternate plants were selected for the harvest. Results for 2010 were published in Hoshika et al. (2013a). Harvesting was done early in the morning to avoid the period of higher transpiration. Before cutting down the trees at the collar, a 20 × 20 × 20 cm soil sample per tree was collected from the side external to the tree lines for collecting fine roots (diameter <2 mm) after carefully removing litter and weeds. Previous samplings showed that fine roots were concentrated in the top 10–15 cm in this post-agricultural soil. These samples were labeled and stored in plastic bags at 4 °C until cleaning in the laboratory. Each soil core was submerged in cold tap water to loosen the soil structure. Roots were carefully washed from soil, and ECM root tips were divided from herbaceous roots and selected for further identification by means of visual inspection. Fine roots were analyzed with a Wild M8 (16×) stereomicroscope by the gridline intersect method (Giovannetti and Mosse, 1980) adapted to ectomycorrhizae (Katanić et al., 2014) for quantification of ECM colonization. Fine root total dry mass was then determined by oven-drying at 60 °C until a constant weight was reached and referred to the unit of soil volume. Coarse roots (diameter >2 mm) per each tree were extracted using an excavator by digging additional soil cores (100 cm depth × 280 cm between the tree lines × 100 cm along a line) localized around the base of a trunk. Roots were separated from the soil and stored in plastic bags at 4 °C until cleaning in cold tap water for removing soil.

Before harvesting, stem diameter was measured at breast height (DBH) by means of a caliper (Mod. Mantax, Italgein SrL, Italy) with a 1-mm accuracy. After harvesting, total tree height and diameter at 50-cm intervals along the stem were measured by a 30-m surveyor tape (Am-Tech, UK) with a 5-mm accuracy, and a caliper. Sixty leaves per each tree were randomly collected for measuring the individual leaf area and dry weight. Leaf area of these samples was determined using an AM300 area meter (ADC, BioScientific Ltd, Herts UK). The remaining leaves and the lateral branches were then collected. Lateral branches longer than 16 cm (Dickson et al., 2001) were counted.

Dry mass of each plant organ was determined by a scale (Mod. PTF35 DHR, Gibertini, Milan, Italy; 0.1 g accuracy) after oven-drying at 60 °C (for leaves and fine roots) and at 103 °C (for woody parts), until a constant weight was reached. Allometric ratios, i.e. shoot/root ratio (leaf plus stem and branch dry weight/root dry weight; S/

R), leaf weight ratio (total leaf dry weight/total plant dry weight; LWR), shoot weight ratio (leaf plus stem and branch weight/total plant weight; SWR), root weight ratio (root weight/total plant weight; RWR), leaf/branch ratio (leaf dry weight/branch dry weight; L/B) and stem height/DBH ratio (H/B) were finally calculated, under the assumption that 80% of roots was within the excavated soil volume (Xi et al., 2011).

### 2.3. *Determination of wood traits*

At the harvest time, woody stem discs of 8–10 cm in thickness were collected from the main stems at 30, 130, and 300 cm from the collar, kept in plastic bags and transported to the laboratory at 4 °C. For each disc, two prismatic sub-samples were collected out from the central part of the sample (excluding pith and bark) following the radial, tangential and longitudinal directions (20 × 20 × 20 mm). Each sub-sample was composed of three annual rings (growth year 2010, 2011, 2012). The samples were immediately weighted (Green Weight, GW) and the fresh volume (Green Volume, GV) was measured by water displacement (Berta et al., 2010). The oven dry weight (Dry Weight, DW) was determined after 96 h at 103 °C. Measurements were carried out by a balance (Mod. BP110S, Sartorius, Goettingen, Germany; 0.1 mg accuracy). The stem disc biomass was used for the calculation of the stem biomass described above. Basic Density (BD, kg $m^{-3}$), Green Density (GD, kg $m^{-3}$) and Moisture Content (MC, %) were calculated as:

$$\mathrm{BD}\left(\mathrm{kg\ m^{-3}}\right) = \mathrm{DW/GV},$$
$$\mathrm{GD}\left(\mathrm{kg\ m^{-3}}\right) = \mathrm{GW/GV},$$
$$\mathrm{MC}(\%) = [(\mathrm{GW} - \mathrm{DW}) : \mathrm{DW}] \times 100$$

The volumetric fractions occupied by cell wall (Wood Substance, WS), Bound Water (BW) and Free Water (FW) were determined according to Chan et al. (2010) assuming the following standard densities: 1500 kg $m^{-3}$ for dry wood, 1000 kg $m^{-3}$ for free water, and 1018 kg $m^{-3}$ for bound water. Volumetric fractions (WS) were calculated as:

$$\mathrm{WS} = (\mathrm{BD}/1500) \times 100$$
$$\mathrm{BW} = (\mathrm{BD}/1018) \times 30$$
$$\mathrm{FW} = (\mathrm{BD}/1000)/(\mathrm{MC}\% - 30)$$

### 2.4. *Statistics*

Percentages were transformed in arcsin $\sqrt{x}$ and normality of the distribution was verified by Levene test ($p \leq 0.05$). In case of non-normal distribution, Mann Whitney's non-parametric test was applied to test the effect of the treatment or of the year. For normally-distributed data, a Student *t*-test was applied. The difference in the slopes of the relationship between diameter and relative height was tested by analysis of covariance (ANCOVA). For wood traits, the effects of $O_3$ and position were assessed by a two-way analysis of variance (ANOVA) followed by Tukey's test. All statistical analyses were performed using Statistica 7.0 software for Windows.

## 3. Results

### 3.1. *Ozone exposure and environmental conditions*

Annual daily $O_3$ averages (M24), peak hourly values of $O_3$ (Max) and $O_3$ exposure above 40 ppb (AOT40) were calculated over the six years (from 2008 to 2013) (Table 1). Data from 2008 to 2010 were from Hoshika et al. (2013a). M24 and AOT40 showed similar values over the first four years of experiment (from 2008 to 2011) and a reduction in 2012–2013. The average values of M24 and AOT40 were 35 ppb and 23 ppm h, respectively, with a maximum $O_3$ concentration of 143 ppb in 2011. These data are similar to those gathered at a nearby station (Settignano, 195 m a.s.l., 9 km away from Antella) over the period 2000–2004 when M24 was 30 ppb, AOT40 was 21.8 ppm h and the peak was 113 ppb (Paoletti, 2006). Also the daily profile at our site (not shown) was similar to that at Settignano (Paoletti, 2006). The coldest year with the highest SWC was 2013. Precipitation was higher in 2010 than in the other years (625 mm in 2010 and 418 mm as mean precipitation over the six years). Overall, the environmental data confirmed that Antella is a typical Mediterranean site *sensu* Köppen-Geiger (Peel et al., 2007).

### 3.2. *Effect of $O_3$ exposure on biomass*

Although ambient $O_3$ exposure decreased all leaf, branch, stem and root parameters relative to EDU-protected plants, the effects were significant only for stem biomass, DBH, and coarse root biomass (Table 2). Ozone effect on stem diameter decreased from the base to the top of the trees, and was significant only up to 2.5 m a.g.l. (Fig. 1). To test whether this response was affected by a change in stem height, diameters were correlated to relative heights (Fig. 1 inset). Significantly different slopes between the treatments confirm that not-protected trees were thinner. The only parameter that was stimulated by $O_3$ exposure was the ECM colonization of fine roots, although the response was not statistically significant (Table 2). As stem and coarse roots represented most part of the tree biomass, the effect of ambient $O_3$ was significant also on total above- and below-ground biomass. The magnitude of their declines was similar, i.e. −51% and −47%, respectively. In fact, the shoot/root ratio as well as the other allometric ratios of biomass did not show a significant response after 6 years of $O_3$ exposure, although the leaf/branch ratio decreased by 31% due to ambient $O_3$. The H/B ratio, i.e. the ratio of tree height and DBH, significantly increased by 19%.

**Table 1**
Mean daily ozone concentration (M24), absolute peak value (Max), accumulated ozone exposure above 40 ppb (AOT40), precipitation, air temperature (T) and soil water content (SWC) over the growing season (April–October) during the six years of experiment.

| Year | M24 [ppb] | Max [ppb] | AOT40 [ppm h] | Precipitation [mm] | T [°C] | SWC [%] |
|---|---|---|---|---|---|---|
| 2008 | 35 | 101 | 24 | 332 | 20.1 | n.a. |
| 2009 | 36 | 105 | 24 | 250 | 21.0 | 20.3 |
| 2010 | 36 | 118 | 26 | 625 | 19.1 | 20.4 |
| 2011 | 41 | 143 | 32 | 380 | 19.5 | n.a. |
| 2012 | 30 | 103 | 18 | 417 | 19.5 | 20.8 |
| 2013 | 32 | 97 | 16 | 504 | 18.7 | 25.9 |
| **Avg** | 35 | 111 | 23 | 418 | 19.7 | 21.9 |
| **±SE** | 2 | 7 | 2 | 54 | 0.3 | 1.4 |

**Table 2**
Biomass and allometric ratios of WAT and EDU trees in Spring 2014 i.e. after six years of ambient $O_3$ exposure (average AOT40 = 23 ppm h) and treatment with EDU (450 ppm), and in a previous assessment in Fall 2010 (data were either published in Hoshika et al., 2013a, or were obtained from new calculations performed within this manuscript). Each value is a mean (±SE). Δ shows the effects of ambient $O_3$ exposure (WAT) relative to protected trees (EDU). Arrows show the direction of change from 2010 to 2014. Statistical significance: ns, $p \geq 0.1$; +, $p < 0.1$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ (Student t or Mann Whitney's test, N = 5).

