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An investigation of ozone uptake by plants and antioxidant effect of plant isoprenoids

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TABLE OF CONTENTS

Legend to Tables

Legend to Figures

Summary

Ozone is a pollutant and oxidant molecule for humans and plants in the atmosphere, present in the range of parts per billion (ppb). Plant ecosystems represent a sink for ozone, although the mechanisms of removal and the damages are still unclear. The aims of this PhD study were a) to investigate the capacity of plants to remove atmospheric ozone, b) to partition between stomatal and non-stomatal components of the ozone flux c) to determine if BVOC (Biogenic Volatile Organic Compounds) emitted by the plant can scavenge ozone, in turn detoxifying the plant. The approach of the study was the use of cuvette systems, namely enclosures. Different of these enclosures were used, containing portions of leaves, entire leaves or the all plants. The uptake of ozone (ozone removed by the plant) was calculated considering the difference in ozone concentration between the inlet and the outlet of the enclosure. The work is articulated in a general introduction on ozone effects on plants and BVOC emission, including the state of the arts on these topics. Following are presented 4 cases of study, each one focussing the attention on different aspects of the plant-ozone-BVOC interactions. Results showed that the main responsible for ozone uptake are stomata, and BVOC emitted by the plants (isoprene and monoterpenes) increase the detoxification and the ozone uptake, thus suggesting that the notion that ozone damage is proportional to ozone uptake is not always correct.

Sommario

L'ozono è una molecola inquinante ed ossidante per le piante e per gli esseri umani, presente in atmosfera nell'ordine di parti per bilione (ppb). Gli ecosistemi vegetali riescono a rimuovere ozono dall'atmosfera, sebbene i meccanismi di rimozione ed i danni che l'ozono provoca non sono ancora stati completamente svelati. Gli obiettivi di questo dottorato sono stati a) verificare la capacità delle piante nel rimuovere ozono in bassa troposfera, b) distinguere tra componente stomatica e non stomatica del flusso di ozono, c) determinare se i BVOC (Biogenic Volatile Organic Compounds – Composti Organici Volatili Biogenici) emessi dalle piante hanno funzione antiossidante, contribuendo a rimuovere ozono prima che questo entri in contatto con le pareti cellulari. Lo studio è stato condotto principalmente mediante l'uso di cuvette contenenti porzioni di foglie, intere foglie o intere piante. La tesi è

articolata in una introduzione in cui vengono descritti gli effetti dell'ozono sulle piante e l'emissione di BVOC, inquadrando lo stato delle conoscenze in questo specifico settore dell'ecofisiologia. A seguire, vengono descritti 4 casi di studio, ognuno dei quali approfondisce un diverso aspetto delle interazioni ozono-BVOC. I risultati mostrano che l'apertura stomatica controlla gran parte del flusso di ozono, ed i BVOC emessi dalle piante (isoprene e monoterpeni) contribuiscono a rimuovere ozono aumentando il flusso totale, ed invalidando la teoria per la quale il danno da ozono è proporzionale al flusso stomatico.

1. Introduction

1.1 Ozone, a threat for forest ecosystems

Ozone (O_3) can be considered as a greenhouse gas, since it may be responsible of an increase of temperature of 0.5 °C after the industrial revolution (IPCC 2001, Shindell et al. 2004). Ozone is a very reactive molecule, which is rapidly formed and destroyed in the atmosphere. Ozone is present in the low troposphere in the range of 0 to more than 100 ppb depending of the abundance of its precursors, mainly NOx (nitrogen oxides) and VOC (biogenic and anthropogenic Volatile Organic Compounds), through a photochemical cycle (Prinn, 2003; Monks, 2005). Reactions below describe this cycle in a simplified way:

$$O_2 + hv \rightarrow O + O \quad \text{(R1)}$$

$$NO_2 + hv \rightarrow NO + O \quad \text{(R2)}$$

$$O + O_2 + M \rightarrow O_3 + M \quad \text{(R3)}$$

$$NO + O_3 \rightarrow NO_2 + O_2 \quad \text{(R4)}$$

being *hv* the UV radiation, M a general molecule absorbing excess energy and allowing the stabilization of O_3, NO and NO_2, respectively, nitrogen monoxide and nitrogen dioxide. High presence of atmospheric NO favours the rate of removal of O_3, but the presence of VOC can increase the ratio NO_2/NO by reacting with hydrossilic radicals ($HO^{\bullet}$) and forming other radical species (alchiperoxyradical, $RO^{\bullet}$, and hydroperoxyradical, $HOO^{\bullet}$):

$$NO + ROO^{\bullet} \rightarrow RO^{\bullet} + NO_2 \quad \text{(R5)}$$

$$NO + HOO^{\bullet} \rightarrow HO^{\bullet} + NO_2 \quad \text{(R6)}$$

Ozone concentration in the troposphere is increasing due to increasing production of precursors (Chameides 1988). In clean air, an equilibrium between ozone formation and in situ photochemical destruction and deposition at the earth's surface is reached (Roelofs and Lelieveld 1997) resulting in a daily "mixing ratio" of 10 to 40 ppb. The background concentration (a level not directly interested by local anthropogenic influence) is continuously increasing (Virganzan 2004) and levels exceeding 60 ppb, considered as

phytotoxic, are predicted to occur in year 2100 on 50% of the global forest area (Fowler et al. 1999). Ozone has also other important chemical interactions, forming reactive hydroxyl radicals (OH), and thus influencing the retention time of many gasses in the atmosphere (Prinn 2003). Ozone is also involved in nucleation events when reacting with VOC, leading to aerosol and particle formation (Bonn and Moortgat 2003).

1.1.1 Ozone effects on plants and the antioxidant mechanisms of plant protection

Since the sixties, scientists started to focus the attention on the negative effects of ozone on vegetation (Berry and Ripperton, 1963). Ozone is now considered the most dangerous oxidant molecule for plants. Chronic stresses with exposure to moderate ozone concentrations usually produce biochemical and physiological changes (Darrall, 1989; Sandermann et al. 1997; Zheng et al. 2002). The attack of ozone to the chloroplast membranes inhibits the ribulose bisphosphate carboxylase/oxygenase enzyme (Rubisco) leading to a limited carbon assimilation by photosynthesis and a decrease in plant growth (Guderian et al. 1985). Exposure to acute tropospheric ozone levels leads to visible injuries (Bussotti et al. 2003; Vollenweider and Gunthardt-Georg 2005).
Plants act as a sink for ozone, adsorbing it by cuticle deposition or absorbing it thorough stomatal apertures (Fig. 1).

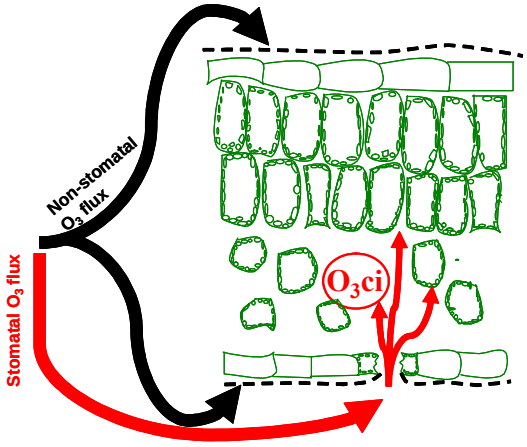

Figure 1: Scheme of a crosscut through one stomatal pore and stomatal and non stomatal fluxes of ozone. O_3ci is the intercellular ozone concentration.

The first process is relevant only under high surface moisture (Altimir et al. 2006). Chronic exposures to ozone can however 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, and thus negatively affecting the water balance (Shreuder et al. 2001). However, Paoletti et al. (2006) observed an increase of wax formation under acute ozone stress, and Vanhatalo et al. (2000) found an increase of wax formation when high chronic ozone was associated to high CO_2 levels. The stomatal absorption of ozone is often dominant (UNECE 2004), because permeability of ozone through the cuticle is very low (0.001 mm s^{-1}, Kerstiens and Lendzian, 1989). This aspect will be analyzed more in detail in the next chapter. In conditions of intense light, ozone damage is increased both when stomata are closed, and when they are fully opened. In the first case, the energy in excess captured by the photosystems is not quenched by the electron transport, due to the limited amount of CO_2 at the carboxylation site. This, and the large amount of reactive oxygen species formed by ozone, rapidly leads to photoinhibition (Guidi et al. 2002). In the second case, the high amount of ozone entered through stomata reacts directly with cell surfaces, and indirectly induces the formation of free radicals, with a cascade of negative effects on plant physiology and biochemistry.

It is still unclear which are the reactions that ozone drives inside the cells, and that lead to cellular damage. A first mechanism of damage is the direct membrane peroxydation which alters membrane permeability (Heath, 1994) and results in an accumulation of end-products of lipid peroxydation such as malonyldialdheyde (MDA) (Heath and Parker 1968). Most probably however 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 (O'_2, OH', and H_2O_2, collectively denoted as ROS (Reactive Oxygen Species)). ROS are per se strong oxidants and also act as signal molecules in plants. ROS start hypersensitive responses, including the activation of ethylene, salycilic acid and jasmonate, and lead to programmed cell death in response to ozone (Kangasjarvi et al. 2005). The main effects of ozone on cell structures are the increase of thylacoid size (Sutinen et al. 1990), the production of plastoglobules (Holopainen et al. 1996), and a collapse of the mesophyll cells with H_2O_2 accumulation in the cytoplasm (Oksanen et al. 2004).

Plants respond to ozone stress with different antioxidant mechanisms. Volatile isoprenoids produced by the leaf may lead to hydroperoxides when reacting with ozone (Hewitt and Kok 1991; Taylor et al. 1999), but other studies have shown that VOC may actually remove ROS

in leaves (Loreto and Velikova 2001; Loreto et al. 2004). The Halliwell-Asada cycle removing ROS formed through the direct photoreduction of ozone by an array of antioxidant enzymes (superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR) and glutathione reductase (GR)), and their metabolites, is one of the most powerful mechanisms of protection against ROS (Foyer et al. 1994; Strohm et al. 2002; Edreva 2005). Ascorbic acid (AA, Vitamin C) is a particularly well studied compound of this defensive line in the apoplast, being capable of scavenging the harmful radicals (especially H_2O_2) produced in endogenous metabolism and in response to environmental stresses (Smirnoff 2000; Arrigoni and De Tullio 2000). AA has been detected in the majority of plant cell types, their organelles, and in the apoplast. The ability to donate electrons in a wide range of enzymatic and non enzymatic reactions makes AA the main reactive oxygen species-detoxifying compound in the aqueous phase. Studies with AA deficient mutants and clones (Conklin et al. 1996, 2000) have related the low concentration of AA in the plant tissue to deficient growth and enhancement of ozone sensitivity. Moreover, AA regenerates tocopherol from tocopherolxyl radical providing further indirect membrane protection (Thomas et al. 1992).

The increase in the synthesis of phenolic compounds is another common response to environmental stresses in plants (Dixon and Paiva, 1995). Phenols constitute several secondary metabolites (flavonoids, tannins, hydroxycinnamate esters and lignin) abundant in plant tissues (Grace and Logan, 2000) and have different protective functions in plants (e.g. protection from grazing, protection from UV light, and biocidal effects against bacteria and fungi). Polyphenols possess ideal structural chemistry for free radical scavenging activity, as they are high reactive as hydrogen or electron donor and can be involved in the hydrogen peroxide scavenging cascade in plant cells (Takahama and Oniki, 1997). Chloroplast-located flavonoids were found to effectively scavenge singlet oxygen (Agati et al. 2007).

Some studies confirmed the hypothesis that ROS act as signal molecules for gene expression of plant metabolites in stress condition. Pellinen et al. (1999) found that H_2O_2 is a signal for cell death and expression of defense genes.

1.1.2 Processes of ozone uptake

Forests act as a sink for ozone. Ozone disappears when reacting in the gas phase or when making contact with cuticles and apoplastic compounds. These reaction processes are controlled by transport phenomena. At a canopy scale, the transport of air masses containing ozone is driven by wind turbulence. At a leaf scale, the transport of ozone consists in the diffusion through stomatal pores (Marrero and Mason, 1972; Laisk et al. 1989). The fate of ozone after entering the stomata is not fully understood. The rate of ozone scavenged by the plant, normalized per time unit and unit area (usually a leaf surface) is termed "ozone flux". This term will be mentioned very often in this thesis, together with the term "ozone uptake". Uptake refers to the ozone concentration scavenged by the leaf (or the plant). It will be expressed as a concentration (ppb) or as a % of the atmospheric ozone concentration impinging the leaf (or the plant), without scaling to time and area.

As shortly described in chapter 1.1.1 (Fig. 1), ozone flux inside leaves has two components. The first component is ozone adsorption (by stems, cuticles, and, in general, external surfaces), also called non-stomatal, dry and wet deposition. At the cuticle level, ozone can react with a multitudes of waxes, salts, ions, biogenic and anthropogenic VOC and many other compounds, especially in conditions of wetness (Altimir et al. 2006). The percentage of non-stomatal ozone flux over the total ozone flux in a Mediterranean ecosystem was found consistent, reaching up to 60 % over the total ozone flux (Gerosa et al. 2005). Similar results were found by Kurpius and Goldstein (2003), which attributed a large part of the non-stomatal ozone flux to gas phase reactions between ozone and VOC outside the leaf. The second component is the absorption of ozone through stomatal pores. This is often the major component of the total ozone uptake by leaves. Stomatal conductance to ozone is also a flux which represents the reciprocal of the sum of an array of resistances that ozone meets along the path from outside the leaf to the reaction site inside the apoplast. The dominant resistances at a leaf level are the boundary layer (the relatively unstirred layer of air surrounding the leaf), the stomata apertures, the mesophyll tissue and, if ozone has not yet been fully scavenged, the chloroplasts. Stomatal conductance to gases is calculated once the flux and the concentration gradient between outside and inside the leaf are measured or calculated. The intercellular concentration of ozone is thought to be zero (Laisk et al. 1989) because of the fast reactions occurring with the cell walls. However, Moldau and Bikele

(2002) demonstrated by exposing only one side of a leaf to high ozone that the intercellular concentration of ozone could be higher than zero, hypothesizing that in conditions of high atmospheric ozone, the molecule does not completely disappear through reactions in the apoplast, thus accumulating in the intercellular spaces (Fig. 1) and decreasing the ozone gradient between intercellular spaces and outside the leaf. If this theory is true, the actual methods to calculate ozone fluxes would lead to an overestimation of the flux. Further details will be given in case study 3. Ozone absorption increases with increasing stomatal opening. The latter is regulated by environmental variables such as light, temperature and water availability in the plant-soil system (Löw et al. 2006). The light dependency of ozone uptake suggests a very high absorption level in comparison to the adsorption level (Fredericksen et al. 1996), although the above mentioned flux studies at ecosystem level lead to the conclusion that the non-stomatal flux is also considerable. Moreover, Musselmann and Minnick (2000) measured high levels of stomatal uptake of ozone during night, when stomatal aperture is supposed to be limited. The mechanisms of nocturnal stomatal uptake of ozone are still unclear. Other studies (Vitale et al. 2007) showed that acute ozone exposures can increase stomatal aperture and maintain stomata open at night.

Although ozone damages plants through deposition on the cuticles (Paoletti et al. 1998), it is reasonable to assume that ozone damage mainly occurs via stomata, and partitioning between the two mechanisms is important for ozone risk assessment. This aspect will be considered in the next paragraph.

1.1.3 Strategies and policies to assess ozone damage

Ozone is the more dangerous oxidant molecule for plant ecosystems, with detrimental effects on forests and crop yield (Karenlampy and Skarby, 1996), in turn decreasing the benefits (economical, social) that natural and cultivated plant ecosystems can offer. Since 1985, several working groups belonging to UNECE (United Nations Economic Commission for Europe) - CLRTAP (Convention on Long Range Transboundary Air Pollution) were set to study air quality and to implement air quality standards. The International Cooperative Programme (ICP) was funded with the aim of monitoring different forest areas over Europe (level I approach), particularly studying crown conditions, chemical composition of leaves

and soils upon ozone exposure. An intensive monitoring activity of ozone damage was successively started, based on many other parameters such as the micrometeorology of the experimental sites, and the deposition of the pollutants on leaves (level II approach). Currently, 39 countries participate to the programme. In Italy, the EC 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-level II approach are applied.

Translating the political scheme into scientific findings, the systematic ozone monitoring started at the end of 80s and the first approach was to determine the effects of ozone on plants, by comparing the damage with the tropospheric ozone concentration measured with passive samplers (Buffoni, 2002). The term "exposure" was used to indicate the amount of ozone concentration occurring near to the experimental sites. A critical level of ozone for vegetation, the concentration of ozone after which damage to vegetation occurs, was established. The most used index of damage was the AOT40 (Accumulated Ozone over a Threshold) (Karenlampy and Skarby 1996; Fuhrer et al. 1997), expressed as the sum of average hourly ozone concentrations exceeding 40 ppb during the day hours in which the global radiation is equal or above 50 W m^{-2} (equation 1).

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

The night hours were excluded since night ozone concentrations are often negligible. The AOT40 for forest ecosystems was fixed at 10000 ppbh averaged on 5 years during the vegetative season.

In several cases, atmospheric concentration not always correlated with the to ozone damage. As a result, the critical dose (level I) approach has been criticised in the last years because it does not take into account the physiology of plants and the effective dose of ozone absorbed by plants. For instance, drought stress in Mediterranean area induce stomatal closure, with the consequent limitation of ozone uptake by stomatal absorption even at high tropospheric ozone concentrations (Emberson et al. 2007). This may result in a much lower damage than predicted by the AOT40 index. In order to establish a cause – effect relationship considering only the effective concentration of ozone entering the leaf, a flux-based index proposed by

the level II approach seems more tenable. A short description of the model and its parameterization is reported in chapter 1.3.4.

A critical level ($AF_{st}Y$, accumulated stomatal flux of ozone (F_{st}) above a flux threshold (Y)) derived as the sum over time of the differences between hourly mean values of F_{st} and Y for the period when F_{st} exceeds Y) can be then calculated. Karlsson et al. (2004) proposed for birch and beech a flux threshold (Y) of 1.6 nmol m^{-2} s^{-1} ($AF_{st}1.6$), and found that a value of cumulative stomatal ozone flux of 4 mmol m^{-2} over a growing season caused a 5% reduction in biomass under the experimental conditions.

The scientific recommendation for planning air quality standards promoted by the UNECE mapping manual in 1996 were originally based on the level I approach, but the last UNECE protocol (2004) underlined the need to adopt the flux-based approach because it is more physiologically relevant. The next 2008 mapping manual will probably adopt the level II approach.

A parallel approach to determine ozone levels and injuries to vegetation is the use of bioindicators. A largely used bioindicator is tobacco, being some cultivars of this plant species (e.g. *Nicotiana tabacum* cv Bel-w3), very sensitive to ozone (Beauchamp et al. 2005). An ICP protocol (2006) suggests the use of two clones (sensitive vs. resistant) of white clover (*Trifolium repens* cv. regal) or brown knapweed (*Centaurea jacea*) to assess the risk of ozone injuries, especially in rural areas where ozone concentration cannot be directly measured. Clover clones were used in experiments simulating exposure to high tropospheric levels of ozone (D'Haese et al. 2005; Ferreira Severino et al. 2007; Crous et al. 2006) and in this study (see case study 2).

1.2 Volatile Organic Compounds, a defensive mechanism against oxidative stresses

Plants emit in the atmosphere Biogenic Volatile Organic Compounds (BVOC), and emissions are estimated at 1-1.5 TgC $year^{-1}$ on a global scale (Guenther et al. 1995, Fig. 2).

These emissions account for no more than 3% of the total carbon exchange between biota and the atmosphere (Kesselmeier and Staudt 1999; Crutzen et al. 1999), but they play an indirect and often 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). Even more importantly, BVOC may contribute, in the

presence of anthropogenic pollutants, namely NOx, to the formation of ozone, photochemical smog, and particulate matter (Chameides et al. 1988; Kavouras et al. 1998; Di Carlo et al. 2004). In non-stressed conditions, isoprenoids are the main constituent of BVOC, and forest plant species are the most important isoprenoid emitters (Kesselmeier and Staudt, 1999).

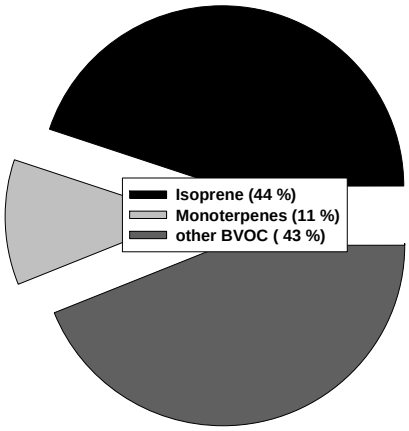

Figure 2: BVOC emission % over the total estimated emission (1150 Tg C y^{-1}) based upon Guenther et al. (1995) inventory.

Isoprene (2 mehyl-1,3 butadiene), is a monomer of 5 carbon atoms and the most abundant isoprenoid, accounting for about half of the total emission of BVOC into the atmosphere (Guenther et al. 1995; Fuentes et al. 2000).

Monoterpenes are 10-carbon atoms very representative emitted by plants, especially oak species (Loreto, 2002) in the regions with Mediterranean climate. The most abundant monoterpenes species 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. Moreover, a large group of plants evolved the capacity to store monoterpenes in specialized organs like ducts or glands. In these plants, monoterpenes are massively emitted when storage organs are broken under mechanical stress (Alessio et al. 2004). Even if monoterpene emission is lower than isoprene at a global scale (Guenther et al. 1995), monoterpenes play a more decisive and important role in the atmospheric chemistry, because they react faster than isoprene with pollutants like ozone and NOx (Atkinson et al. 1997), and because monoterpenes contribute to form particles and secondary organic aerosols (Kanakidou et al. 2005; Lee et al. 2006; Iinuma et al. 2007) .

Sesquiterpenes are 15-carbon BVOC and they are synthesized by plants also emitting isoprene or monoterpenes. Due to their high reactivity, often higher than for monoterpenes, they are very important in the chemistry of the atmosphere, but difficult to measure for their fast reaction in the atmosphere. They have been reported to be emitted by vegetation of southern and central Europe (Seufert et al. 1997).

A second class of BVOC is represented by the oxygenated hydrocarbons (OVOC). Methanol is one of the most abundant compound among the emitted OVOC, especially in developing leaves, being an indicator of leaf expansion (Nemecek-Marshall et al. 1995). Other compounds emitted in response to injury are methyl-jasmonate and ethylene (Farmer and Ryan 1990), acetaldehyde (Kreuzwieser et al. 1999), methyl-butenol (Goldan et al. 1993), and C-6 compounds formed by the breakdown of membrane lipids (Hatanaka, 1993) which are responsible for the "green leaf smell" of cut vegetation.

1.2.1 BVOC formation and environmental control on emissions

The pathway of formation of isoprene and monoterpenes is strictly dependant on photosynthesis. This was postulated by early studies based on the observed light dependence of isoprenoid emission (Loreto and Sharkey, 1990) and on the complete and fast labelling of the skeleton of volatile isoprenoids, reminiscent of the labelling pattern of photosynthesis intermediates (Loreto et al. 1996). Isoprene and monoterpenes are formed through a chloroplastic pathway described by Lichtentaler et al. (1997) on the basis of experiments of intramolecular labelling showing a characteristic pattern for the moiety derived from chloroplastic carbon. This isoprenoids biosynthesis is called "MEP pathway" from the specific intermediate 2-methyl-D-erythritol 4-phopshate formed by reactions between cytosolic pyruvate, and the photosynthesis intermediate glyceraldehyde-3-phosphate (Fig. 3). Further details on the metabolic pathways are given in Laule et al. (2003). The contribution of the cytsolic pathway of isoprenoid formation, often called mevalonic or MEV pathway (Fig. 3), to the formation of volatile isoprenoids in the chloroplasts is still under debate. It appears that a cross-talk between the two pathways exist (Laule et al. 2003), and that the non-chloroplastic pathway may supply considerable amount of carbon for isoprene

formation under heavy stress conditions that inhibit photosynthesis (e.g. drought stress, Brilli et al. 2007).

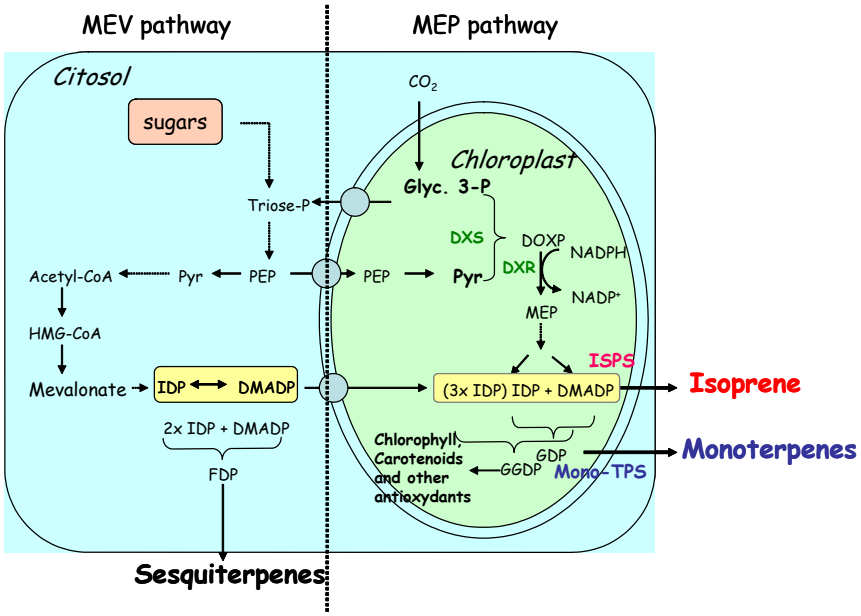

Figure 3: Synthesized overview of isoprenoids metabolic pathways localized into the cytosol (MEV) and into the chloroplasts (MEP) of plants. Evidence was given to the main intermediate biosynthetic products (in black), on the enzymes (coloured, respectively 1-deoxy-D-xylulose 5-phosphate synthase and deoxy-D-xylulose 5-phosphate riductase in green, isoprene synthase in red, and monoterpene synthases in blue).

The last step of isoprene synthesis is under the control of isoprene synthase, a light-activated enzyme that catalyzes the elimination of pyrophosphate from dimethylallyl diphosphate (DMADP, Silver and Fall, 1991; Wildermuth and Fall, 1996). Isoprene emission can be regulated by substrate availability or by enzyme activity, as explained more in detail below.