| | | WAT | EDU | 2014 | | 2010 | | 2010–2014 | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Δ | p | Δ | p | p | |
| | | | | | | | | WAT | EDU |
| Leaf | dw [kg tree$^{-1}$] | 0.9 ± 0.1 | 1.6 ± 0.3 | −44% | ns | −64% | ** | $\uparrow^{*}$ | $\uparrow^{**}$ |
| Branch | dw [kg tree$^{-1}$] | 2.8 ± 0.1 | 3.5 ± 0.3 | −20% | ns | −46% | ** | $\uparrow^{*}$ | $\uparrow^{**}$ |
| Branch | num tree$^{-1}$ | 65.2 ± 1.3 | 69.6 ± 5.0 | −6% | ns | −45% | * | =ns | $\downarrow^{**}$ |
| Stem | dw [kg tree$^{-1}$] | 5.2 ± 0.7 | 12.2 ± 4.7 | −57% | * | −36% | * | $\uparrow^{*}$ | $\uparrow^{**}$ |
| Stem | DBH [cm tree$^{-1}$] | 7.7 ± 0.5 | 10.5 ± 1.7 | −27% | ** | −10% | ns | $\uparrow^{***}$ | $\uparrow^{***}$ |
| Stem | height [m tree$^{-1}$] | 7.4 ± 0.5 | 8.4 ± 0.6 | −12% | ns | −7% | ns | $\uparrow^{***}$ | $\uparrow^{***}$ |
| Fine root | dw [g L$^{-1}$ soil tree$^{-1}$] | 0.29 ± 0.06 | 0.43 ± 0.09 | −32% | ns | −52% | *** | =ns | =ns |
| Coarse root | dw [g L$^{-1}$ soil tree$^{-1}$] | 2.44 ± 0.30 | 4.22 ± 0.54 | −42% | * | −40% | * | $\uparrow^{*}$ | $\uparrow^{**}$ |
| ECM colonization | [%] | 62 ± 9 | 53 ± 5 | +17% | ns | +40%[a] | ns | $\uparrow^{*}$ | $\uparrow^{*}$ |
| Total biomass | Above ground [kg tree$^{-1}$] | 10.2 ± 0.7 | 20.9 ± 3.0 | −51% | * | −44% | * | $\uparrow^{*}$ | $\uparrow^{**}$ |
| | Below ground [g L$^{-1}$ soil tree$^{-1}$] | 2.5 ± 0.5 | 4.7 ± 0.4 | −47% | * | −44% | * | $\uparrow^{*}$ | $\uparrow^{**}$ |
| S/R | Shoot/Root | 2.7 ± 0.1 | 2.8 ± 0.2 | −4% | ns | −14% | ns | $\uparrow^{*}$ | $\uparrow^{**}$ |
| LWR | Leaf weight ratio | 0.061 ± 0.002 | 0.056 ± 0.001 | +9% | ns | −36% | ** | $\downarrow^{*}$ | $\downarrow^{**}$ |
| L/B | Leaf/Branch | 0.31 ± 0.05 | 0.45 ± 0.20 | −31% | ns | −33% | * | $\uparrow^{*}$ | $\uparrow^{**}$ |
| SWR | Stem weight ratio | 0.73 ± 0.01 | 0.74 ± 0.01 | −0.4% | ns | +3% | ns | $\uparrow^{*}$ | $\uparrow^{**}$ |
| RWR | Root weight ratio | 0.27 ± 0.01 | 0.26 ± 0.01 | +1.5% | ns | +9% | ns | $\downarrow^{*}$ | $\downarrow^{**}$ |
| H/D | Height/DBH [m cm$^{-1}$] | 0.96 ± 0.04 | 0.81 ± 0.04 | +19% | * | +12% | ns | $\downarrow^{**}$ | $\downarrow^{***}$ |

[a] Data from a sampling in September 2011 (see Katanić et al., 2014).

A comparison with an assessment carried out in Autumn 2010 showed interesting similarities, i.e., all parameters declined due to $O_3$ exposure while ECM colonization increased, and the above- and below-ground declines of biomass were similar. In 2010, however, the higher above-ground declines were in the leaves and lateral branches, while in 2014 the stem showed the most significant impact as a possible carry-over effect. As a result, in 2010, LWR, i.e. the ratio of leaf and total biomass, and L/B, i.e. the ratio of leaf and branch biomass, significantly decreased. Changes from 2010 to 2014 within each treatment usually showed an increase of biomass and allometric ratios, while the number of branches per tree in the EDU-protected trees, LWR, RWR and H/D in both treatments decreased, and fine roots per unit soil volume and the number of branches per tree in the not-protected trees did not significantly vary.

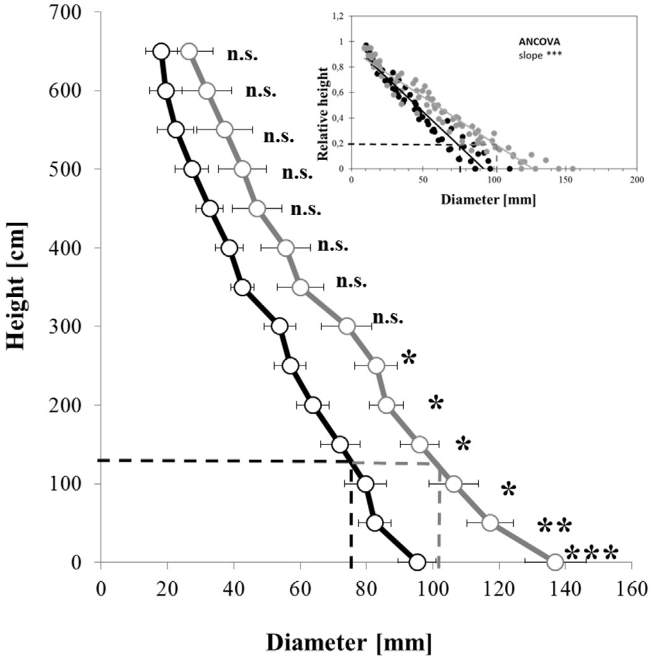

**Fig. 1.** Stem diameter at different heights. Values at heights >650 cm are not shown. WAT trees (black line) were not protected from ambient $O_3$; EDU trees (gray line) were protected by irrigating with ethylenediurea. Dashed lines mark the Diameter at Breast Height. Each value is a mean (±SE). Statistical significance: n.s., $p \geq 0.1$; +, $p < 0.1$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ (Student t test, N = 5). The inset shows that the slopes of the relationship between diameter and height (relative to the total height) were significantly different between the treatments.

### 3.3. *Effect of $O_3$ exposure on wood traits*

The long term $O_3$ exposure did not induce changes in BD, while significant increases were recorded for GD, MC and FW (Table 3). In addition, GD significantly decreased along the stem (from the collar to 300 cm in height). When the effect of $O_3$ on GD, MC and FW was evaluated at different positions along the stem, no significant interaction was found.

## 4. Discussion

Current surface $O_3$ concentrations have the potential to reduce the carbon sink strength of forests (Sitch et al., 2007; Fares et al., 2013). This effect has been quantified by controlled-conditions experiments (summarized in a meta-analysis by Wittig et al., 2009) and by a few open-air fumigation experiments (e.g. Karnosky et al., 2007; Matyssek et al., 2010b; Watanabe et al., 2013). For long-living organisms like trees, however, there is still a need of long-term experiments. We used the chemical $O_3$-protectant EDU to quantify the effects of a 6-year exposure to ambient $O_3$ relative to not-protected plants. The experiment was carried out at a field site in central Italy, where $O_3$ pollution was high. AOT40, in fact, exceeded by ~4 times the critical level recommended for the protection of European forests, i.e. 5 ppm h (UNECE, 2010). Environmental conditions were typically Mediterranean, with a mean temperature of ~20 °C over the growing season and limited precipitation (~420 mm). As trees were drip-irrigated, however, they were not subject to the serious water limitations that are typical of

**Table 3**

Wood traits of WAT and EDU trees after six years of ambient $O_3$ exposure (average AOT40 = 23 ppm h) and treatment with EDU (450 ppm Each value is a mean (±SE) for: Basic Density (BD); Green Density (GD); Wood Substance (WS); Bound Water (BW); Moisture Content (MC); and Free Water (FW). Samples were collected at 30, 130, 300 cm above the collar level (Position). Δ shows the effects of ambient $O_3$ exposure (WAT) relative to protected trees (EDU) as an average of the three positions. Statistical significance: ns, $p \geq 0.1$; +, $p < 0.1$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ (two-way ANOVA, N = 5).

| Trait | Position (cm) | WAT treatment | | | EDU treatment | | | Δ | ANOVA | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 30 | 130 | 300 | 30 | 130 | 300 | | Treatment (T) | Position (P) | T × P |
| BD [kg $m^{-3}$] | | 401.9 ± 12.3 | 398.5 ± 22.7 | 386.9 ± 6.3 | 415.1 ± 16.6 | 401.4 ± 16.7 | 399.0 ± 12.6 | −2.3% | ns | ns | ns |
| GD [kg $m^{-3}$] | | 843.9 ± 58.1 | 804.7 ± 22.4 | 775.8 ± 46.5 | 806.4 ± 56.6 | 763.1 ± 28.5 | 735.3 ± 41.0 | +5.2% | * | ** | ns |
| WS [%] | | 26.8 ± 0.8 | 26.6 ± 1.5 | 25.8 ± 0.4 | 27.7 ± 1.1 | 26.8 ± 1.1 | 26.0 ± 0.8 | −1.9% | ns | ns | ns |
| BW [%] | | 11.8 ± 0.4 | 11.7 ± 0.7 | 11.4 ± 0.2 | 12.2 ± 0.5 | 11.9 ± 0.5 | 11.8 ± 0.4 | −2.8% | ns | ns | ns |
| MC [%] | | 110.0 ± 14.9 | 102.3 ± 9.6 | 100.5 ± 11.2 | 94.5 ± 15.0 | 90.4 ± 11.4 | 84.2 ± 5.6 | +16.2% | ** | ns | ns |
| FW [%] | | 32.1 ± 5.6 | 28.7 ± 2.7 | 27.3 ± 4.4 | 26.7 ± 5.7 | 24.1 ± 3.8 | 21.7 ± 2.7 | +21.5% | ** | ns | ns |

Mediterranean climates (Paoletti, 2006) as SWC was close to the field capacity.