The biosynthesis of monoterpenes and other chloroplastic isoprenoids (e.g. carotenoids) occurs by successive addition of branched chains, each chain consisting of one molecule of DMADP and of its isomer, IDP (isopentenyl diphosphate). Monoterpenes are synthesized by the head to tail condensation of DMADP and IDP to form geranyl diphosphate (GDP, Ruzika, 1953). GDP is then transformed in monoterpenes. Interestingly, while isoprene is the main C-5 compound emitted by the MEP pathway, the blend of monoterpenes emitted by the plant is highly variable and species-specific. The monoterpenes are produced under the catalytic action of specific enzymes, each one responsible for the biosynthesis of only one product or of a blend of different products. Many of these enzymes have been found, but other still wait for proper identification and description of their catalytic properties (Tholl et

al. 2005; Laule et al. 2003). The most abundant monoterpenes emitted from *Quercus ilex* were measured in this study and are listed in table 3.

As for isoprene, also monoterpene biosynthesis is light-dependent. The light dependence reflect the availability of the precursor, and, ultimately, the availability of photosynthetic carbon that enter the biosynthetic pathway. Both isoprene and monoterpene emissions are temperature dependent. Temperature primarily affects the activation state of the enzymes involved in isoprenoid biosynthesis, rather than substrate production. Being the Q10 of enzyme activity typically between 2 and 4 (that is, an increase of 10°C results in a 2 to 4 time higher production of the final compound), isoprenoid emission is mainly controlled by temperature, especially at temperatures between 20 and 45 °C. The latter is the optimal temperature of isoprene synthase, being the enzyme rapidly denatured at higher temperatures (Monson et al. 1992).

The emission of isoprenoids seems to be insensitive to small variations of atmospheric CO_2 (Loreto and Sharkey, 1990; Loreto et al. 1996) but other experiments have shown that growth at elevated temperatures may inhibit isoprene (Rosenstiel et al. 2004; Scholefield et al. 2004) and monoterpene (Loreto et al. 2001) emission. Why rising CO_2 negatively affects isoprenoid biosynthesis and uncouples the process of isoprenoids formation from photosynthesis is unknown. It has been hypothesized that at high CO_2 respiration could more successfully compete for a common metabolic precursor in the cytosolic, pyruvate (Rosenstiel et al. 2004), although recent experiments do not support this view (Loreto et al. 2007). It should be mentioned that the concurrent increase of temperature caused by CO_2 accumulation in the atmosphere (IPCC, 2001) may offset the CO_2-driven reduction of isoprenoids, eventually leading to an increase of the net isoprenoid emission.

The biosynthesis of sesquiterpenes occurs in the cytosol, and can be shortly described as a further addiction of 2 IDP molecules to the DMADP to form Farnesyl diphosphate (FDP), successively transformed to sesquiterpenes through enzymatic reactions (Laule et al. 2003).

The oxygenated volatile organic compounds (OVOC) are formed through processes which are not as directly related to photosynthesis as in the case of isoprene and monoterpenes. OVOC emissions are often caused by biotic and abiotic stresses. Methanol is formed from the demethylation of pectins in cell walls (Galbally and Kirstine, 2002). Large but transient releases of methanol in the atmosphere may be associated to cell wall damage occurring because of wounding (Karl et al. 2001). However, methanol can be also released by rapidly

expanding leaves (Nemecek-Marshall et al. 1995) and by senescing leaves, perhaps again because of irreversible decomposition of cell walls (Fall, 2003).

C-6 compounds (primarily hexenal, hexanal, hexenol) are formed from the breakdown of membrane lipids, predominantly those made by unsaturated fatty acids, under the action of lipoxygenase and hydroperoxide lyase enzymes (Hatanaka, 1993). C-6 emissions are always observed after mechanical stresses (Loreto et al. 2006). Recently, however, emission of C-6 compounds has been also reported as a consequence of insect feeding, high temperature (Loreto et al. 2006), or ozone exposure (Heiden et al. 2003). Beauchamp et al. (2005) observed a good relationships between ozone uptake and C-6 compound emissions, indicating that the emission was probably related to the ozone damage to membranes.

Acetaldehyde is predominantly formed by the enzymatic oxidation of ethanol, which is formed in roots under anoxic conditions and is then translocated to leaves through the transpiratory stream (Kreuzwieser et al. 1999). Acetaldehyde is also emitted following wounding (Fall et al. 1999) and ozone stress episodes (Cojocariu et al. 2005). The release of acetaldehyde is associated to the amount of ozone to which the leaves have been exposed, but the biochemical origin of this pool of acetaldehyde is still elusive (Cojocariu et al. 2005).

1.2.2 The antioxidant role of volatile isoprenoids

Why isoprenoids are emitted by plants giving away a considerable fraction of the fixed carbon, especially under stress conditions? It was observed that isoprenoid emission is stimulated in plants that have been exposed to stress conditions, namely drought (Sharkey and Loreto, 1993) and ozone (Loreto et al. 2004). In 1995, Sharkey and Singsaas found that isoprene is involved in a mechanism of thermal protection. They observed that the photosynthetic machinery of isoprene-emitting leaves was more resistant to high temperature than in non-emitting leaves. More recently, Loreto et al. (2001), attributed to isoprene a more general antioxidant role after observing improved protection from ozone damage in isoprene emitting leaves or in leaves exposed to exogenous isoprene during ozone treatment. Further experiments demonstrated that reactive oxygen species (H_2O_2, singlet oxygen) are reduced, and membranes are better preserved in isoprene emitting leaves than in non-emitting leaves, after an ozone treatment (Loreto and Velikova, 2001). Sharkey and Yeh (2001) raised the

hypothesis that the lipophylic isoprenoids interact with lipids in membranes, strengthening membrane resistance to stresses. Theoretical confirmation of this action has been recently obtained (Siwko et al. 2007). This action may usefully defend leaf structure against both high temperature and ozone stress. Isoprenoids may also scavenge pollutants in the intercellular spaces reducing the potential level of reactive oxygen species that impinge on membranes causing their oxidation (Loreto et al. 2001). Whether this occurs by direct reaction or indirectly is not known, although a direct quenching of singlet oxygen in presence of isoprene has been reported (Velikova et al. 2004). Finally, Velikova et al. (2005b) found that, in isoprene-emitting leaves that were exposed to ozone, the accumulation of NO and H_2O_2 in the mesophyll was highly quenched. Since NO and H_2O_2 are the signalling molecules that start the sequence of biochemical events leading to programmed cell death, it was surmised that isoprene could modulate molecular signalling in leaves. The observation that high ozone exposure can stimulate the emission of isoprenoids was also made in tobacco leaves by Beauchamp et al. (2005) and in poplar leaves (this study). However, other studies observed a reduction of isoprene emission during and after the ozone treatment (Velikova et al. 2005a; Calfapietra et al. 2007). It was hypothesized that when ozone largely affects photosynthesis, also the metabolic pathway of isoprenoid formation is negatively influenced.

Isoprenoids play a defensive role also against biotic stress. They can deter insects, attact their parasitoids and block the colonization of wounding by bacteria and fungi (Parè and Tumlison, 1997). While these effects have been found to be elicited by monoterpenes and sesquiterpenes, isoprene role in biotic stress defense remains more elusive. In any case, the interaction between isoprenoids and biotic stresses is beyond the scope of this study and will not be further addressed.

1.3 Methods to measure BVOC and ozone fluxes

Various methods and modelling approaches can be used to detect ozone flux at leaf, plant, ecosystem scale. The purpose of a research and the instruments available should assist with the selection of the most appropriate technique to use for measurements. The precision of the measurements unavoidably decreases with increasing level of observation. In the present study, I mostly concentrated on measurements of BVOC and ozone fluxes at leaf level, using

the enclosure technique, with technical adjustments to further render this technique suitable for accurate measurements of these very reactive gases. Special enclosures were also used to upscale measurements at whole plant level. Details of the enclosure system will be given in the next section, and in the Materials & Methods section of each case of study. The most used micrometeorological techniques for canopy scale fluxes, as well as models to calculate ozone and BVOC fluxes from vegetation will also be mentioned, but further information on these experimental techniques can be retrieved on the cited literature.

1.3.1 Proton Transfer Reaction Mass Spectrometry (PTR-MS) for BVOC measurement

This section is dedicated to the novel approach (PTR-MS) used in this study to measure BVOC emission. The Proton Transfer Reaction-Mass Spectrometer (PTR-MS) was developed by Lindinger et al. (1998) and consists of a discharge ion source to produce H_3O^+ ions, a drift-tube reactor, in which the proton-transfer reactions between H_3O^+ and BVOC take place, and a quadrupole mass spectrometer for the detection of reagent and product ions. The ionization reaction is described as follow:

$$H_3O^+ + BVOC \rightarrow BVOCH^+ + H_2O \qquad \text{(R7)}$$

The ion source consists of a hollow cathode discharge in water vapour and provides a source of H_3O^+ ions. In a small intermediate chamber, ions from the hollow-cathode are then converted into H_3O^+ ions while being transported towards the drift tube. The latter is separated from the second intermediate chamber by a small orifice, and most of the air is pumped away by a turbo pump. A fraction of the ions is extracted through the nose cone into the quadrupole mass spectrometer, which consists of a commercial Vacuum mass filter and an electron multiplier for ion pulse counting. In the present experiments, the mass spectrometer was run in selected-ion mode, in which only certain ions ($BVOCH^+$) of interest are analyzed rather than scans over a range of masses.

These reactions are exothermic if the proton affinity (PA) of R is higher than the PA of water (166.5 kcal mol^{-1}), in which case the reaction proceeds at a rate close to the collision rate of 10^{-9} cm^3 $molecule^{-1}$ sec^{-1}. Isoprenoids and oxygenated compounds studied in this work have

a proton affinity much higher than water. For VOCs that have a PA only slightly higher than that of water, the exothermicity of reaction is small and the rate coefficient of the reversed reaction 7 is not-negligible. This is the case, for example, for formaldehyde, and such compounds were not investigated in this study.

The $BVOCH^+$ ions produced from reaction 7 can undergo fragmentation, especially when exceeding a molecular weight above 100. This is the case of monoterpenes (m/z 136). Fragmentation is function of the humidity of the sampled air and the voltage of the drift tube influencing the electron impact between protons and VOCs (Tani et al. 2004). However, the masses and patterns of monoterpene fragmentation are well known (Rinne et al. 2005), and it is possible to measure the concentration of monoterpenes simply measuring their main fragment with protonated m/z 81.

Due to the continuing sampling and the short residence time of sample gas in the drift tube, the response of the PTR-MS is very fast, varying from 0.2 to 5 sec (de Gow and Warneke, 2007). Moreover, the high sensitivity of the quadrupole allows a low detection limit of 10 to 50 ppt, thus making the PTR-MS a powerful detector for trace gases.

1.3.2 Enclosures

Enclosure systems, namely "cuvettes", have become increasingly popular in the field of on-line analysis of gases exchanged by the plant (Tholl et al. 2006). In association with suitable detection systems, cuvette measurements allow precise and rapid monitoring of the air composition and the release or uptake by plants of gaseous compounds such as CO_2, H_2O, ozone and BVOC enabling accurate *in vivo* studies of the relationships between primary metabolism (photosynthesis) and secondary metabolism (BVOC synthesis). Cuvette systems also allow the investigation of physical constraints to ozone and BVOC fluxes such as those caused by stomatal opening (Niinemets et al. 2004) or mesophyll resistances.

Three types of cuvettes are in general used: leaf, branch and plant cuvettes. Leaf cuvettes are made of a frame in which a leaf, or a portion of it, is enclosed. They generally have a very small and fixed volume, rarely exceeding 0.5 liters. Cuvettes used for measuring BVOC emissions from tree branches have a larger volume (>2 l) and are often inflatable (with or without solid frames). A selection of different leaf and branch cuvettes is shown in Figure 4.

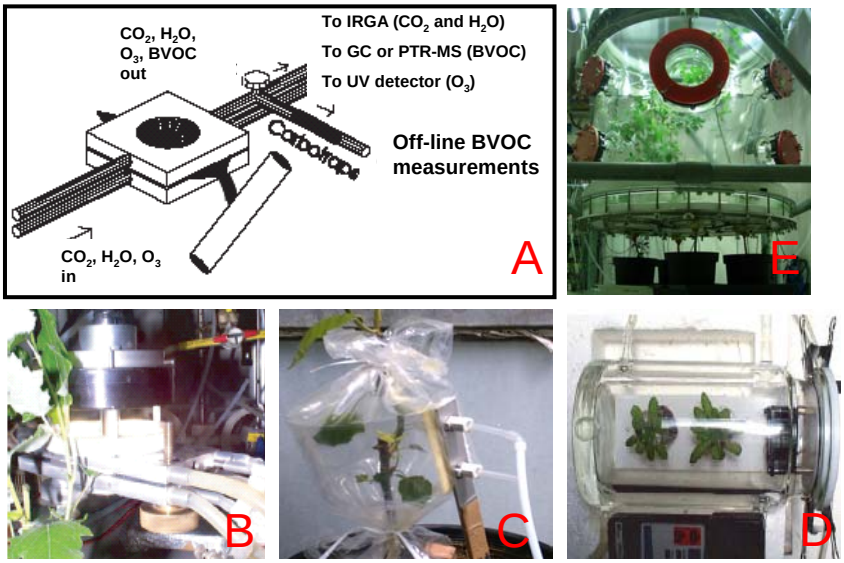

Figure 4: Examples of cuvette systems for measurements of plant BVOC and physiological parameters.
(A) A simplified sketch of a typical cuvette allowing measurements of the concentration of CO_2 and H_2O (by infrared analysis) and BVOC emissions by on-line analysis via PTR-MS or GC, or off-line analysis via cartridge trapping and subsequent analysis by GC-MS.
(B) Special cuvette for simultaneous measurements of gas exchange and fluorescence properties of the leaf.
(C) Cuvette system used to monitor gas exchanges from entire branches under field conditions.
(D) Cuvette system for gas exchange analysis from whole small plants.
(E) Plant enclosures allowing to enclose one or more transplants.
All cuvettes (except (C)) allow full control of the principal environmental parameters (temperature, light intensity and quality, wind speed and relative humidity).

Cuvettes have transparent windows of glass or plastic material to allow illumination of the enclosed plant specimens on the adaxial and, occasionally, also on the abaxial side of the leaf. Light is provided by external, generally artificial, sources such as light-emitting diodes (LEDs) with wavelengths needed for physiological light reactions to occur (Tennessen et al. 1994). As a cold and easy modular light source, LEDs can generate very high and uniform light intensities without overheating the leaf cuvette. However, LEDs only provide few wavelengths and do not generally reconstitute the solar spectrum. Moreover, LEDs are currently difficult to use in branch cuvettes due to insufficient and inhomogeneous illumination over large and uneven surfaces. When using branch or whole plant cuvettes the solar spectrum is provided by batteries of powerful lamps (e.g. the Osram, Hg vapour, Power Star series) which however also generate large infrared radiation. Overheating must be then

avoided by intercepting the infrared radiation with appropriate filters (e.g. water layers circulating to remove the heat).

Leaf cuvettes are generally thermoregulated by Peltier resistances or, more simply but less effectively, by water circulating in the body walls at the desired temperature. Water regulation of temperature is slower and less homogenous than the regulation by thermoelectric devices. In both cases, the actual leaf temperature is constantly measured by thermocouples attached to the side of the leaf which is not directly exposed to incident light. Leaf temperature may be considerably different than air temperature, and generally lower when latent heat is dissipated by stomatal transpiration. Large-volume branch cuvettes are often not thermostated and temperature is simply monitored by one or more thermocouples close to the leaves in strategic positions (e.g. in the shaded areas and close to air stirring devices). In all cuvette systems, air circulates inside the cuvette entering/exiting from one or more inlet/outlet holes. The geometry of the cuvette must favour rapid and turbulent air motion to avoid air stagnation, which may in turn cause condensation of water on cold spots of the cuvette and the formation of a boundary layer resistance restraining the exchange of gases between the leaf and the atmosphere. Therefore, built-in fans are often used to stir the air inside the cuvettes. Fan engines however often generate ozone and for this reason their use should be avoided when working on ozone measurements. Ozone-free fan engines are however available on the market if the volume is too high or uneven to be satisfactorily exchanged without the help of fans. In general, the volume of the cuvette must be replaced at least twice per minute to avoid the above-mentioned condensation and low boundary layer conductance problems.

The humidity of the air in the cuvette can be easily controlled when working with laboratory equipment by bubbling, prior to insertion in the cuvette, air into water and passing then the air stream in a condenser. Condensation of excess humidity is generally achieved placing the condenser in a water bath maintained at a temperature lower than air temperature. Humidity can be also removed by using water chemical adsorbants, such as drierite, silica gel or magnesium chloride. Air humidity can be calculated from psichrometric tables, knowing exactly the air temperature before the cuvette and in the cuvette, or can be actually measured with capacitors (e.g. Vaisala condenser).

Laboratory equipment also allows excellent control of the composition of the air, especially when mixing air components from gas cylinders and regulating the concentration of each

gas precisely, with the assistance of mass flow controllers. When using contaminat-free mixtures of N_2, O_2 and CO_2, to reconstitute the desired air composition, measurements of trace-gases released by plants are easy and clean. Single pollutants (e.g. ozone or hydrocarbons) may also be added to the mixture at ppb level, and their uptake can be detected very effectively when the background is that clean. It should be noted, however, that such measurements under contaminant-free conditions may not reflect 'real life' conditions, in which gases may e.g. react before coming in contact with the leaf (e.g. BVOC + ozone). On the positive side, cuvettes are ideal to study the effect of one single pollutant (e.g. ozone) on the leaf physiology, or to focus the attention on the species-specific emission rates of BVOC, and for the parameterization of these compounds on the basis of single environmental or physiological factors.

Beside using ozone-free synthetic air from cylinders, rapid reactions of BVOC with ozone present in the inflowing air can be avoided scavenging ozone from ambient air with appropriate chemical traps or physical devices, such as a simple piece of metal.

Like other devices used in the collection of BVOC, cuvettes should be entirely constructed with materials which are inert to BVOC and ozone. Glass and Teflon, also manufactured as a transparent film (Fig. 4) are suitable materials. If metal is to be used, the metal parts (generally the cuvette frame) can be wrapped in Teflon film or sprayed with a liquid layer of Teflon. When fumigating leaves with very reactive compounds, fittings and pipelines external to the cuvette should also be made of inert materials (Fig. 4). In all cases, a measurement of the uptake of ozone, BVOC, or other reactive materials by the empty cuvette must be performed, and this blank measurement can be subtracted to the leaf uptake. To ensure a correct estimation of the cuvette wall contribution, the cuvette uptake measurement should be repeated at each condition set for leaf uptake measurements (e.g. at different temperatures, light intensities, etc.) and memory effects (persistent emission of sticky compounds once the leaf has been removed by the cuvette, or fumigation has ended) should be properly measured.

In this work, I measured gas exchange adopting the theory of diffusion in isotropic substances (Nobel, 1974), which is based on the physical law stating that the rate of transport of diffusing substances through unit area cross section is proportional to the concentration gradient normal to that section:

$$\Phi_x = -D\frac{\partial x}{\partial z} \quad (2)$$

where Φ is a flux, the rate of transfer for a diffusing substance through a unit area of cross-section, x is the concentration of the different gases (CO_2, BVOC, and ozone in this study), z is the space coordinate measured normal to the section and D is the diffusion coefficient. When assuming that $\partial z / D$ is equal to a resistance, the inverse of resistance is a conductance (g_x), which can also be measured according to equation 3:

$$g_x = \frac{F}{A^L} \cdot \frac{[x] - [x]_{in}}{[x]_i - [x]} \quad (3)$$

where F is the air flux passing at a certain amount through the cuvette, A^L is the leaf area inside the cuvette, $[x]$ and $[x]_{in}$ are the concentrations of a gas inside and outside the cuvette. The term $[x] - [x]_{in}$ is considered an uptake. The term $[x]_i$ is the intercellular concentration of a gas in the cuvette, assumed to be zero in the case of ozone (Laisk et al. 1989) and 100% in case of water.

When substituting equation 3 to the g_x term of equation 2, the flux (Φ_x) of a generic gas is given by:

$$\Phi_x = \frac{F}{A^L} \cdot [x] - [x]_{in} \quad (4)$$

Cuvette measurements are a favorite *in vivo* method for studying at the same time the BVOC emission, photosynthesis, transpiration, and ozone uptake. Simultaneous on-line measurements are best performed when air leaving the cuvette is split to reach an infrared gas analyzer for CO_2/H_2O detection, an UV detector for ozone (Loreto et al. 2001), and a fast detector for BVOC emissions such as PTR-MS (Lindinger et al. 1998). Off-line measurements can also be performed by entrapping air samples in canisters or solid adsorbent and successively analyzed with a GC-MS (Gas Chromatograph-Mass Spectrometer (Helmig, 1999)).

Finally, special cuvettes allow rapid freeze-clamping of the enclosed leaves for biochemical analysis of enzyme activities and metabolites with very rapid turnover. In a system

developed by Sharkey (Loreto and Sharkey, 1993) the leaf enclosed in the cuvette is frozen extremely quickly (<0.1 sec) by smashing it between the plastic windows of the cuvette under high pressure (>100 bar) with metallic drums previously frozen in liquid nitrogen. This system was effectively used, with slight modification, to determine pools of dimethylallyl diphosphate (DMADP), the last intermediate in isoprene biosynthesis (Loreto et al. 2004), and to measure the activities of isoprene and monoterpene synthases (Loreto et al. 2001).

1.3.3 Micrometeorological techniques

When studying the fluxes of ozone and/or BVOC at ecosystem level rather than at leaf level, it is necessary to use suitable methods which do not rely on enclosures. Measurements are made under uncontrolled environmental conditions and are averaged over long time periods. For ozone, the most used method to determine fluxes exchanged by vegetation is the Eddy Covariance (Hicks and Matt, 1998; Bauer et al. 2000). This method allows to calculate the flux of a scalar (energy, mass) at a point centered on instrument placed at a certain height above the canopy. The flux (Φ_x) is then calculated multiplying the gas concentration at the set height (X) by the vertical wind velocity (w), as shown in equation 5:

$$\Phi_X = \overline{w' X'} \qquad (5)$$

The prime symbols indicate the instantaneous deviations from the mean, and the overbar indicates the time averaging, usually 30 min. Fluxes for BVOC are generally positive, indicating emission from the canopy to the atmosphere; ozone fluxes are always negative, indicating an uptake by vegetation. The value is expressed in mol m^{-2} s^{-1} or g (DW) m^{-2} s^{-1}, the same unit of the fluxes measured with enclosures, but is referred to m^{-2} of surface, not foliar area. This makes comparisons between leaf and whole ecosystem measurements difficult, unless introducing a term for biomass density (g m^{-2}) of the specific site of measurement.

The Eddy Covariance approach requires fast response sensors for gases and wind, usually collecting data with a frequency around 10 Hz. For BVOC a reasonably fast sensor is the

PTR-MS, but this instrument can often collect data at frequencies of around 1 Hz, especially when several masses are measured simultaneously. The Disjunct Eddy Covariance technique (Rinne et al. 2001; Karl et al. 2002) allows to measure BVOC using an approach similar to the EC, but in which BVOC are sampled at lower frequency and are correlated with the wind value along a continue time series. This method needs very accurate calculation of the exact timing of BVOC sampling, since a wrong correlation would give misleading results. It also needs to average data collected on a rather long period and to use a rather complicate statistical package, to smooth data, eliminating measurements that are biased by occasionally uneven meteorological conditions.

A second and indirect method to determine fluxes is the "Gradient diffusion" approach (Ciccioli et al. 1997). This method assumes that a hydrocarbon flux can be characterized by the product of the concentration gradient ($\partial\chi/\partial z$) defined between two levels above the source, and an atmospheric eddy diffusivity ($K\chi$) of the hydrocarbon constituent (equation 6).

$$\Phi_X = -K_x \frac{\partial \chi}{\partial z} \tag{6}$$

K_χ can be obtained by invoking the Monin and Obukhov (1954) similarity hypothesis, as shown below:

$$K_X = \frac{u_* k(z-d)}{\phi_h} \tag{7}$$

where ϕ_h denotes the diabatic influence function for heat, u_* signifies the friction velocity, k is the von Kármán constant (= 0.4), and d represents the forest zero plane displacement. An advantage of this method is that the gas measurement is very detailed, especially for BVOC, and can be done off-line by analyzing with a GC-MS the gasses previously trapped in canisters. However, this technique cannot be applied for ozone, since it is impossible to store ozone in canisters or traps.

A third method to measure fluxes of gases by vegetation is the "Relaxed Eddy Accumulation" (REA), which involves the separation of gas samples into two reservoirs for

ascending and descending air. The flux (equation 8) is related to an empirical coefficient $b(\zeta)$, the standard deviation of the vertical wind speed σ_w, the difference in the mean gas concentration stored in updrafts (χ_u) and downdrafts ((χ_d) Businger and Oncley, 1990):

$$\Phi_X = b(\zeta)\sigma_w(\chi_u - \chi_d) \quad (8)$$

The vertical wind speed provided by sonic anemometers is used to divert through fast solenoid valves air to reservoirs associated with updrafts and downdrafts. An adsorbent cartridge can also be used to store samples in lieu of reservoirs (Ciccioli et al. 1997). This last technique is very labour intensive, but it gives very specific information about BVOC species, since the off-line analysis can be performed with a GC-MS. However also in this case ozone cannot be measured, because of the too high reactivity of this compound inside reservoirs or cartridges.

1.3.4 Models to estimate and predict ozone and BVOC fluxes

Modelling ozone fluxes has to consider the high gas reactivity, and the still unknown deposition processes that can remove ozone. The most relevant processes involved in ozone uptake by plants are related to transport in gas and liquid phases, through surfaces, until the reaction sites. The transport of a molecule is a physical phenomenon occurring in the atmosohere (Seinfeld and Pandis, 1989).Until reaching the reaction site, a molecule is transported by turbulent air masses, and meets an array of resistances. The most successful models are those used to predict ozone deposition on a large, regional scale considering the deposition on different surfaces, in relation to environmental parameters: light, temperature, rain, snow. At a plant scale, the environmental inputs are required to simulate the stomatal behaviour influencing the stomatal ozone flux (Vautard et al. 1995; Simpson et al. 2003). There is evidence, however, that stomatal uptake at ecosystem level cannot explain alone ozone deposition. In some field measurements (Gerosa et al. 2005; Kurpius and Goldstein, 2003) the modelled non-stomatal contribution based on resistance analogies and water exchange accounted for 50-70 % of the total flux. It is important to understand the purposes of the model, if the aim is to assess the total ozone deposition, or it is necessary to quantify

the stomatal ozone flux. If the model needs to serve for risk assessment, the accuracy in determining the stomatal flux is fundamental (Grünhage et al. 2000; Erisman et al. 2004; Emberson et al 2000, 2007). The parameterisation of stomatal ozone flux from the model of Emberson et al. (2000) and also present in the UNECE mapping manual (UNECE, 2004) considers the stomatal flux of ozone as the final product between the atmospheric ozone concentration at canopy top ($[O_3]$) assuming intercellular ozone concentration = 0, and the stomatal conductance to water vapour (g_{sto}) including the deposition rate to the leaf through resistances:

$$\Phi_{O3} = [O_3] * g_{sto} * \frac{r_c}{r_b + r_c} \quad (9)$$

where r_c the leaf surface resistance, abd r_b is the quasi laminar resistance. The core of this ozone flux model is the multiplicative algorithm to calculate g_{sto} according to the following formulation:

$$g_{sto} = g_{\max} * [\min(f_{phen}, f_{O3})] * f_{light} * \max\{f_{\min}, (f_{temp} * f_{VPD} * f_{SWP})\} \quad (10)$$

Where g_{max} is is the species-specific maximum stomatal conductance, and the f parameters are all expressed in relative terms (taking values between 0 and 1) for leaf phenology (f_{phen}), the influence of ozone concentration on stomatal conductance (f_{O3}), the light conditions (f_{light}), the minimal estimated levels of g_{max} (f_{min}), temperature (f_{temp}), Vapour Pressure Deficit (f_{VPD}) and soil water potential (f_{SWP}). This model is applied to sunlit leaves of canopies in ecosystems, and is actually used to assess ozone risk on crops, and the ongoing research activity is performing this methods for forest ecosystems (UNECE "level II" approach, see section 1.1.3). This method is accurate when a high number of ground-based measurements on the physiology of plants and environmental variables is performed.