Under these conditions, ambient $O_3$ exposure reduced above and below ground biomass in not-protected trees with major declines in stem, leaves, and coarse roots. The declines were of much larger magnitude than those reported in a meta-analysis at similar average $O_3$ exposure (40 ppb), e.g. −51% and −47% for above and below ground biomass, respectively, relative to −6% and −5% in the meta-analysis (Wittig et al., 2009). This discrepancy is justified by the fact that most of the experiments in Wittig et al. (2009) were with potted plants and exposure durations <1 year. This suggests a greater impact of $O_3$ when in-ground plants are exposed in ambient air in long-term experiments than when potted plants are exposed to short-term controlled-conditions experiments. In fact, soybean biomass was decreased more under open-air treatment than in chamber experiments (Morgan et al., 2003, 2005) and stem and root biomass in the field were higher due to EDU application, relative to potted plants (Feng et al., 2010).

Consistently with Wittig et al. (2009), however, our shoot/root ratio was not significantly affected by growing at 35–40 ppb $O_3$. A decline may occur only at elevated $O_3$ concentrations (on average 97 ppb in Wittig et al., 2009) when the reduction in root biomass exceeds the decrease in shoot biomass. The finding in Wittig et al. (2009) supports the common knowledge about $O_3$ decreasing the partitioning of carbon to roots (Andersen, 2003) and thus increasing the predisposition of trees to drought. Such a response, however, may have been overestimated in the literature. In our highly polluted site, in fact, the $O_3$-sensitive clone of a fast-growing poplar species with no soil water limitation to stomatal uptake did not show a change in the allocation of shoot/root biomass after six growing seasons of exposure. Also Andersen et al. (2010) did not observe changes in carbon allocation below ground in adult *Fagus sylvatica* and *Picea abies* exposed to long-term $O_3$ enrichment in free-air.

Interestingly, a higher ECM colonization was observed in not-protected plants, which is a quite common response to $O_3$ in field experiments with trees (Haberer et al., 2007; Grebenc and Kraigher, 2007a). Decreased allocation of belowground carbon, in contrast, is expected to impair fine roots and root symbionts (Grebenc and Kraigher, 2007b). Cudlin et al. (2007) suggested that an increased number of active ECM root tips in adult trees after several years of $O_3$ exposure may indicate a transient response to different source–sink relationships in the ecosystem.

The effect of ambient $O_3$ on all plant-level allometric ratios was not significant, suggesting that both EDU-protected and not-protected trees maintained the same within-plant distribution of carbon or relative weight of different plant organs. Worth mentioning, however, is the 31% decrease of the L/B ratio. Although not significant, in fact, this decrease suggests an allometric change within the crowns and is consistent with the significant 33% decrease found after three growing seasons of exposure. Dickson et al. (2001), in two trembling aspen clones planted in the ground in open-top chambers and exposed for only 98 days, found similar effects of $O_3$ (58–71 ppm h AOT0) on all allometric ratios but L/B, likely because they investigated current-year branches only. In our experiment, both leaf and branch biomass decreased with $O_3$, but the relative weight of leaves decreased even more. This response occurred both in 2010 (Autumn) and in 2014 (Spring), and was thus not affected by early leaf abscission. Ozone, in fact, did stimulate premature leaf loss in our experiment (Hoshika et al., 2012). The severe reduction of leaf biomass in 2010 also implied a dramatic decline of LWR i.e. the ratio of leaf and total biomass.

Actually, a major difference between the assessments carried out after three and six years of exposure was the pattern of aboveground allocation. In younger trees, the major declines were in the leaves (−64%) and lateral branches (−46%), while in older trees the stem showed the most significant impact (−57%). As a consequence, the significant declines in the amount of carbon allocated to the leaves relative to that in the branches (L/B) and the entire plant (LWR) in younger trees, were not confirmed in older trees. As a general rule, growth allocation shifts from branches to coarse roots with increasing tree size/age (Vanninen and Mäkelä, 2005). As discussed in Hoshika et al. (2013a), a rapidly growing species such as poplar preferentially allocates recently fixed carbon to new leaf and shoot growth (Dickson et al., 1998). When $O_3$ impairs leaf photosynthesis, the total carbon budget is reduced and the leaf + branch sink is affected at first. It is not surprising that $O_3$ impact on stem growth increased with tree aging. Ritter et al. (2015) showed that allocation of recent photo-assimilates to stems of *F. sylvatica* in a phytotron study was decreased by 2× ambient $O_3$ before total tree biomass was affected. The reduction in root biomass in not-protected trees may induce a limitation of water and nutrient supply and thus limit new leaf formation for compensatory response to $O_3$ injury (Shipley and Meziane, 2002; Hoshika et al., 2013a).

In poplar, over 90% of the photo-assimilates from mature leaves is allocated to lower stem and roots (Coleman et al., 1995). Because $O_3$ impacts are greatest in mature poplar leaves (Dickson et al., 2001), the response to $O_3$ exposure was actually greatest in lower stem and roots. A significant decrease of stem diameter occurred up to 2.5 m a.g.l. in not-protected trees, that corresponded to around 1/3 of the stem height. While tree height was also reduced, this effect was not significant. As a result, $O_3$ exposure significantly increased H/B, i.e. the ratio of tree height to diameter. This ratio is used to predict tree susceptibility to storm damage (Wonn and O'Hara, 2001). Higher ratios under $O_3$ pollution suggest higher probability of damage. As this response was not significant in younger trees, we conclude that the risk of storm damage to trees under $O_3$ pollution increases with increasing $O_3$ exposure and effects on carbon allocation to the stem. Ozone effects on stem shape, however, are species-specific, as demonstrated in a mature stand of *P. abies* and *F. sylvatica* exposed in free-air to 2× ambient $O_3$ for 8 years (Pretzsch et al., 2010; Matyssek et al., 2010a). Ozone fumigation induced a shift in the resource allocation into height growth at the expense of diameter growth. This change in allometry, however, still results in reduced stem stability.

Variability in carbon allocation and growth rate may lead to differences in wood properties that can be evaluated through basic density and related parameters such as the percentage of solid fraction and cell bounded water. After six-years, we did not observe significant changes in the physical wood structure. Woody samples collected along the stem from not-protected and protected trees showed similar basic density, which was consistent with no impact on the solid fraction forming the cell wall matrix (WS) as well as the water fraction bounded to the wall (BW). No effect on basic density may be due to the reduced sensitivity of the developing xylem to $O_3$ during the late summer (September–October) when visible foliar $O_3$ injury usually occurred on not-protected trees (Hoshika et al., 2012). In poplar, the basic density is closely correlated with the vessel lumen area (Cocozza et al., 2011) which in turn depends on rate and duration of the cell expansion phase (Deslauriers et al., 2009). In Mediterranean environment, the maximum rate of cambium activity and cell expansion in poplar takes place in mid May-mid June and then decreases rapidly (Deslauriers et al., 2009). We hypothesize that the plant water-carbon imbalance in our $O_3$-exposed poplar trees took place when the majority of the xylem elements had reached the final size. These results emphasize the central role of cambium phenology and the xylem cell maturation processes in the modulation of plant response to environmental stresses (Pallara et al., 2012).

Interestingly, however, ambient $O_3$ increased wood moisture, free water and thus green density. The reasons of such an increased wood moisture may be related to impaired stomatal control over water losses, that is an important effect of $O_3$ (Paoletti and Grulke, 2010; Hoshika et al., 2013b, 2014) and was demonstrated to occur also in our poplar trees (Hoshika et al., 2012). Further investigations, however, are warranted, because a high wood moisture implies a low net calorific value and thus reduced yield of wood fuel for energy generation (Brand and De Muñiz, 2010) as well as longer drying schedule to reach the lumber standard because the maximum moisture content allowable for construction lumber is ~20%.

In conclusion, this long-term EDU experiment showed that 1. $O_3$ effects on biomass allocation above ground depended on the tree ontogenetic stage, 2. Ambient $O_3$ exposure reduced biomass of roots and stem, but did not change biomass allocation, 3. The stem shape changed due to $O_3$, so that tree susceptibility to storm damage is increased, 4. Moisture content of wood was increased by $O_3$ exposure. Two basic types of physiological mechanisms contribute to growth decline: (i) mechanisms leading to the reduction of carbon assimilation and (ii) mechanisms leading to modification of the resource economy. Investigating root biomass in adult trees planted in the ground is obviously challenging and thus the processes relating to whole-plant carbon allocation have been little investigated as compared to the processes governing carbon assimilation (Genet et al., 2009). Knowledge about the differential response of tree organs to $O_3$ and overall resource allocation at the whole-plant level under stress impact is a prerequisite for understanding ecological performance and economic yield (Matyssek et al., 2005). Our result warrant more research on age-related changes in carbon allocation and wood properties of trees under future climate and $O_3$ pollution.

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# *III. BVOC responses to ozone and nitrogen in silver birch*

Carriero G., Brunetti C., Fares S., Hayes F., Hoshika Y., Mills G., Tattini M., Paoletti E.

*Environmental Pollution* (Under revision)

# BVOC responses to nitrogen and ozone in silver birch

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## Abstract

Emission of BVOC (Biogenic Volatile Organic Compounds) from plant leaves in response to ozone ($O_3$) and nitrogen (N) exposure is poorly understood. For the first time, BVOC emissions were explored in a forest tree species (silver birch, *Betula pendula*) exposed for two years to realistic levels of $O_3$ (36, 49 and 70 ppb as daylight average) and N (10, 30 and 70 kg $ha^{-1}$ $yr^{-1}$, applied weekly to the soil as ammonium nitrate). The main BVOCs emitted were: α-pinene, β-pinene, limonene, ocimene, (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and hexanal. Ozone exposure increased BVOC emission and reduced total leaf area. The effect on emission was stronger when a short-term $O_3$ metric (concentrations at the time of sampling) rather than a long-term one (AOT40) was used. The effect on leaf area was not able to compensate for the stimulation of emission, so that responses to $O_3$ at leaf-unit and entire-plant level were similar. Nitrogen fertilization increased total leaf area, decreased α-pinene and β-pinene emission, and increased ocimene, hexanal and DMNT emission. The increase of leaf area changed the significance of the emission response to N for most compounds. Nitrogen fertilization mitigated the effects of $O_3$ exposure on total leaf area, while the combined effects of $O_3$ and N on BVOC emission were additive and not synergistic. In conclusion, $O_3$ and N pollution have the potential to affect global BVOC via direct effects on plant emission rates and changes in leaf area.