At a leaf level, there were some efforts not described in this work (Chameides et al. 1987; Cape et al. 1998) to parameterize ozone uptake considering the reactions occurring on the cuticula and inside the leaf, and also considering the leaf wetness and resistance analogies.

In the field of BVOC emissions, large efforts have been made to model the emission of isoprenoids, the most abundant and reactive compounds. Isoprenoid emissions are controlled

by light and temperature (Monson et al. 1992; Tingey et al. 1991). Based on these dependencies, an algorithm was elaborated by Guenther et al. (1995, 1999), which applies two correction factors to a standard emission rate (*SE*) measured in basal conditions (T = 30°C and PPFD (Photosynthetic Photon Flux Density) = 1000 μmol m^{-2} s^{-1}). A simplified form of the equation is reported in equation 11:

$$E_{Isoprenoids} = D * SE\left[\frac{\alpha C_L PPFD}{\sqrt{1+\alpha^2 PPFD^2}}\right] * \left[\frac{\exp\left(\frac{C_{T1}(T-T_S)}{RT_S T}\right)}{1+\exp\left(\frac{C_{T2}(T-T_M)}{RT_S T}\right)}\right] \quad (11)$$

where C_L, α, C_{T1}, C_{T2}, and T_M are empirical coefficients, R is the universal gas constant, D is the foliar density, T is the leaf temperature and T_S in the leaf temperature at standard conditions (303 °K). It was found that the basal emission is changing with the seasonality and with rising CO_2 concentartion in the atmosphere, and the algorithm has been modified introducing a seasonality factor (Ciccioli et al. 2003) or a CO_2-dependent factor (Arneth et al. 2007) in the algorithm.

2. Cases of studies

2.1 Impact of high ozone on isoprene emission and on anatomical and physiological parameters of developing *Populus alba* leaves directly or indirectly exposed to the pollutant.

2.1.1 Aims of the study

In this study, I wanted to investigate ozone direct and indirect impact on *Populus alba* leaves, that is, whether ozone fumigation to some leaves only, also affecting the physiology, anatomy and biochemistry of other leaves that were growing without being directly exposed to the pollutant.

Branch cuvettes were designed, in which enclosed leaves were exposed to high ozone levels while leaves above the cuvettes developed at ambient ozone levels. Photosynthesis parameters, isoprene emission, isoprene synthase mRNA expression and changes of anatomical parameters, were investigated to specifically understand whether ozone could indirectly affect the anatomy and physiology of leaves.

2.1.2 Methods

2.1.2.1 Plant material

The location of the experimental site was CNR-IBAF, Monterotondo Scalo (Roma). Two-year-old transplants of *Populus alba* were used for this study. Plants were potted in 50-L pots filled with commercial soil and grown and maintained in the greenhouse of the Institute under controlled conditions. Experiments were run in late spring 2005 when the air temperature was 24-30°C, the air humidity was around 50%, and the light intensity at the canopy level was around 850 μmol photons m^{-2} s^{-1}. Plants were irrigated daily and fertilized weekly to avoid drought and nutritional stress during the experiments.

2.1.2.2 The cuvettes and the ozone fumigation system

Branch cuvettes (3 L of internal volume) especially developed for this experiment (Fig. 4 C) were carefully installed at the bottom of the stem including the basal (3-4) leaves (Fig. 5).

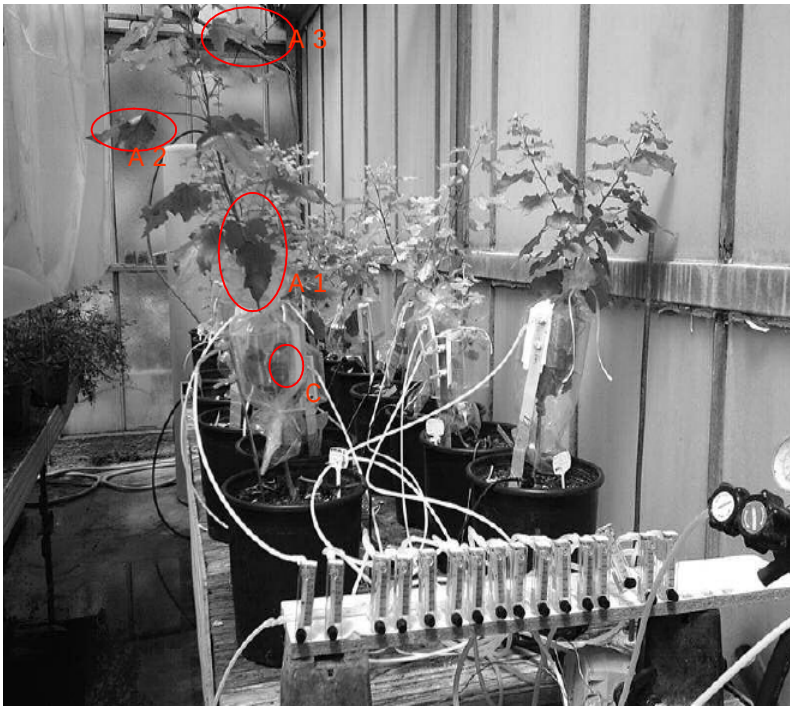

Figure 5: View of the experimental system used for ozone fumigation. In this figure, the 3-L branch Teflon cuvettes developed for this experiment are visible, as well as the Teflon lines to circulate ozonated or non-ozonated air inside the cuvette at controlled concentrations and flow rates. Details about the system are reported in the text. C = leaves grown at high levels of ozone (100 ppb), A = leaves grown outside the cuvette in ozone-free air, 1-3 = level of leaf expansion (1 = mature, 3 = young).

The cuvettes were made of soft Teflon film fastened to a rigid ring of PVC to avoid the direct contact with leaves. The top part of the cuvettes was then gently wrapped on the stem and firmly tied to it to avoid leaks. The stems exiting the top part of the cuvettes were cut to allow the sprouting of one bud only per stem during ozone fumigation. New buds appeared after one week and new shoots grew within three weeks after cutting. The lateral part of the cuvettes housed two holes and connectors to allow the flux of air in and out of the cuvette. All connecting pipelines were made by Teflon to avoid high ozone uptake by other plastic materials and early wearing out of the materials. Air was pumped in the cuvettes with a

compressor at a flux of 6 L min^{-1}. This high flux allowed to avoid condensation of transpired water vapour inside the cuvette and also inflated and self-sustained the cuvette walls distant from the rigid PVC frame. Sensors of light and temperature were also placed in the cuvettes through the air inlet hole. The enclosures caused an attenuation of 15% of the light intensity and a max increase of air temperature of 2°C with respect to the temperature measured in the greenhouse during the hottest hours of the day.

The air entering the cuvette was enriched with ozone in half of the cuvettes (five plants referred to as treatment). Ozone was generated by flowing part of the air, pushed by a diaphragm pump, through a winding quartz glass illuminated with an UV light source (Helios Italquartz, Milan, Italy) placed at short distance from the cuvette inlets. This method offered the possibility to change the amount of ozone generated by changing the air flow through the winding quartz glass or by changing the intensity of the UV light source. The lamp was set to generate ozone at a concentration of 150 ppb, and fumigation was carried out for 11 hours per day (h. 07.00 – 18.00) and for one month. Five other plants thereafter referred to as controls, were grown in cuvettes as for the ozone treated plants but the air in was not enriched with ozone. In control cuvettes the ozone concentration followed a daily trend. Ozone concentration was 0 ppb at night but built up photochemically to a max of 40 ppb during the central hours of the day. The buds developing on the stem out of the cuvettes grew at ambient ozone concentration both in treated and control plants. The ozone concentration in the cuvettes of treated and control plants was monitored with a Photometric O_3 analyzer (1008 Dasibi Environmental Corp., Glendale, California, USA) connected with the cuvette inlet and outlet and periodically switching between cuvettes with Teflon valves (Velikova et al. 2005). The ozone-enriched air outflow from the cuvettes was convoyed out of the greenhouse. In the greenhouse the ozone concentration was similar to that recorded in the control cuvettes.

Measurements were carried out on the following leaf material: a) the first three leaves expanding out of the cuvettes. These leaves will be named A1 (first leaf after the cuvette), A2 (second leaf) and A3 (third leaf); b) the leaves growing inside the cuvette since the beginning of the experiment. These leaves will be named B; c) the leaves expanding from buds inside the cuvettes during the experiment. These leaves will be named C.

2.1.2.3 Gas exchange and fluorescence measurements

The exchange of CO_2 and H_2O between leaves and air were measured with a Li-6400-40 portable gas exchange system (LI-COR, Lincoln, Neb, USA), and photosynthesis (Pn), transpiration, stomatal conductance to CO_2 and H_2O, and intercellular CO_2 concentration (Ci) were calculated with the instrument software. Chlorophyll fluorescence was also measured in vivo and simultaneously to gas exchanges with the same apparatus. In particular, I measured the quantum yield of PS2 in darkened leaves (the ratio between variable and maximal fluorescence, Fv/Fm) and in illuminated leaves (the ratio between maximal – steady state fluorescence and maximal fluorescence, ((Fm'-Fs)/Fm' = □F/Fm'). A modulated fluorimeter (Plant Efficiency Analyser (PEA) Hansatech Ltd, UK) was used. Leaves were dark-adapted for 40 min, and then exposed to a sequence of illumination to detect the basal fluorescence (F_0) under a weak modulating red light, and the maximum fluorescence (Fm), under a saturating bright white light pulse (> 10000 μmols photons $m^{-2}s^{-1}$) overimposed to the modulating light. Details about nomenclature and connotation of fluorescence parameter, and about the protocol of fluorescence measurements are given in van Kooten and Snel (1990). Measurements were carried out on the central part of the leaf (2 cm^2) which was clamped in the Li-6400 gas-exchange cuvette and exposed to a flux (0.45 L min^{-1}) of a synthetic air (20% O_2, 80% N_2, 380 ppm CO_2) free of ozone, BVOCs and other pollutants. During all gas-exchange measurements the leaf temperature was maintained at 30°C and the leaf was illuminated with a light intensity of 800 μmol photons $m^{-2}s^{-1}$. The CO_2 concentration in the air was modulated to generate a response of photosynthesis to CO_2 concentrations between 40 and 1000 ppm. Isoprene emission from leaves was detected simultaneously to CO_2 and H_2O gas exchange measurements, connecting the outflow of the Li-6400 cuvette to a proton-transfer-reaction mass-spectrometer (PTR-MS, Ionicon, Innsbruck, Austria). Details on the instrument functioning can be found in section 1.3.1.
Measurements were done at the same time of the day (h 10.00 – 11.00) to minimize physiological changes driven by environmental factors or by accumulation of photosynthates during the day. Measurements of B and C leaves were carried out temporary removing the plants from the fumigation cuvettes. Measurements were carried out on B leaves at d 0, 6, 14 and 21 of the experiment. Measurements of leaves A and C were carried out at d 30-35 of the experiment.

2.1.2.4 Histological observations of leaf anatomy

Histological observations were carried out with the help of a team of the University of Perugia, Plant Biology and Biotechnology Department, on leaves at d 30 of the experiment. Leaf tissue pieces (1–2 mm^2) were excised at the same hour of the day (h 12.00) to avoid diurnal variability of sugar accumulation in leaves. Leaf tissues were immediately fixed in 5% (w/v) glutaraldehyde in 0.075 M sodium cacodylate buffer, pH 7.2, for 12 h. Samples were then washed three times for 15 min each in 0.1 M phosphate buffer, pH 7.2, and were post-fixed in 1% (w/v) OsO_4. Samples were then dehydrated in increasing concentrations of ethanol and included in resin (Epon 812 resin, 2-dodecenylsuccinic anhydride, and methylnadic anhydride mixture, Sigma Aldrich, S.Louis, USA). A pre-inclusion at room temperature in increasing concentrations of resin dissolved in propylene oxide was followed by the final inclusion in freshly prepared resin followed by the polymerization at 35°C for 12 h, 45°C for 12 h, and at 60°C for 12 h. Semi-thin (1–2 □m) and ultra-thin (< 1 □m) sections were cut with an ultramicrotome (Om U2, Reichter, Heidelberg, Germany) equipped with a glass blade. The semi-thin sections were stained with toluidine blue and were observed under a light microscope (DMR HC, Leica, Wetzlar, Germany) equipped with a system to take micrographs. Histo-anatomical parameters were measured with an image analysis software (IM1000, Leica, Cambridge, UK). Ultra-thin sections were mounted on uncoated copper grids (200 mesh) and were contrasted by adding uranyl acetate and an aqueous solution of lead nitrate before observation with a transmission electron microscope (TEM 400 T, Philips, Monza, Italy).

2.1.2.5 Isoprene synthase (PaISPs) RNA isolation and analysis

Total RNA was extracted from frozen, homogenized leaf tissue (0.1-0.15 g FW) of control and ozone-treated poplar plants with the NucleoSpin® RNA Plant kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instruction. A given amount of total RNA (1-2 ug) was reverse transcribed for 1 h at 42°C using 200 units Superscript II RT

(Invitrogen, Carlsbad, CA) with 1x corresponding buffer, 10 mM DTT, 0.4 mM each dNTPs, 0.5 µg μl^{-1} oligo dT (5'-$T_{(25)}$-VX-3') primer (Invitrogen, Carlsbad, CA). The cDNA was used for PCR with 1 unit *Taq* polymerase (Amersham Bioscience, Uppsala, Sweden), 1x corresponding buffer, 0.2 mM each dNTP and 10 µM of the *actin* and *PaIspS* primers (Invitrogen, Carlsbad, CA, USA). The authenticity of the PCR products was checked by two directional sequencing using ABI Prism 310 Genetic Analyzer (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA).

2.1.3 Statistics

The data presented are means from at least four replications for each data-point. Mean values ± standard errors are shown for gas-exchange, fluorescence measurements and anatomical features. Anatomical microphotograph show only one sample but they were repeated on at least 20 samples yielding comparable results. Differences of isoprene emission and PaISPs mRNA expression between controls and ozone-treated leaves of the same classes were tested with a t-test and asterisks (*** or *) indicate differences significant at $P < 0.01$ or 0.10, respectively. A Multiple Range Test was used to compare the means of anatomical features and fluorescence data in an ANOVA scheme, and means significantly different were separated by different letters ($P < 0.01$ for anatomical features and $P < 0.05$ for fluorescence data).

2.1.4 Results

The high ozone level was rapidly sensed by the leaves B inside the cuvette. Visual signs of necrosis caused from ozone exposure were observed after three days of treatment (Fig. 6 A). All physiological parameters, including photosynthesis, stomatal conductance and isoprene emission decreased rapidly, within three weeks of treatment (Fig. 6 B) and were undetectable in further measurements. Many of the leaves were shed by d 30 of the experiment.

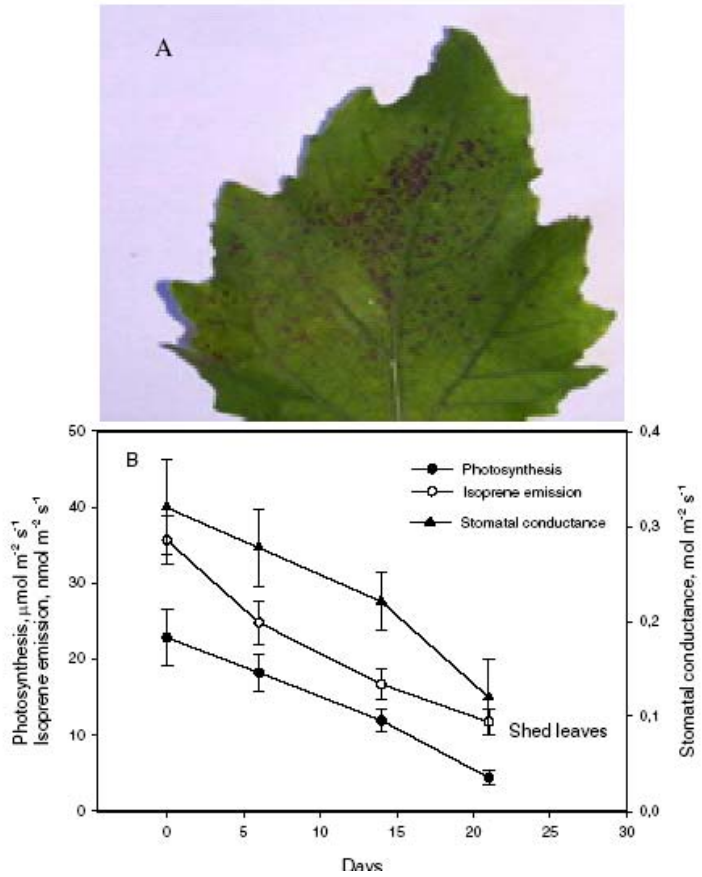

Figure 6: Effects of ozone fumigation on fully expanded leaves (class B) directly exposed to the pollutant for 3 d (A). In (B) photosynthesis, isoprene emission and stomatal conductance of these leaves were followed until leaf abscission, which occurred between 21 and 30 d after beginning the ozone treatment. Means + s.e. (n = 4).

The first leaves which developed outside the cuvette within one week from the beginning of the ozone treatment (A1) showed peculiar morphology and anatomy. The leaf laminas were small (< 5 cm) and lost the typical shape of poplar leaves being oval and with regular edges (data not shown). The lamina was much thicker than in control leaves and in all other leaves of class A and C, and of the corresponding controls growing in plants which were not fumigated with ozone (Tab. 1). All components of the lamina (parenchyma and the two layers of palisade tissues) contributed to make A1 leaves thicker and their mesophyll more packed than in controls (Tab. 1). These features were rapidly lost in the A leaves developing after A1 which were very similar to control leaves. New leaves developed inside the cuvettes during the experiment (C leaves) and were clearly resistant to ozone. C leaves developed with a time frame similar to A3 leaves (they started to expand 15 d after beginning the ozone treatment) and were thinner in ozone-exposed plants than in control plants, especially

because of a reduced height of the palisade parenchyma (Tab. 1 and Fig. 7). The mesophyll of ozone-exposed C leaves was densely packed and palisade cells were smaller in C leaves growing in plants not exposed to ozone.

Leaf class	**Thickness of leaf lamina (µm)**	**Height of palisade parenchima (µm)**	**Height of palisade tissue (1st layer) (µm)**	**Height of palisade tissue (2nd layer) (µm)**	**Diameter of palisade cells (µm)**
Controls					
C	126 ± 2b	51 ± 1b	28 ± 1ab	25 ± 1b	17 ± 1b
A1	128 ± 2b	66 ± 1cd	29 ± 1b	32 ± 2c	14 ± 2a
A2	130 ± 1b	64 ± 1c	31 ± 2c	34 ± 1c	14 ± 1a
A3	103 ± 1a	51 ± 1b	27 ± 1a	24 ± 1ab	14 ± 1a
Ozone treated					
C	106 ± 2a	46 ± 1a	25 ± 1a	22 ± 1a	14 ± 1a
A1	147 ± 3c	68 ± 2d	35 ± 2d	36 ± 2cd	17 ± 1b
A2	128 ± 2b	62 ± 1c	31 ± 1c	31 ± 1c	15 ± 1a
A3	114 ± 2ab	54 ± 1b	29 ± 1b	26 ± 1b	15 ± 1a

Table 1: Anatomical features of leaves expanding above the cuvettes (A), and of leaves developing inside the cuvettes since the beginning of the ozone treatment (C). The number following class A legend denotes the sequence of expansion, being A1, A2, A3 the first, second and third leaf to expand above the cuvettes (Fig.5). Leaves growing inside or above the cuvettes where ozone fumigation was carried out (ozone-treated) are compared to leaves of the two classes growing in absence of ozone fumigation (controls). A Multiple Range Test was used to compare the means within the same row in an ANOVA scheme, and means significantly different were separated by different letters ($P < 0.01$, $n = 20$).

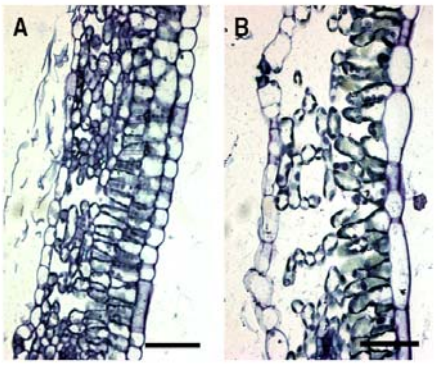

Figure 7: Light microscopy images of sections of leaves developing in the cuvettes during the experiment (referred to as C leaves in the text). In leaves growing in cuvettes under high ozone (A) the total thickness is lower and the mesophyll is more packed than in leaves growing in control conditions, i.e. in cuvettes without ozone enrichment (B). Bars represent 50 µm in both images.

Measurements of the relationship between photosynthesis and intercellular CO_2 concentration (Ci) are often used to reveal photosynthesis limitations. This approach clearly indicated a difference between C and A leaves in ozonated samples, the latter behaving as the corresponding control, while photosynthesis response to increasing Ci was less steep, indicating a lower Rubisco activity, in C leaves (Fig. 8). There were also differences between A1 and A2 leaves of ozone treated plants in the response of photosynthesis to Ci. However, in this case the difference was observed at Ci higher than ambient, where photosynthesis response to Ci was not linear and should be limited by the regeneration rate of RuBP. Moreover, control leaves of comparable age behaved as A1 and A2 leaves, respectively, which indicated a strong developmental control on the response of photosynthesis to Ci, rather than a direct effect of the ozone treatment. For instance C leaves of control plants and A3 leaves of ozone treated and control plants, ontogenetically similar, showed the same response of photosynthesis to Ci (Fig. 8).

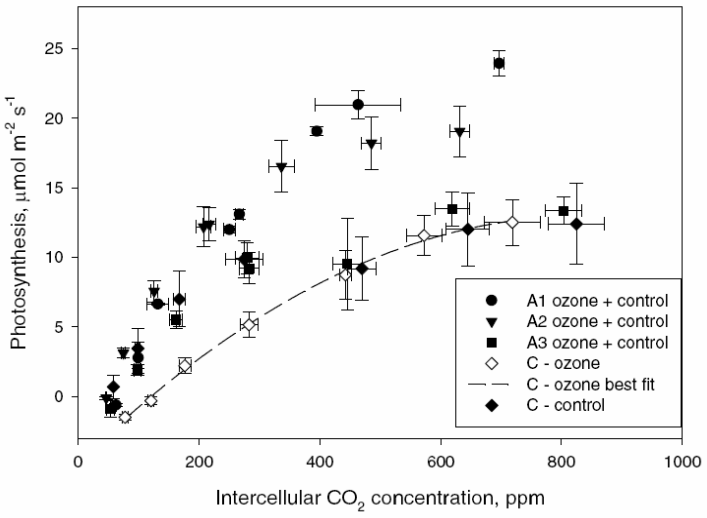

Figure 8: Photosynthesis response to intercellular CO_2 concentration in leaves expanding above the cuvettes (A), and in leaves developing inside the cuvettes since the beginning of the ozone treatment (C). The number following class A legend denotes the sequence of leaf expansion, being A1, A2, A3 the first, second and third leaf to expand above the cuvettes. In class A leaves no difference was observed in the response of ozone fumigated and control plants and the two treatments are pooled (n = 8). The ozone treated plants of class C leaves (n = 4) are shown separately from controls (n = 4), and the different response of ozone-treated C leaves has been interpolated with a best fit line. Means + s.e. are shown.

No differences were found in the quantum yield of dark-adapted leaves measured by the fluorescence parameter Fv/Fm (Tab. 2), but in illuminated leaves the quantum yield of C leaves was clearly affected by growth under high ozone. Moreover, A leaves again showed a very clear developmental control. The ΔF/Fm' parameter was higher in control and ozone treated A1 leaves than in control and ozone treated A2 and A3 leaves (Tab. 2). This confirmed the indications supplied by gas exchange data (Fig. 8) that photosynthesis of developing leaves was limited by the electron transport driven regeneration of RuBP, irrespective of the ozone treatment.

Leaf class	**Fv/Fm**	**ΔF/F'm**
Controls		
C	0.79 ± 0.02a	0.23 ± 0.5b
A1	0.81 ± 0.04a	0.22 ± 0.4b
A2	0.77 ± 0.02a	0.16 ± 0.3a
A3	0.77 ± 0.04a	0.14 ± 0.3a
Ozone treated		
C	0.8 ± 0.02a	0.11 ± 0.5a
A1	0.77 ± 0.05a	0.21 ± 0.5ab
A2	0.79 ± 0.03a	0.14 ± 0.3a
A3	0.78 ± 0.01a	0.13 ± 0.4a

Table 2: Quantum yield of PS2 in darkened leaves (the ratio between variable and maximal fluorescence, Fv/Fm) and in illuminated leaves (the ratio between maximal 2 steady-state fluorescence and maximal fluorescence (Fm'-Fs)/Fm'=ΔF/Fm'). Fluorescence parameters were collected in leaves expanding above the cuvettes (A), and in leaves developing inside the cuvettes since the beginning of the ozone treatment (C). The number following class A legend denotes the sequence of expansion, being A1, A2, A3, the first, second and third leaf, respectively, to expand above the cuvettes (Fig. 5). Leaves growing inside or above the cuvettes where ozone fumigation was carried out (ozone treated) are compared with leaves of the two classes growing in absence of ozone fumigation (controls). A multiple range test was used to compare the means within the same row in an analysis of variance scheme, and means significantly different were labelled by different letters ($P < 0.05$, $n = 5$).

Electron micrographs showed the accumulation of many starch granules in A leaves of control plants. In A3 leaves of ozone treated plants, however, no starch accumulation was observed (Fig. 9). Starch accumulation in A2 leaves of ozone-treated plants was about 50 % that observed in A2 controls. The accumulation of starch in A1 leaves of controls and ozone-treated plants was not significantly different (data not shown).