## 1. Introduction

Elevated tropospheric ozone ($O_3$) concentrations and nitrogen (N) deposition are main drivers of global change (Bytnerowicz *et al.*, 2007). High solar radiation, hydroxyl radicals, nitrogen oxides ($NO_x$), carbon monoxide (CO), methane ($CH_4$) and volatile organic compounds (VOC) from man-made (anthropogenic) and natural sources (biogenic) favor $O_3$ formation (Jenkin and Hayman, 1999). Ground-level $O_3$ concentration has been increasing in the Northern Hemisphere since the pre-industrial age to a present annual average of 35-40 ppb in the mid-latitudes of the Northern Hemisphere (Fowler *et al.*, 2009). Deposition of reactive N has increased globally from 32 Mt $y^{-1}$ in 1860 to ~112-116 Mt $y^{-1}$ at present (Reay *et al.*, 2008). Current projections estimate a further increase of the global annual N deposition to around two times the current levels by 2050 (Galloway *et al.*, 2004), while estimates of future $O_3$ trends are more complex and depend on emission scenarios of precursors (VOC, $NO_x$, $CH_4$, CO) and climate change (Young *et al.*, 2013; IPCC, 2013). These enhanced $O_3$ pollution and N deposition are already affecting the structure and function of plant ecosystems (Bobbink *et al.*, 2010; Ainsworth *et al.*, 2012). Plants release biogenic VOC (BVOC) for protection against stress including $O_3$ exposure (Loreto *et al.*, 2004), wounding (Loreto and Velikova, 2001), water deficit (Ormeño *et al.*, 2007) and heat (Holopainen and Gershenzon, 2010). At a global scale, the emission of BVOC from plants greatly exceeds the emission from anthropogenic sources (Peñuelas and Llusià, 2003). BVOC are involved in plant growth, reproduction and defense and contribute to atmospheric chemistry, such as $O_3$ and aerosol formation (Peñuelas and Staudt, 2010). BVOC include isoprenoids (isoprene and monoterpenes) as well as alkanes, alkenes, carbonyls, aldehydes, alcohols, esters, ethers and acids (Kesselmeier and Staudt, 1999). $C_6$ and $C_9$ aldehydes and alcohols, also known as GLVs

(Green Leaf Volatiles), derive from the “lipoxygenase (LOX) pathway”. The main substrates of LOX enzymes are free fatty acids which may be generated from cell membranes in response to stress agents (Dudareva *et al.*, 2013). GLVs are thus emitted from mechanically damaged plant tissues (Holopainen, 2004; Holopainen and Gershenzon, 2010) such as when $O_3$ damages growth or cellular structures (Fares *et al.*, 2010). Ozone affects plant metabolic processes leading to reductions in carbon assimilation (Fares *et al.*, 2013), growth (Hoshika *et al.*, 2013), leaf area (Wittig *et al.*, 2009), water control (Hoshika *et al.*, 2014, 2015), and tolerance to other stressors such as drought (Wilkinson *et al.*, 2010). The effect of elevated $O_3$ exposure on BVOC emission have been shown to be both positive and negative depending on experimental conditions (e.g. temperature and $O_3$ exposure), plant species, type of BVOC and season (Peñuelas and Staudt, 2010). Two previous studies reported that $O_3$ fumigation did not significantly affect BVOC emission of silver birch (2× ambient and 1.3–1.4× ambient $O_3$ over one and two years in Vuorinen *et al.*, 2005 and Hartikainen *et al.*, 2012, respectively).

Eutrophication caused by human activities (Galloway *et al.*, 2008; Peñuelas *et al.*, 2013) has an important long-term impact on the functioning and biodiversity of terrestrial plant communities (Janssens *et al.*, 2010). Elevated levels of N deposition increase plant production but may also render trees more vulnerable to other air pollutants and natural environmental stressors (Lindner *et al.*, 2010). Nitrogen fertilization usually stimulates the production of new leaves and thus enhances total leaf area per plant (Meier and Leuschner, 2008). The effects of elevated N deposition on BVOC emission are still unclear. Ormeño and Fernandez (2012) reported that N fertilization favors terpenoid production and can indirectly influence carbon-based defense metabolism. Litvak *et al.* (1996) found increasing isoprene emission with increasing leaf N concentration, supporting the existence of mechanisms that link leaf N status to isoprene synthase activity. Blanch *et al*. (2007) found that N fertilization stimulated the emissions of BVOC in *Pinus halepensis* and *Quercus ilex*. When the effects of a combination of $O_3$ and N were investigated, high nutrition was usually beneficial under $O_3$ stress because of improved metabolism, growth and leaf formation (Pääkkönen and Holopainen, 1995; Landolt *et al*., 1997; Maurer and Matyssek, 1997). Research on the concurrent effect of $O_3$ and N on BVOC emission, however, is still very limited. One, possibly the only recent study reported that both $O_3$ and N reduced terpene emissions in two leguminous species of Mediterranean pastures (*Ornithopus compressus* and *Trifolium striatum*) (Llusià *et al*., 2014).

Our goal was to explore the effects of realistic exposure to $O_3$ and N, single and in combination, on BVOC emission by a forest tree species. Silver birch (*Betula pendula*) is a widespread broadleaf tree in temperate and boreal forests (Hynynen *et al.*, 2010) and a significant monoterpene emitter (Lindfors *et al.*, 2000; Vuorinen *et al.*, 2005). We tested the hypotheses that: (*i*) N deposition ameliorates the effects of $O_3$ exposure on BVOC emission and vice versa; (*ii*) long-term and short-term exposure to $O_3$ induce different effects on BVOC emission; (*iii*) N and $O_3$ effects on total leaf area compensate for the effects on BVOC emission at leaf-unit level. Such information is needed for estimating BVOC emission and carbon load to the atmosphere under projected climate conditions.

## 2. Materials and methods

### *2.1. Plant material and treatments*

Three year old saplings of silver birch (*Betula pendula*) obtained from a commercial tree nursery (British Hardwood Plants, UK) were grown in 6.5 l pots, filled with John Innes No 1 compost (J. Arthur Bowers). All plants were inoculated with mycorrhiza (obtained from Buckingham Nurseries, UK) at the time of planting and were watered twice a week or as required to maintain soil moisture content at near field capacity. Plants were placed in solardomes i.e. hemispherical glasshouses (3 m diameter, 2.1 m height) located at a rural site near Bangor, North Wales, UK. The experiment started on April 13th, 2012, and $O_3$ exposure started after 17 days of adaptation. The saplings were exposed to $O_3$ from 30 April to y September, 2012 and 17 May to 16 September, 2013. Each one of the seven solardomes had a different $O_3$ target regime following a weekly $O_3$ profile based on an episodic profile recorded at a rural $O_3$ monitoring site (Aston Hill, Wales, UK, 52_500N, 3_030W), with treatments designed to reflect future $O_3$ scenarios, with peak concentrations reduced by more than the background (for details see Hewitt *et al*., 2014). The solardomes were ventilated at a rate of two air changes per minute with charcoal-filtered air injected with controlled amounts of $O_3$. Ozone was provided by a G11 $O_3$ generator equipped with oxygen generator (Dryden Aqua, UK). Concentrations were determined by a computer-controlled $O_3$ injection system (Lab VIEW version 8.6, National Instruments, Texas, US). Ozone was distributed to each solardome via PTFE tubing, with the concentration inside each solardome measured for 5 min every 30 min using two $O_3$ analyzers (400a, Enviro Technology Services, Stroud, UK) of matched calibration. Four N treatments were applied weekly in each solardome as ammonium nitrate in 200 ml water with added nutrients (P, K, Na, Fe, S, Mn, Mo, Ca, Mg, Cu, Zn, B) per pot to avoid nutrient deficiency overriding the N effect. Nitrogen treatments were 10, 30, 50 and 70 kg N $ha^{-1}$ $yr^{-1}$, and were structured as a split-plot factor inside the main $O_3$ factor. Nitrogen doses were selected to reflect the range of N in the soil and expected atmospheric N deposition (Dise and Wright, 1995; Goulding *et al.*, 1998; Rodà *et al.*, 2002). In one solardome, ambient air temperature, photosynthetically active radiation (PAR) and vapor pressure deficit (VPD) were continuously monitored by an automatic weather station (Skye Instruments Ltd, Llandridod Wells, UK). In this experiment, because of the need to sample under comparable environmental and plant growth stage conditions, a subset of the total $O_3$/N treatments was used and three solardomes with three representative $O_3$ and N levels were selected. Mean daily $O_3$ levels during the sampling period were: low (weekly average of daylight concentrations was 36 ± 0.2 ppb), medium (49 ± 1.1 ppb) and high (70 ± 1.8 ppb), treatments 1, 5 and 7 in Hewitt *et al.* (2014). The levels of N used for this experiment were: low, 10 kg $ha^{-1}$ $yr^{-1}$; medium, 30 kg $ha^{-1}$ $yr^{-1}$; and high, 70 kg $ha^{-1}$ $yr^{-1}$.