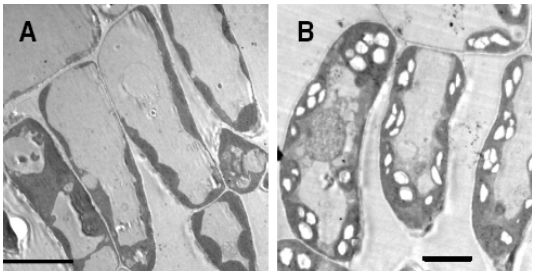

Figure 9: Electron microphotograph of cells of the third leaves (A3) expanding above the cuvettes in which ozone was (A) or was not fumigated (B). Chloroplasts of control A3 leaves present numerous white starch grana which are absent in chloroplasts of A3 leaves developing from ozone fumigated cuvettes. Bars represent 10 µm in panel A and 5 µm in panel B.

The ozone treatment caused a stimulation of the emission of isoprene by poplar leaves. This increase was statistically significant in C leaves and, particularly, in A1 leaves, when compared with control leaves of similar age (Fig. 10). In A1 leaves, the stimulation of isoprene emission was associated to a larger expression of *Populus alba* PaISPs with respect to the control of correspondent age (Fig. 11). PaISPs expression also matched the age dependent reduction of isoprene emission in A leaves (Fig. 10). In C leaves, a very low expression of PaISPs was detected without significant differences between ozone treated and control leaves.

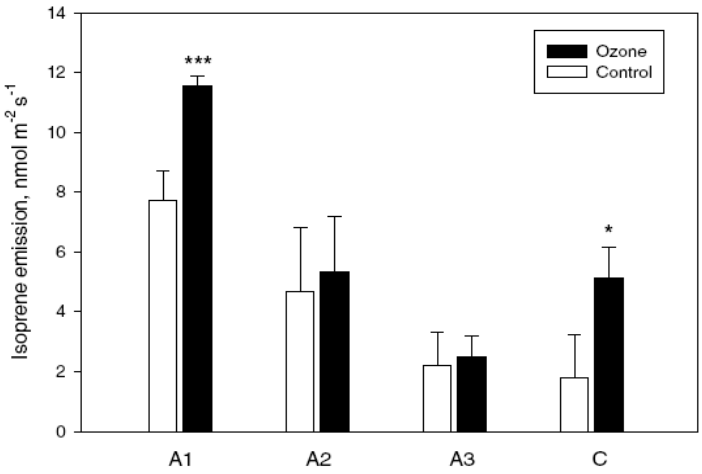

Figure 10: Emission of isoprene in leaves expanding above the cuvettes (A), and in leaves developing inside the cuvettes since the beginning of the ozone treatment (C). The number following class A legend denotes the sequence of expansion, being A1, A2, A3 the first, second and third leaf to expand above the cuvettes. Means +

s.e. (n = 4) are shown. Differences between controls and ozone-treated leaves of the same classes were tested with a t-test and asterisks (*** or *) indicate differences significant at P <0.01 or 0.10, respectively.

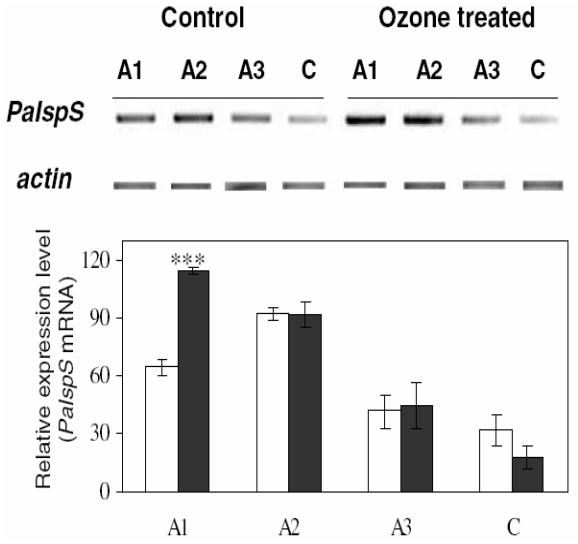

Figure 11: Expression analysis of isoprene synthase mRNA (*PaISPs*) in *Populus alba* leaves expanding above the cuvettes (A), and in leaves developing inside the cuvettes since the beginning of the ozone treatment (C). The number following class A legend denotes the sequence of expansion, being A1, A2, A3 the first, second and third leaf to expand above the cuvettes. One to two µg of total RNA were reverse transcribed and amplified by semi-quantitative RT-PCR. mRNA levels loaded in each lane were determined by co-amplification and normalization with an internal standard (actin). Close to the blot, a graph with the relative intensities of the signals (means + s.e., n = 4) is shown. Differences between controls (empty bars) and ozone-treated leaves (filled bars) of the same classes were tested with a t-test and asterisks (***) indicate differences significant at P <0.01 level.

2.1.5 Discussion

Ozone damage occurred fast and was severe in *Populus alba* leaves directly exposed to ozone, confirming that this plant species is sensitive to ozone (Bortier et al. 2000). My task was however to detect whether ozone could affect the morphology and physiology of leaves of the same plant but which were developing in condition of indirect exposure to the stressor. This was possible with the innovative design of the cuvette system. In fact, the anatomical changes of the first leaves developing at ambient ozone level (A1) were dramatic and indicated that ozone can strongly impact on their development. However, the impact of

ozone was rapidly lost in leaves developing after A1, suggesting that ozone only affects relevantly the closest leaf to the ozone source. The signalling system which brings to these interesting developmental changes is still unknown and deserves further investigation. Interestingly, despite ozone impact on leaf anatomy, A1 leaves revealed a very well preserved physiology, being the photochemistry and biochemistry of photosynthesis very similar to that observed in control leaves of corresponding developmental stage. However, a very low accumulation of starch was noted in leaves developing after A1 in ozone-treated plants. This indicates that ozone may affect starch biosynthesis on expanding leaves. The results suggest that the biosynthesis of starch is delayed, as this would explain why no differences were found in fully expanded leaves (e.g. A1) while newly developing (A3) leaves of ozone-treated plants were unable to accumulate starch. However, it cannot be excluded that starch was in fact produced, but exported very rapidly from A3 leaves to stronger sinks of ozone-treated plants. The perturbed starch biosynthesis may ultimately affect plant growth and productivity in plants experiencing ozone exposure. More experiments are needed to investigate whether this strong starch depletion also occurs in response to less dramatic ozone exposure, which may indicate its very common occurrence in nature. Starch depletion has been observed by other authors in leaves directly exposed to ozone, but was associated to photosynthesis inhibition and never independent on photosynthetic competence (Oksanen 2003).

Ozone also had a very strong impact on isoprene emission in the first leaf developing at ambient ozone. The observed isoprene stimulation in A1 leaves developing above those fumigated with ozone expands the observation that leaves recovering from ozone stress emit more isoprene (Velikova et al. 2005) or monoterpenes (Loreto et al. 2004). This seems in contrast with the results obtained from Calfapietra et al. (2007) from a FACE (Free-Air CO_2 Enrichment) experimental site with *Populus* spp. exposed to elevated ozone. These authors observed a drop in ISPS mRNA and isoprene emission due to ozone exposure. However, they also observed strong photosynthetic limitations, due a heavier and more prolonged chronic exposure to the pollutant, which might have limited considerably the substrate availability for isoprene formation. As in the case of other physiological and anatomical features, also isoprene emission was not affected in leaves developing at more distance from ozone fumigated leaves, although a residual but not significant stimulation was also noted in A2 leaves. It should be considered that isoprene emission is under a strong developmental

regulation, the low emission of young leaves being regulated at a transcription and transduction level because of the low amount of isoprene synthase mRNA and protein (Wiberley et al. 2005). Our finding confirms that PaISPs mRNA is very low in leaves which start to develop and show that indirect exposure to ozone does not affect early stages of isoprene induction.

The biochemical steps which are involved in isoprene stimulation under oxidative stress are unknown. Isoprene emission is often associated to the availability of photosynthetic intermediates and this is also true for B leaves growing in the cuvettes at high ozone. However, this is not the case in A leaves which showed no photosynthesis stimulation with respect to A leaves of control plants. Isoprene stimulation in A1 leaves was associated with a greater expression of isoprene synthase gene, indicating that there might have been an increase of the protein amount, and that isoprene emission of leaves developing above those which are exposed to ozone can be regulated at the transcriptional level, as also observed in leaves exposed to light and heat stress (Sasaki et al. 2005).

The observation that new leaves (C leaves) developed inside the cuvettes under high levels of ozone, while already developed leaves (B leaves) showed larger damage and were eventually shed, reveals adjustments leading to the acquisition of ozone resistance. This is in our view an important observation whose biochemical basis should be further investigated. The mesophyll of C leaves exposed to ozone was more packed than in C leaves of controls and in other leaf classes, suggesting that ozone diffusion might be reduced and that this may be related to the observed resistance. Ozone exposure often results in a reduction of mesophyll thickness and leads to more packed cell structure (Oksanen et al. 2005). Probably C leaves also have a more developed antioxidant system protecting them from reactive oxidative species formed by ozone (Pell et al. 1997; Diara et al. 2005).

Isoprene was described to have an antioxidant action protecting leaves from ozone (Loreto and Velikova, 2001). I have detected a stimulation of isoprene emission in C leaves directly exposed to ozone in comparison to the emission of corresponding control leaves. However, the emission of ozone-treated C leaves was lower than in A1 leaves and in B leaves at the beginning of the stress treatment. Two aspects should be considered when analyzing this data-set. First, C leaves were analyzed at a very young stage, comparable to those of A3 leaves. This is reflected in a yet low developmental capacity to produce isoprene as also indicated by the very low level of PaISPs mRNA detected in C leaves. Second, despite being

resistant to ozone, the photosynthetic metabolism of C leaves was perturbed by direct ozone exposure and this may have further limited isoprene production. A very large limitation of photosynthesis was revealed in ozone exposed C leaves by the response of photosynthesis to increasing intercellular CO_2 concentration. The limitation was particularly evident at low CO_2 levels at which photosynthesis responds linearly to CO_2 and is limited by Rubisco activity (von Caemmerer and Farquhar 1981). Rubisco is known to be negatively affected by ozone, being a very common cause of photosynthesis limitation in ozone-stressed leaves (Pell et al. 1997). This study suggests that Rubisco remains a parameter sensitive to ozone, even in leaves which acquire resistance to the pollutant.

2.2 Ozone uptake by leaves of different species, and the implications on BVOC emitted

2.2.1 Aims of the study

In order to investigate the role of plants as ozone scavengers in the atmosphere (see section 1.1.2), it is important to measure the ozone uptake by leaves in response to changing environmental parameters, and to determine the stomatal and non-stomatal component of this sink. This was the first objective of this study. As a second objective, I investigated whether volatile isoprenoids protecting membranes under high ozone exposure, also play a role in scavenging ozone or reactive oxygen species, overall reducing ozone injuries in leaves. I also wanted to test the dogma that ozone uptake is directly associated to ozone sensitivity, under the assumption that, if isoprenoids remove ozone through secondary reactions in the mesophyll, ozone uptake by the leaf increases while less damage is exerted.
To fulfil the above aims, isoprenoid emitting and non-emitting species were used. In order to investigate the impact of isoprenoids in naturally emitting species, a chemical inhibitor of isoprenoid biosynthesis was used. In the case of non-emitting species, the same objective was reached adding isoprene exogenously. In a different experiment with non-emitting plants, two clover clones (*Trifolium Repens* cv "Regal") previously characterized for their contrasting sensitivity to ozone were exposed to chronic levels of the pollutant to investigate whether the different resistance to ozone was associated to stomatal uptake or detoxification of ozone through biochemical defence mechanism. Finally, a chronic exposure to high ozone was also performed on isoprene-emitting plants of *Populus alba*, with the aim to study how the continuous exposure to the pollutant influences ozone uptake, and isoprene, methanol, and lipoxygenation product emissions along plant profile.

2.2.2 Methods

2.2.2.1 Plant material

The experimental site was located at CNR-IBAF, Monterotondo Scalo (Roma). As forest species, three-year-old plants of *Populus alba, Populus nigra, Quercus ilex, Carpinus betulus, Betula pendula* were used for this study. Plants were potted in 50-L pots filled with

commercial soil and grown in the greenhouse under controlled conditions. As non-forest species, I used well developed plants of *Nicotiana tabacum* and *Trifoliun repens,* the latter being obtained by the International Cooperative Programme (ICP). Clover seeds were germinated at the University La Sapienza, Rome, Dipartimento di Biologia Vegetale, and successively planted in the greenhouse of the Institute. A third herbaceous plant species, *Phragmites australis*, a well characterized isoprene emitter, was also used. Phragmites were reproduced through cuttings and kept in an vermiculite pots immersed in water and fed with Hoagland solution. Experiments were performed during spring and summer of 2005 and 2006 when the air temperature was 24-30°C and the air humidity was around 50%, the light intensity at the canopy level during the day above 850 μmol photons m^{-2} s^{-1}. Plants were irrigated daily and fertilized weekly to avoid drought and nutritional stress during the experiments.