### *2.2. Sampling of BVOC emission*

BVOC sampling was carried out from 10 am to 4 pm from 19 – 26 July 2013 (9 -10 weeks after the start of the second exposure period) during optimal environmental conditions and repeated three times for each solardome within one week. In each solardome, three saplings per each N treatment were randomly selected. Average air temperature, PAR and

VPD inside the solardomes during the sampling were 29.9 ± 0.3 °C, 704 ± 39 μmol $m^{-2}$ $s^{-1}$, 2.00 ± 0.09 kPa. Soil moisture content was determined after every sampling with a hand-held soil moisture probe (ML2xThetaProbe, HH2 Moisture Meter; Delta T Services, Cambridge UK) and ranged 23.4 to 32.3 $m^3$ $m^{-3}$. A portable gas exchange system equipped with a controlled-environment 6-$cm^2$ broadleaf cuvette (LI6400, Li-Cor, Lincoln, USA) was used to measure net photosynthesis (A) and stomatal conductance ($g_s$) at constant conditions of PAR (1000 μmol $m^{-2}$ $s^{-1}$), leaf temperature (25 °C) and $CO_2$ concentration (400 ppm). Per each sapling, 1-2 fully expanded, healthy leaves of the same leaf order (4th-6th from the tip of a shoot) were sampled. According to the methodology in Loreto *et al.* (2001), when A reached a steady-state, the outlet tube from the leaf cuvette was replaced with a teflon tube, and the air stream exiting from the cuvette was used as a sampling port for BVOC by a silico-steel cartridge packed with 200 mg of tenax (Superlco, PA, USA). Tenax is a very hydrophobic and adsorbent material with high thermal stability generally used for trapping BVOC (Dettmer and Engewald, 2002). The flow rate through the leaf cuvette was maintained at 500 μmol $s^{-1}$, and a subsample of 200 mL $min^{-1}$ (130 μmol $s^{-1}$) was pumped through the cartridge with an external pump (AP Buck pump VSS-1) for a total volume of 6 L of air. Blank samples of air without a leaf in the cuvette were collected every day in each solardome before and after the BVOC samplings. Finally, the cartridges were sealed and stored at 4 °C until the analysis.

### *2.3. BVOC analysis*

The cartridges were analyzed using a Perkin Elmer Clarus 580 gas-chromatograph coupled with a Clarus 560 Mass-Detector and a thermal-desorber Turbo Matrix (Perkin Elmer Inc., Waltham, MA, USA). The gas-chromatograph was equipped with an Elite-5-MS capillary column (30 m length, 250 μm diameter and 0.25 μm film thicknesses). The carrier gas was helium. The column oven temperature was kept at 40 °C for 5 min, then increased with a 5 °C $min^{-1}$ ramp to 250 °C and maintained at 250 °C for 5 min. Mono- and homo-terpenes (C10 and C11) and compounds other than terpenes (including GLV) were identified using the NIST library provided with the GC/MS Turbomass software. GC peak retention time was substantiated by analysis of parent ions and main fragments of the spectra. Commercially available reference standards were used to create the calibration curves and to quantify the emissions. The reference standards for DMNT and (E)-β-ocimene were not available, therefore the concentration of these compounds were calculated using the calibration curve of (Z)-ocimene. To normalize the BVOC results, the quantities of terpenes collected from the empty cuvette (blanks) were subtracted from the plant emission results. The quantification of total BVOC emission was performed using authentic gaseous standards (Rivoira, Milan, Italy) or liquid standards (Sigma Aldrich, Milan, Italy).

### *2.4. Leaf harvest and BVOC calculation*

After BVOC sampling, all leaves (longer than 5 mm) of each sampled plant were harvested and leaf area was estimated from a leaf weight: leaf area relationship established using spare plants during the growing season. Total leaf area per plant ($m^2$) was calculated with the aim of scaling up from the leaf-unit level to the entire-plant level under the assumption that reduced BVOC emission from shaded leaves is negligible in three years old saplings of silver birch. BVOC emission was finally expressed per leaf-unit (ng $m^{-2}$ $s^{-1}$) and per entire-plant (ng $plant^{-1}$ $s^{-1}$).

### *2.5. Statistical analyses*

Before analysis, data were checked for normal distribution using Kolmogorov-Smirnov test. While the N factor was replicated, the $O_3$ factor was not. Therefore, a two-way ANOVA (N x $O_3$) could not be applied, and the effect of $O_3$ was analyzed using linear regression analysis within each N level. As $O_3$ metric, we used either hourly $O_3$ concentrations at sampling time or seasonal AOT40 (from the start of exposure to sampling), i.e. the accumulated $O_3$ hourly concentrations during daylight hours, above a threshold of 40 ppb (CLRTAP, 2010). Leaf area was correlated only with seasonal AOT40, because this parameter cannot change in the short term of an hour. When the regression lines were significant, i.e. when an $O_3$ effect was recorded, the effect of N was analyzed by ANCOVA (analysis of covariance) to test differences in slope or y-intercept among the regression lines. When no $O_3$ effect was recorded, i.e. when the regression lines showed $p>0.05$, data of each N level were grouped and one-way ANOVA (analysis of variance) was applied for testing the main effect of N. Differences were tested by the Bonferroni test. Results were considered significant at $p\leq 0.05$. All statistical analyses were performed using STATISTICA 7.0 and SPSS software for Windows.

## 3. Results

### *3.1. Effects of nitrogen and ozone on total leaf area and gas exchange*

Nitrogen increased total leaf area per plant (Fig. 1, right column), as the average value at high N was significantly higher than the values at low and mid N. Long term $O_3$ exposure, expressed as AOT40, significantly decreased leaf area only at low N conditions (Fig. 1, left column). Neither

$O_3$ exposure nor N fertilization significantly affected instantaneous A and $g_s$ as recorded at the time of sampling (data not shown). The average values of A and $g_s$ were 10.2 ± 0.5 μmol $m^{-2}s^{-1}$ and 0.20 ± 0.01 mol $m^{-2}$ $s^{-1}$, respectively.

### *3.2. Effects of nitrogen and ozone on BVOC emission*

Monoterpenes i.e. α-pinene, β-pinene, limonene and ocimene (sum of (E)-β-ocimene and (Z)-ocimene), the homoterpene (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and the GLV hexanal were the main compounds emitted by silver birch. Limonene was the most abundant, reaching values up to 60 ng $m^{-2}$ $s^{-1}$. BVOC emission significantly increased with increasing $O_3$ concentrations at sampling time (Table 1; Fig. 2). Short-term $O_3$ exposure significantly increased α-pinene, limonene, ocimene and DMNT in all N treatments (from ≈6.8, 19, 2.0 and 0.11 to ≈13.2, 45, 4.6 and 0.25 ng $m^{-2}$ $s^{-1}$, respectively), while the increase in the emission of β-pinene and hexanal (from ≈2.2 and 9.4 to ≈2.7 and 14.1 ng $m^{-2}$ $s^{-1}$, respectively) was not significant at low N. When we up-scaled at the entire-plant level, the results were similar, although with different statistical significance for hexanal and DMNT. Seasonal AOT40, i.e. long-term $O_3$ exposure, still increased BVOC emission, although few statistically significant responses were recorded (Table 1). AOT40 increased DMNT emission in all N treatments, β-pinene at low and high N, and hexanal at medium and high N. Up-scaled emissions revealed that AOT40-induced increases were significant only in few cases, i.e. for β-pinene (high N), ocimene (mid N), hexanal (low N) and DMNT (mid and high N). Individual monoterpene species showed a compound-specific response to N (Table 2; Fig. 2). Both α-pinene and β-pinene decreased with increasing N (from ≈12 and 3.4 to ≈6 and 1.2 ng $m^{-2}$ $s^{-1}$, respectively), limonene and DMNT did not change (27 ng $m^{-2}$ $s^{-1}$, on average), while ocimene and hexanal increased (from ≈1.6 and 10.6 to ≈3.6 and 11.5 ng $m^{-2}$ $s^{-1}$, respectively). When emissions were calculated at leaf-unit or entire-plant level, few changes occurred. At total-plant level, N had no effect on α-pinene, significantly increased DMNT, and had a stronger effect on ocimene emission.

## 4. Discussion

Our study confirmed previous findings that silver birch is a monoterpene emitter (Vuorinen *et al.,* 2005; Ibrahim *et al.*, 2010), releasing mainly limonene and α-pinene. In particular, silver birch emissions are dominated by monoterpenes during the summer, when leaves are fully expanded and under high light and temperature conditions which favor BVOC biosynthesis (Hakola *et al.*, 1998), by homoterpenes at late summer (Hartikainen *et al*., 2012), and by sesquiterpenes at early summer (Zhang *et al.*, 1999; Vuorinen *et al.*, 2005). A recent study also pointed out that BVOC emission changes across a latitudinal gradient in Finland (Maja *et al.*, 2015), thus suggesting that *B. pendula* has a high degree of genetic variability and BVOC emission is an adaptive feature in response to local climate conditions. Hexanal and DMNT were less abundant compared to monoterpenes. Hexanal is a GLV usually formed from the breakdown of membrane lipids under the action of lipoxygenase and hydroperoxide lyase enzymes (Heiden *et al.*, 2003; Fares *et al.*, 2010), while DMNT is an homoterpene especially released when plants are under stress e.g. by elevated $O_3$ exposure (Vuorinen *et al*., 2005) and herbivore attack (Dicke, 1994). Constitutive BVOC can be stored and emitted at optimal conditions as well as under stress, while induced BVOC are typically produced and emitted upon stress (Joó *et al*., 2011). Knowledge on BVOC emission under stress is still imperfect, in particular when $O_3$ and N impacts on vegetation are investigated.

Ozone is well known to co-occur with N pollution (Tjoelker and Luxmoore, 2006; Bassin *et al*., 2008), but despite the multitude of studies in which $O_3$ and N effects on vegetation were individually studied, the combined effect have poorly been investigated. Therefore, we initiated a long term experiment where silver birch saplings were exposed to realistic levels of $O_3$ combined with different levels of N fertilization. Our results showed that N increased total leaf area per plant and ameliorated the negative effects of $O_3$ on total leaf area. Under increased N availability, in fact, the resource allocation to leaf production is prioritized (Ibrahim *et al.*, 1998; Cooke *et al.*, 2005; Li *et al.*, 2012). Also Pääkkönen and Holopainen (1995) reported that high N availability not only increased mean leaf size, but also conferred birch trees more resistance to $O_3$ stress in terms of increased biomass production. An $O_3$-induced decrease in leaf area is in agreement with impaired growth, accelerated leaf senescence and decreased mean leaf size in silver birch plants exposed to $O_3$ (Riikonen *et al.*, 2004). While the effects of N and $O_3$ on leaf area are well known, i.e. increase and decrease, respectively, this is the first study to quantify how such changes affect BVOC emission. Ozone exposure increased foliar emission of BVOC, and this effect was still appreciated when we up-scaled leaf emissions to the entire-plant level even if the plant biomass decreased in response to $O_3$ exposure. Although the $O_3$ effect was statistically weaker for hexanal and DMNT at entire-plant level, no changes occurred for the other compounds, suggesting that the $O_3$–induced decline of total leaf area was not able to compensate for the $O_3$–induced stimulation of leaf-unit BVOC emission. Similar to our study, Vuorinen *et al.* (2004) found that $O_3$

activates defence responses in plants under biotic stress, and triggers the emission of inducible BVOC from several defence pathways. The increased emission of the GLV hexanal under $O_3$ fumigation may be considered as a direct effect of the lipoxygenase cleavage of fatty acids in the plant membranes exposed to $O_3$ (Paoletti, 2007). GLV are typically released from damaged plant tissues (Holopainen, 2004; Holopainen and Gershenzon, 2010). The homoterpene DMNT is among the main inducible BVOC emitted by plants (Holopainen, 2004), which is consistent with the enhanced emission recorded in our study in response to $O_3$ exposure (from ≈0.12 to ≈0.25 ng $m^{-2}$ $s^{-1}$). Peñuelas and Staudt (2010) reviewed the studies on $O_3$ and isoprenoids and found that negative effects were more frequent for isoprene than for monoterpenes, for which mostly positive or no effects were reported. Peñuelas and Staudt (2010) justified the different responses of isoprene and monoterpenes by the fact that monoterpene-emitting plants usually store terpenes in specialized organs, which are under complex metabolic controls. Interestingly, our short-term $O_3$ exposure stimulated the emission of both constitutive BVOC i.e. pinenes and limonene, and induced BVOC, i.e. ocimene, hexanal and DMNT. In addition, it is worth noting that most of the previous studies used unrealistically high $O_3$ concentrations (150-300 ppb), whilst our study used instantaneous $O_3$

concentrations (36, 49 and 70 ppb as daylight average) that are well within the ambient range in mid latitudes of the northern hemisphere (EEA, 2007). Such concentrations ranged from present annual averages of 36-48 ppb at mid latitudes of the Northern Hemisphere (Fig. 1.1 in The Royal Society, 2008) to 60 ppb over the summer 2030 according to the A2 scenario in the same geographical region (Fig. 5.9a in The Royal Society, 2008). As we simulated a weekly profile of ambient $O_3$, peaks of 80-110 ppb occurred at the time of BVOC sampling in the high $O_3$ treatment. In order to test the effects of $O_3$, we used two metrics: the average concentrations recorded at the time of BVOC sampling, and AOT40, i.e. the accumulated exposure above 40 ppb from the start of $O_3$ exposure to the time of BVOC sampling. The former metric was representative of short-term exposure, while the latter summarized seasonal exposure. We expected that the cumulative impacts of $O_3$ on plant performance over the season affected BVOC emission differently from the exposure in the short term. In contrast, the direction of change did not change in response to short- and long-term exposure, as increased emission was always recorded. However, the effects of $O_3$ on BVOC emission were more significant when the short-term metric was used. This response is consistent with instantaneous leaf membrane damage and stimulation of defence mechanisms (Castagna and Ranieri, 2009). Due to reactive oxygen species generation following $O_3$ exposure, plant membranes can undergo lipid peroxidation (Paoletti, 2007). All processes located on plant membranes, including photosynthesis, can thus be affected, although no significant effects on photosynthesis were detected at the time of the BVOC sample collection. Stronger responses to an episodic $O_3$ metric than to a seasonal one are consistent also with the most common form of the hormesis principle (Fig. 1A in Calabrese, 2005), where low doses of a toxicant have a stimulatory effect while high doses gradually translate into an inhibitory effect. Controversial responses of secondary metabolism to $O_3$ have been frequently reported, and may be also due to length and level of exposure, occurrence of visible foliar $O_3$ injury, as well as to the $O_3$ metric used. A stimulation of plant defence mechanisms at low $O_3$ concentrations or in the initial phases of $O_3$ stress has been frequently reported (e.g. Pääkkönen *et al.*, 1996b), while long-term exposure usually results in a decline of biomass production (Hoshika *et al.*, 2013).

A compound-specific variability in the response to N was observed, that was likely due to different biosynthetic formation pathways of the emitted compounds (Kesselmeier and Staudt, 1999; Niinemets *et al.*, 2004). The monoterpenes α-pinene and β-pinene decreased with increasing N availability (from ≈12 to ≈6 ng $m^{-2}$ $s^{-1}$ and from ≈3.4 to ≈1.2

ng $m^{-2}$ $s^{-1}$, respectively), in line with previous findings which associated $CO_2$ fertilization in a poplar plantation with a decrease of isoprene emission (Rosenstiel *et al.,* 2003). The reduction of α-pinene and β-pinene emission under N or $CO_2$ enrichment may be explained by the carbon/nutrient balance hypothesis (CNBH) (Bryant *et al*., 1983): when a resource is abundant, plants allocate less carbon toward carbon-based secondary compounds and more carbon toward growth (Lerdau *et al.*, 1995, Penuelas and Estiarte 1998). This is also why monoterpene emissions were reported to significantly decrease in terpene-storing *Pinus halepensis* grown in a soil fertilized with nitrogen and phosphorous, with limited effect for the non-storing *Quercus ilex* (Blanch *et al*., 2007). In our experiment, limonene emission was 3-4 and 3-15 times higher than emission of α-pinene and β-pinene, respectively. Although these three compounds are the most volatile monoterpenes contained in birch leaves (Hakola *et al.,* 2001; Vuorinen *et al.,* 2005) and their synthesis is under enzymatic control (Loreto *et al.,* 1998), the effect of N fertilization on limonene was not significant. Nitrogen fertilization significantly stimulated the emission of the inducible BVOC ocimene, hexanal and DMNT (from ≈1.6, 10 and 0.18 to ≈3.6, 12 and 0.17, respectively), suggesting that excess N acted as a stress factor for our birch saplings. LOX products and homoterpenes (ocimene and DMNT) are released as a result of activation of different defence pathways, suggesting a stress response similarly to herbivory feeding (Holopainen and Gershenzon, 2010). The response to N fertilization changed for most compounds (α-pinene, DMNT, ocimene) when emissions were up-scaled at entire-plant level, confirming that leaf area is a critical input for estimating global BVOC emissions. However, our up-scaling procedure did not take into account that BVOC emission is typically reduced in response to low levels of irradiation and cooler temperatures, such as in shaded leaves, and VOCs emission is higher in mature leaves than in senescent leaves. Therefore we expect that in a dense forest such N-induced effect may be reduced. Nitrogen fertilization mitigated the effects of $O_3$ exposure on total leaf area, while no significant $O_3$ x N interaction affected BVOC emission, i.e. the effects were additive and not synergistic.

In conclusion, our results pointed out that $O_3$ exposure increased BVOC emission in silver birch saplings, while N had a compound-specific effect, i.e. inhibition in the case of pinenes and stimulation in the case of inducible BVOCs. The effects of $O_3$ and N were additive and not synergistic (hypothesis *i*, rejected). Effects on emission were stronger when a short-term $O_3$ metric (concentrations at the time of sampling) rather than a long-term one (AOT40) was used (hypothesis *ii*, rejected). Effects on leaf area changed the emission significance only in the case of

N (hypothesis *iii*, partly confirmed). We conclude that $O_3$ and N pollution have the potential to affect global BVOCs via direct effects on plant emission rates and changes in leaf area. These results warrant more research for incorporating the effects of these global change factors into future emission algorithms, and unravel their effects on atmospheric chemistry and plant ecology. Emissions of BVOC are a chief uncertainty in calculating the burdens of important atmospheric compounds like tropospheric $O_3$ itself, and monoterpene estimates show significantly larger model-to-model variation than isoprene estimates (Arneth *et al*., 2008).

**Table 1.** Statistical significance of ozone impacts on emission of α-pinene, β-pinene, limonene, ocimene, hexanal and DMNT at leaf-unit level and at entire-plant level, within each nitrogen treatment, i.e. 10 (low), 30 (mid) and 70 kg ha$^{-1}$ yr$^{-1}$(high). Ozone effects were tested by linear regression analysis versus ozone concentrations at sampling time (Fig. 2) or versus seasonal AOT40 (accumulated $O_3$ exposure above 40 ppb during daylight hours from sprouting to sampling, data not shown) (n.s., not significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$). Arrows summarize the direction of BVOC emission change.

| | | **Leaf-unit** [ng m$^{-2}$ s$^{-1}$] | | | **Entire-plant** [ng plant$^{-1}$ s$^{-1}$] | |
|---|---|---|---|---|---|---|
| **vs. $O_3$ concentration at sampling time** | **Low N** | **Mid N** | **High N** | **Low N** | **Mid N** | **High N** |
| α-pinene | * ↑ | ** ↑ | ** ↑ | * ↑ | ** ↑ | * ↑ |
| β-pinene | n.s. | * ↑ | ** ↑ | * ↑ | * ↑ | ** ↑ |
| limonene | * ↑ | * ↑ | * ↑ | * ↑ | * ↑ | * ↑ |
| ocimene | * ↑ | ** ↑ | * ↑ | * ↑ | ** ↑ | * ↑ |
| hexanal | n.s. | *** ↑ | ** ↑ | ** ↑ | ** ↑ | n.s. |
| DMNT | *** ↑ | * ↑ | ** ↑ | * ↑ | ** ↑ | * ↑ |
| **vs. seasonal AOT40** | | | | | | |
| α-pinene | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| β-pinene | * ↑ | n.s. | * ↑ | n.s. | n.s. | * ↑ |
| limonene | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| ocimene | n.s. | n.s. | n.s. | n.s. | * ↑ | n.s. |
| hexanal | n.s. | ** ↑ | ** ↑ | * ↑ | n.s. | n.s. |
| DMNT | ** ↑ | * ↑ | ** ↑ | n.s. | * ↑ | * ↑ |

**Table 2.** Statistical significance of nitrogen impacts on emission of α-pinene, β-pinene, limonene, ocimene, hexanal and DMNT at leaf-unit level and at entire-plant level. As the effects of ozone concentrations at sampling time were significant (Table 1), nitrogen effects were tested by analysis of covariance (ANCOVA) to determine significant differences among slopes or y-intercepts of the regression lines showed in Fig. 2 (n.s., not significant; *, *p*≤0.05; **, *p*≤0.01; ***, *p*≤0.001). Arrows summarize the direction of BVOC emission change.

| | **Leaf-unit** [ng $m^{-2}$ $s^{-1}$] | | **Entire-plant** [ng $plant^{-1}$ $s^{-1}$] | |
|---|---|---|---|---|
| | **slope** | ***y*-intercept** | **slope** | ***y*-intercept** |
| α-pinene | n.s. | ** ↓ | n.s. | n.s. |
| β-pinene | *** ↓ | | n.s. | *** ↓ |
| limonene | n.s. | n.s. | n.s. | n.s. |
| ocimene | n.s. | * ↑ | n.s. | *** ↑ |
| hexanal | * ↑ | | n.s. | *** ↑ |
| DMNT | n.s. | n.s. | n.s. | * ↑ |

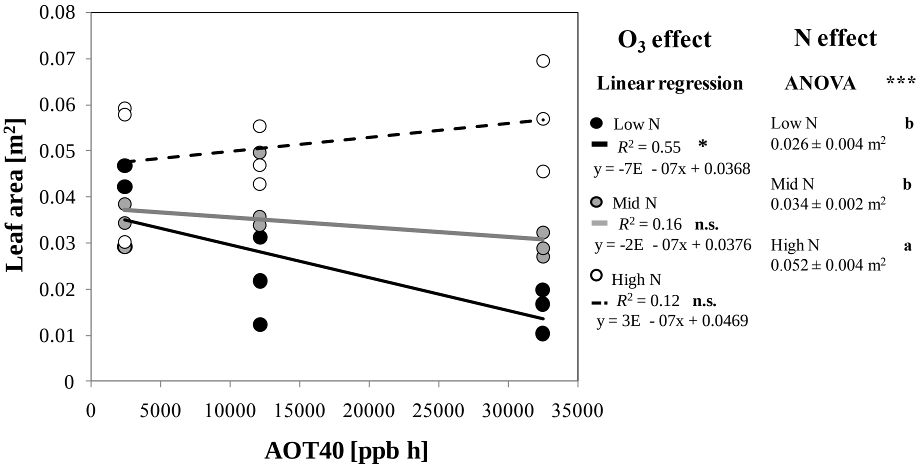

**Fig. 1** Total leaf area per plant as a response to seasonal AOT40 (accumulated $O_3$ exposure above 40 ppb during the daylight hours from sprouting to sampling) at three nitrogen (N) conditions in the soil, i.e. 10 (low), 30 (mid) and 70 kg ha$^{-1}$ yr$^{-1}$(high). Ozone effects were tested by linear regressions within each N level. Results are shown in the first column on the right. As $O_3$ effects were significant only at low N, N effects were tested by analysis of variance (one-way ANOVA). The second column on the right shows mean total leaf area (±SE) at each N level. Different letters show significant differences among N levels (Bonferroni's test, $p < 0.001$, N= 3).

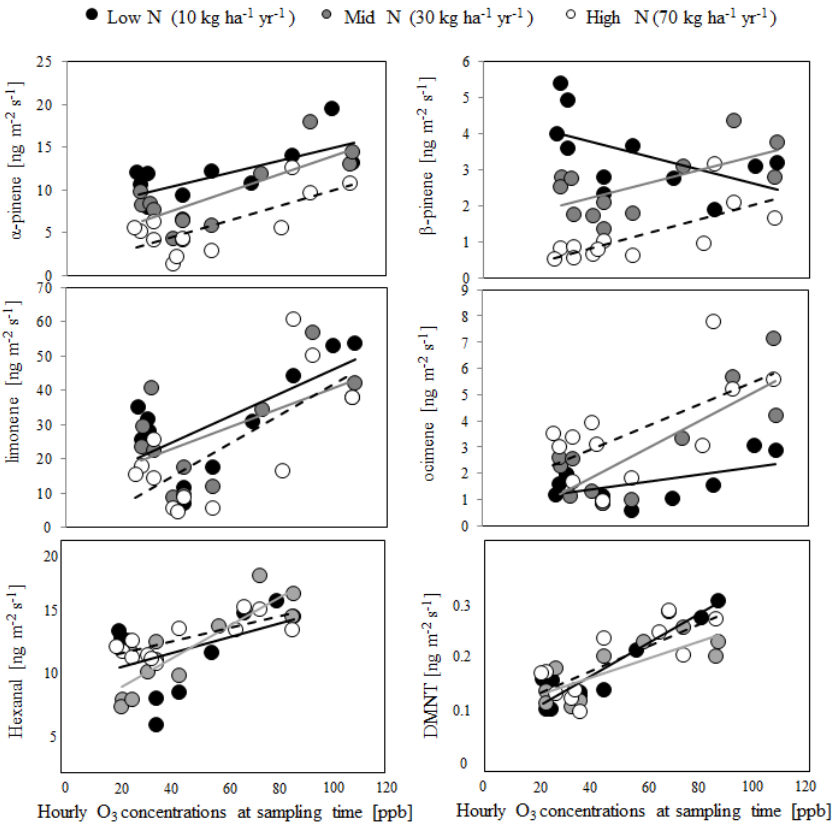

**Fig**. **2**. Emission of α-pinene, β-pinene, limonene, ocimene, hexanal and (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) at leaf-unit level as affected by mean hourly $O_3$ concentrations at sampling time. Ozone effects were tested by linear regressions at each nitrogen fertilization level (10, 30 and 70 kg $ha^{-1}$ $yr^{-1}$). Statistical results are in Tab. 1 and 2.

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# 2. GENERAL DISCUSSION

Main findings of the experiments included in this thesis are summarized in Table 1.

**Table 1.** Main results of the experiments reported in the original publications of the thesis

| | |
|---|---|
| **Paper I** | • Ozone exposure slowed down the closure of stomata<br>• Stomatal sluggishness was correlated to the ratio of stomatal ozone flux to net photosynthesis<br>• Stomatal sluggishness depended on ozone flux and on the capacity for detoxification and repair |
| **Paper II** | • Long-term ambient ozone exposure significantly reduced stem and coarse roots biomass<br>• Control plants were protected by EDU irrigation<br>• Ambient ozone decreased diameter of the lower stem and increased wood moisture content along the stem<br>• Ozone effects on biomass partitioning to above-ground organs depended on tree ontogenetic stage |
| **Paper III** | • Ozone exposure increased BVOC emission and reduced total leaf area<br>• Responses to an episodic ozone metric were stronger than to a seasonal one<br>• Nitrogen fertilization had a compound specific effects on BVOC emission and increased total leaf area<br>• Ozone and nitrogen impacted BVOC emissions individually with no significant interactions |

## 2.1. Ozone effects on steady-state leaf gas exchange and dynamic stomatal response in deciduous tree species

Ozone concentrations are increasing globally and affect physiological and biochemical processes of tree species. Physiological studies suggested that ozone decreases productivity primarily by lowering photosynthesis (Dizengremel, 2001; Long and Naidu, 2002). Significant damage occurs when ozone enters the leaf trough stomata (Wittig *et al.*, 2007). Ozone causes stomatal impairment, decreasing the ability to close, referred to as ozone-induced stomatal sluggishness (Wittig *et al.*, 2007; Paoletti and Grulke, 2010; Hoshika *et al.*, 2012). In our study, elevated ozone exposure slowed stomatal response in different Chinese tree species. Photosynthesis rate and stomatal conductance had a different response after ozone exposure. Ozone exposure decreased net photosynthetic rate in all three species (*A.altissima, F. chinensis* and *P. orientalis*) while slowed stomatal closing during severe water stress after leaf cutting and the effect was species-specific. This effect was greater in *F. chinensis* and smaller in *P.orientalis*. Accordingly also Paoletti (2005) reported impairment in stomatal function, i.e. sluggish closure after ozone exposure of *Arbutus unedo*. Such slower stomatal response was explained by accelerated senescence in the cell physiological processes (Paoletti *et al.*, 2009), reduced sensitivity to ABA (Mills *et al.*, 2009), or ozone-induced ethylene emission (Wilkinson and Davies 2010). There was no significant relationship between stomatal sluggishness and stomatal density. The slightly faster stomatal responses associated with higher stomatal density were not enough to reduce ozone injury to stomatal control. *A.altissima* and *F.chinensis* had higher stomatal ozone flux and higher impairment in stomatal control while *P.orientalis* had a slower stomatal ozone flux but had the lowest decrease in stomatal conductance after leaf cutting. This was explained by the more efficient limitation of stomatal ozone flux of *P. orientalis* which contributed to reduce injury to stomatal control under elevated ozone exposure. *F.chinensis* showed higher relationship between stomatal sluggishness and indices of ozone flux (stomatal ozone flux per net photosynthetic rate) compared to *A.altissima*. The ratio of stomatal ozone flux to net photosynthesis was the best parameter explaining the impairment of stomatal control induced by ozone. Such impairment was related not only to diffusion of ozone into leaves but also to biochemical detoxification capacity and repair (Paoletti *et al.*, 2008). Biochemical detoxification capacity is related to LMA because greater LMA implies higher density of mesophyll tissues, suggesting higher capacity of repair or detoxification (Wieser *et al.*, 2002). However, there was no difference in LMA between ozone treatments for any species. In conclusion, the physiological leaf traits affected stomatal sluggishness more than anatomical leaf traits. This ozone impairment to stomatal control may increase leaf water loss and stomatal ozone flux under rising ozone levels (Hayes *et al.*, 2012). Although the mechanisms of the sluggish response of stomata are still unclear, this study contributed developing knowledge into the mechanisms of plant response to ozone induced-stress.

## 2.2. Biomass allocation from adult trees under long-term ambient ozone stress

Ozone is the major pollutant currently responsible for visible foliar injury and reduced growth in trees (Karnosky *et al.*, 2007; Matyssek *et al.*, 2007). We investigated long-term effect of ambient ozone exposure on biomass of a poplar plantation comparing results with those obtained after three years of ozone exposure in the same experiment (Hoshika *et al.*, 2013; Katanić *et al.*, 2014). Trees were exposed for six years to ambient ozone and irrigated with EDU, as chemical ozone protectant for quantifying the effects of long-term ozone relative to non-protected plants. Plants were grown in a Mediterranean field site where AOT40 was found to exceed the critical level recommended for the protection of forests (UNECE, 2010). Under these conditions, ozone reduced above and below-ground biomass in EDU non-protected trees with major declines especially in stem and coarse roots. Accordingly ozone was shown to decrease biomass in open air conditions (Morgan *et al.*, 2003; 2005), decrease the partitioning of carbon to roots (Andersen, 2003; Wittig *et al.*, 2009) while EDU application increased stem and root biomass (Feng *et al*., 2010). Surprisingly, our experimental plants did not show any change in the allocation of shoot/root biomass after long-term ambient ozone exposure. The higher ectomychorrhizal (ECM) colonization was found in non-protected plants which indicated a transient response to different source-sink relationships in the ecosystem (Cudlin *et al.*, 2007). Both EDU protected and not-protected trees maintained the same within-plant distribution of carbon or relative weight of different plant organs. Leaf biomass decreased with ozone but was not affected by early leaf abscission (Hoshika *et al.*, 2012) and this response occurred both in Autumn 2010 (Hoshika *et al*., 2013) and in Spring 2014. The pattern of above ground biomass allocation changed after three and six years of ambient ozone exposure. After three years the major declines was in leaves and in lateral branches while after six years the major effect was on stem. Usually when ozone impairs leaf photosynthesis, the total carbon budget is reduced and leaf and branches are affected at first (Dickson *et al.*, 1998). Growth allocation shifts from branches to coarse roots with increasing tree age (Vanninen and Mäkelä, 2005) and ozone impact on stem growth also increases with tree aging. This demonstrated that ozone effect on above ground biomass allocation depended on tree ontogenetic stage. A significant decrease of stem diameter occurred up to 2.5 m a.g.l. in EDU non-protected trees. Ozone increased the ratio of tree height to diameter suggesting higher probability of storm damage to trees with increasing ozone pollution and effects on carbon allocation to the stem. However this ozone effect on stem shape was previously demonstrated in *Picea abies* and *Fagus sylvatica* exposed to ambient ozone for eight years (Pretzsch *et al.*, 2010; Matyssek *et al.*, 2010) and was related to a reduction of carbon sink strength induced by ozone (Sitch *et al.*, 2007). Ozone did not affect the wood structure after six years while interestingly increased wood moisture, free water and green density. The increased wood moisture can be explained by impaired stomatal control over water losses induced by ozone (Paper I) also in our poplar trees (Hoshika *et al.*, 2012). Processes relating to whole-plant carbon allocation have been poorly investigated as compared to the processes governing carbon assimilation (Genet *et al.*, 2009). We contributed enhancing research on carbon allocation and wood properties of trees under ambient ozone pollution.

### 2.3. Effect of ozone and nitrogen on plant BVOC emission

Enhanced ozone pollution and nitrogen deposition are affecting forest ecosystems (Bobbink *et al.*, 2010; Ainsworth *et al.*, 2012). Although the individual effects of ozone exposure and nitrogen on BVOC emissions have been studied (Peñuelas and Staudt, 2010; Ormeño and Fernandez, 2012), research on the concurrent effect of ozone and nitrogen on BVOC emission is still very limited. We tested the hypothesis that nitrogen deposition ameliorates the effects of ozone exposure on BVOC emission; we investigated long-term or short-term effect of ozone exposure on BVOC emission and we aim understanding if nitrogen and ozone effects on total leaf area compensate for the effects on BVOC emission at leaf-unit level. Monoterpenes, i.e. α-pinene, β-pinene, limonene, ocimene (Vuorinen *et al*. 2005), homoterpene, i.e. DMNT, and GLV, i.e. hexanal, were the main compounds emitted by silver birch. Nitrogen increased total leaf area conferring to birch trees more resistance to ozone stress in terms of increased biomass production (Pääkkönen and Holopainen, 1995) and compensating the effect on BVOC emission at leaf-unit level. Nitrogen had a compound-specific effect on BVOC emission related to the different biosynthetic formation pathway (Kesselmeier and Staudt, 1999), i.e. inhibition in the case of pinenes and stimulation in the case of inducible BVOC. α-pinene and β-pinene decreased with increasing nitrogen in the soil and this reduction was well explained by the carbon nutrient balance hypothesis (CNBH) (Bryant *et al*., 1983; Blanch *et al*., 2007). On the contrary nitrogen increased the emission of ocimene, hexanal and DMNT as a result of activation of different defence pathways (Holopainen and Gershenzon, 2010) suggesting that excess of nitrogen acted as stress factor. We used two ozone metrics for testing short term and seasonal effect of ozone exposure: the average concentrations recorded at sampling time and the AOT40, the accumulated ozone exposure above 40 ppb from the start of exposure to the time of BVOC sampling. The effect of ozone on BVOC emission was significant when the short-term metric was used and this was explained by the hormesis principle (Calabrese, 2005) where low doses of a toxicant have a stimulatory effect (Pääkkönen *et al*., 1996) and high doses have an inhibitory effect (Hoshika *et al*., 2013). Ozone decreased leaf area, accelerated leaf senescence and decreased mean leaf size (Riikonen *et al*., 2004). Ozone exposure increased foliar emission of BVOC but the ozone-induced decline of total leaf area was not able to compensate for the ozone-induced stimulation of leaf-unit BVOC emission. Short-term ozone exposure increased constitutive BVOC emission, i.e. pinenes and limonene, and induced BVOC, i.e. ocimene, hexanal and DMNT. Indeed ozone increased hexanal emission as a direct effect of plant damage (Holopainen and Gershenzon, 2010) and enhanced DMNT and ocimene with increasing ozone concentrations (36, 49 and 70 ppb as daylight average). In conclusion nitrogen fertilization mitigated the effect of ozone on total leaf area and there was no significant interaction of nitrogen and ozone on BVOC emission. Both ozone and nitrogen had the potential to affect BVOC via direct effects on plant emission rates and changes in leaf area.

# 3. CONCLUSIONS AND IMPLICATIONS

Ozone has the most widespread negative impact on vegetation than any other air pollutant (Ashmore, 2005). Forests are negatively affected by the present ozone levels which affect physiological and biochemical processes of tree species (Ainsworth *et al.*, 2012). Significant gaps in knowledge remain about the interactions of rising tropospheric ozone concentrations and variables associated with climate change. Increasing atmospheric ozone concentrations will negatively impact plant production, reducing the ability of ecosystems to sequester carbon. In this thesis I discussed the interactions of plant responses to ozone and soil nutrients considering implications for future climate change. Ozone deposition to vegetation represents the major sink for ozone and stomata play a fundamental role in determining ozone flux into the leaves (Kangasjarvi *et al.*, 2005). Enhanced ozone levels slow stomatal control which is related to stomatal ozone flux per net photosynthesis (Paper **I**). The impairment in stomatal function enhances leaf water loss and stomatal ozone flux with increasing ozone pollution. Such impairment is related not only to diffusion of ozone into leaves but also to biochemical detoxification capacity, repair and carbon assimilation strategy (Paper **I**). Indeed ozone reduces plant productivity and causes oxidative stress which decreases photosynthesis, plant growth and biomass accumulation. When ozone impairs gas exchange traits in leaves, the total carbon budget is reduced and plant growth is affected at first (Paper **II**). Ozone reduces plant biomass and the pattern of above and below-ground biomass allocation is related to tree ontogenetic stage. Leaf and branches are affected at first and with increasing tree age ozone affects coarse roots and stem growth. Ozone also increases wood moisture along the stem

(Paper **II**) as a result of impaired stomatal control over water losses (Paper **I**). Individual biotic and abiotic stresses, i.e. ozone and nutrient availability are well known to influence the emission of biogenic volatile organic compounds from plants which play a central role in atmospheric reactivity, particularly in ozone-forming reactions. Plants release BVOC for alleviating oxidative stress induced by ozone (Paper **III**). The cumulative impacts of ozone on plant performance over the season affect BVOC emission differently from the exposure in the short-term. Short-term ozone exposure significantly stimulates BVOC emission as plant defense mechanism while long-term ozone exposure results in a decline of biomass production (Paper **II**). Elevated levels of nutrient in the soil, such as nitrogen availability, increase plant production but render trees more vulnerable to other air pollutants and natural environmental stressors. Nitrogen has a compound-specific effect on BVOC emission as a result of different biosynthetic pathways of the compounds, i.e. inhibition in the case of constitutive BVOC (pinenes) and stimulation in the case of inducible BVOC (GLV, homoterpenes). The inhibition of constitutive BVOC is related to the different allocation of carbon. Indeed, when a resource is abundant plants allocate less carbon toward carbon-based secondary compounds and more carbon toward growth (CNBH Carbon nutrient balance hypothesis). The stimulation of inducible BVOC is related to the activation of different defense pathways as a stress response induced by nitrogen similarly to ozone exposure (Paper **III**). A mechanistic understanding of BVOC emissions may have importance for estimating future alterations of emissions because of climate change.

The main conclusion of this study is that the current ambient ozone concentrations and climate factors are already having extensive impacts on vegetation. Ozone causes impairment of leaf gas exchange and shift in biomass allocation especially after long-term exposure. In addition ozone enhances BVOC emission, while soil nutrient availability has a compound-specific effect on emissions which depends on different biosynthetic pathways of each compound. The increase of tropospheric ozone pollution is affecting forest ecosystems as climate change. Thus, there is an urgent global need for coordinating effort to reduce the emissions of the precursors of ozone pollution to benefit security of food supplies, improve human health and help reduce global warming. This study contributed enhancing knowledge on effects and mechanisms of action of ozone and climate change on forest tree species both in the field and in controlled conditions.

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