2.2.2.2 Measurement of physiological parameters and ozone injuries

Net photosynthesis (A), leaf transpiration (E) and stomatal conductance (Gs) were measured by a portable open-system infrared gas analyzer Li-Cor 6400 (LI-COR, Lincoln, NE, USA). Leaf temperature was set at 30°C, and light intensity at 1000 □mol m^{-2} s^{-1}. Further information on the gas exchange system are given in case study 1. An additional and custom-made system was used to detect gas exchange. In this system, certified tanks of pure N_2, O_2, and CO_2 were connected to mass-flow controllers (Brooks 5860, Rosemont, USA) modulated by a computer software in order to obtain a mix of synthetic air (80% N_2, 20% O_2, 380 ppm of CO_2) and a flux of 800 ml/min to be convoyed to the cuvettes through a Teflon tubing system. The same source of synthetic air was also convoyed to the portable system for a more accurate measurement. The light intensity inside the cuvettes was controlled manually switching on a light lamp placed at short distance above the cuvettes to reach an intensity at the leaf surface of 1000 μmol m^{-2} s^{-1}. Air humidity of the air entering the cuvette was set at 50 % by bubbling the N_2+O_2 mixture, prior CO_2 insertion, into water and passing then the stream in a custom made condenser. The cuvettes were thermostated at 30°C with a custom-made Peltier resistances. The outlet of the cuvettes was convoyed to a LI-COR 6262 Infra Red Gas Analyzer (LI-COR, Lincoln, NE, USA) for CO_2 and water

measurements, and to a PTR-MS for BVOC measurement. Different sized, custom made leaf cuvettes (Fig. 4 A, B) were used depending on the size of the leaves to be enclosed. Specifically, for this experiment a glass, 3 L cuvette (Fig. 4 D) was used for cut leaves. When testing the isoprenoid effect on ozone uptake, the petiole of leaves was fed with fosmidomycin (30 mM for 60 min), an isoprenoid inhibitor dissolved in water solution (further details are given by Velikova et al. 2005). In cases where an exogenous source of isoprene was needed, a tank of isoprene (10 ppm) was connected to the main tubing line of the isoprene-free air stream (800 ml min^{-1}) prior to cuvette inlet. The air flow from the tank was regulated with a mass-flow controller (Brooks 5860, Rosemont, USA) and diluted with the isoprene-free synthetic air to reach a final concentration in the cuvette of 3 ppm. For *Populus nigra*, isoprene, methanol, acetaldehyde, and hexanol emissions were measured on 8 leaves along the plant profile, in the scheme shown in Figure 5. I tested the emission of these same BVOC blend from two clones of *Trifolium repens* exposed to chronic concentrations of ozone (see next chapter) during the intermediate period of the experiment (7-8 days after the fumigation). Ozone damage to plants was assessed by measuring photosynthesis and the maximal photochemical efficiency of dark-adapted leaves, as monitored by the ratio between variable and maximal fluorescence (Fv/Fm). The occurrence of visible damages caused by ozone 7 d after the ozone treatment, was assessed by photographing the leaf lamina exposed to the pollutant with a digital camera (DC 120, Eastman-Kodak). Ozone-induced necroses of the leaf lamina in presence or in absence of isoprene were compared by separating the damaged areas with a computer software (DS1D Scientific Imaging System, Kodak).

2.2.2.3 Ozone fumigation and measurement of ozone uptake

When assessing chronic ozone exposure on *Populus nigra* and *Trifolium repens* leaves, plants grown in growth chambers were fumigated with 100 ppb of ozone for 10 days, 6 hours per day (09:00 – 15:00 h, when plants showed the highest physiological activity), during three consecutive weeks (poplars) and 10 consecutive days (clovers). Ozone was generated through photochemical reactions by pure O_2 (see reactions 1-4 in section 1.1) flowing into a box containing two UV lamps (Helios Italquartz, Milan, Italy). This flow was

kept constant (0.3 L min^{-1}) by using an electronic mass flow controller (MCF-D-5111, M+W Instruments, Leonhardsbuch, Germany). Instantaneous O_3 concentration was continuously monitored by UV photometric O_3 detector (Model 1008, Dasibi Environmental Corp., Glendale, California, USA) and automatically saved every 7 min on a PC linked to the ozone detector.

When measuring the ozone uptake, ozone was generated by flowing the synthetic air entering the cuvette through a winding quartz glass illuminated with a second UV light source (Helios Italquartz, Milan, Italy) placed at short distance from the cuvette inlet. The lamp was set to generate ozone at a concentration of 100 ppb. The ozone concentration in the cuvette was monitored with a Photometric O_3 analyzer (1008 Dasibi Environmental Corp., Glendale, California, USA) periodically switching between the cuvette inlet and outlet with Teflon valves as explained elsewhere (Velikova et al. 2005). The difference in ozone concentration between inlet and outlet of the cuvette was considered to be taken up by the leaf. Measurements with empty cuvette were also routinely performed before and after inserting the leaves inside the cuvette, in order to ascertain that ozone uptake by the cuvette material was constantly low. The ozone flux into the leaf (Φ_{O_3}, (nmol m^{-2} s^{-1})) was calculated according to equation 4. In the case of *Populus nigra*, ozone uptake was measured on 8 leaves along the plant profile, with the scheme shown in Figure 5.

2.2.2.4 Biochemical measurements

These assays were performed in collaboration with other researchers of CNR-IBAF (Isabel Nogues, Csengele Barta). Destructive assays on the two clones of *Trifolium repens*, were performed on leaf discs (one cm^2) that were freeze-clamped under two brass drums pre-chilled in liquid nitrogen immediately after the end of physiological measurements (h. 16.00), and were stored at -80 °C until processed.

Hydrogen peroxide (H_2O_2) content was determined according to Velikova et al. (2000).

For measurements of lipid peroxydation in leaves, the thiobarbituric acid (TBA) test, which determines malonyldialdehyde (MDA), an end product of lipid peroxidation, was used as described by Heath and Parker (1968).

For total phenolics, leaf material (100 mg) stored in liquid nitrogen was extracted twice with 80 % methanol (1.5 ml) for 3 minutes in ultrasonic bath. The extracts were centrifuged and combined in a single volume. The amount of total extractable polyphenols (low and intermediate weight phenols) was determined with the Folin-Ciocalteau reagent as described by Singleton et al. (1999).

Ascorbate content was determined using the spectrophotometer method described by Takahama and Oniki (1992).

2.2.3 Results and discussion

2.2.3.1 Stomatal conductance is the key driver of ozone uptake

A clear relationship was observed between ozone uptake and stomatal conductance for all species (Fig. 12). This relationship was best fitted by a second order curve in both clones, and suggested that the increments of ozone uptake are reduced when stomatal conductances are very high. Such a curvilinear relationship may indicate that, at high stomatal conductance, a saturation of the scavenging capacity of the pollutant inside leaves is reached. This may in turn cause ozone accumulation inside the mesophyll, reducing the gradient with external ozone concentration and its further uptake (Fig. 1).

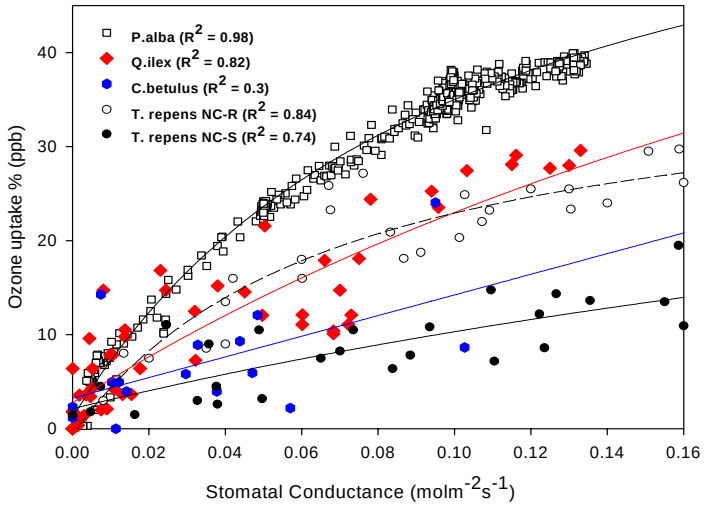

Figure 12: Ozone uptake (% of 100 ppb of ozone entering the cuvette) related to the stomatal conductance of different plant species. The Pearson coefficient is reported.

The concentration of ozone inside the leaves is believed to be zero, as ozone instantaneously react with chemical components of cell walls and membranes (Laisk et al. 1989) but this may not be always the case, at least at the level of intercellular spaces. This argument will be developed in case study 3.

Ozone flux was low when stomatal opening was also reduced by decreasing the PPFD (Fig. 12). Stomata effectively regulate ozone entry in the leaves and this is often the reason why sclerophyllous (Manes et al. 1998) and drought stressed plants (Nali et al. 2004) suffer less ozone damage in comparison with other vegetation types and with well-irrigated plants. The best fits for the ozone-treated species extrapolated at ozone fluxes around 2 ppb when stomatal conductance was zero (*Populus alba* = 1.17, *Quercus ilex* = 2.76, *Carpinus betulus* = 3.5, *Trifolium repens* NC-S = 0, *Trifolium repens* NC-R = 2.13). This may be considered as the non-stomatal uptake, at least when stomata are completely shut. Non-stomatal uptake seems to play a minor role in setting the total ozone flux at leaf level, accounting for about 10% of the uptake measured when stomata are open. However, we do not know at this stage whether the non-stomatal uptake is higher when stomata are open and transpiration is high. A substantial contribution of non-stomatal uptake to total ozone uptake by the leaves has been observed when the leaf surface is wet (Altimir et al. 2006) and can be driven by the humidity released when stomata are open (Zheng et al. 2002). The possibility that stomatal and non-stomatal ozone fluxes are strictly dependent was indeed postulated by Altimir et al. (2006). The contribution of BVOC emitted by the plant to the non-stomatal uptake could be important. This issue will be treated in the case of study 3.

A direct relationship between ozone flux and stomatal conductance was also observed in *Populus nigra* (Fig. 13 panel D, E). In this experiment, a chronic exposure to the pollutant did not significantly affect the stomatal uptake, photosynthesis, and emission of oxygenated compounds along the plant profile in comparison with a control plot (Fig. 13 panel B, C, D, F) suggesting that a fumigation with 100 ppb of ozone for a prolonged time did not have any detrimental effects on plant physiology. Oxygenated compounds as methanol and acetaldehyde were also inhibited in ozone-treated plants (Fig. 13 A, B), maybe because ozone induced senescence, thus limiting the production of molecules formed during leaf expansion, especially methanol (Nemecek-Marshall et al. 1995). This experiment also demonstrated that leaf age is an important parameter which influences the magnitude of

ozone uptake. Indeed, youngest leaves of treated plants showed a higher stomatal conductance than oldest leaves, with higher levels of ozone uptake.

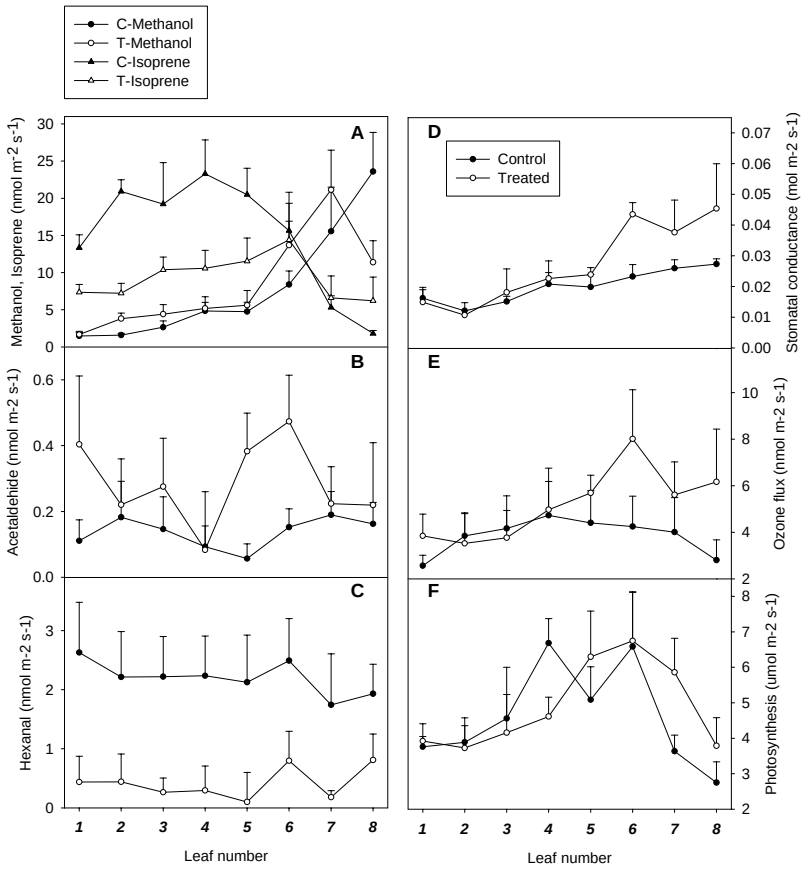

Figure 13: Emission of isoprene and methanol (A), acetaldehyde (B), Hexanal (C), stomatal conductance (D), ozone flux (F) and photosynthesis (G) of two sets of *Populus nigra*. A first set was treated with chronic exposure to 100 ppb ozone (white circles), a second set was treated with ozone-free air (black circles).

In the experiment with *Trifolium repens*, ozone treatment with 100 ppb damaged both the resistant (NC-R) and the sensible (NC-S) clone. Photosynthesis and stomatal conductance of both clones rapidly decreased after exposure to ozone (Figure 14 A, B). However, photosynthesis dropped more rapidly in the NC-S clone (-0.156 $\mu molCO_2\ m^{-2}\ day^{-1}$) than in the NC-R clone (-0.103 $\mu molCO_2\ m^{-2}\ day^{-1}$) during the first 7 d of ozone exposure. Photosynthesis was significantly lower in NC-S than in NC-R plants until 10 d of treatment, but no differences were observed between clones 3d after ozone fumigation, indicating that the damage caused by ozone to the photosynthetic apparatus cannot be rapidly recovered.

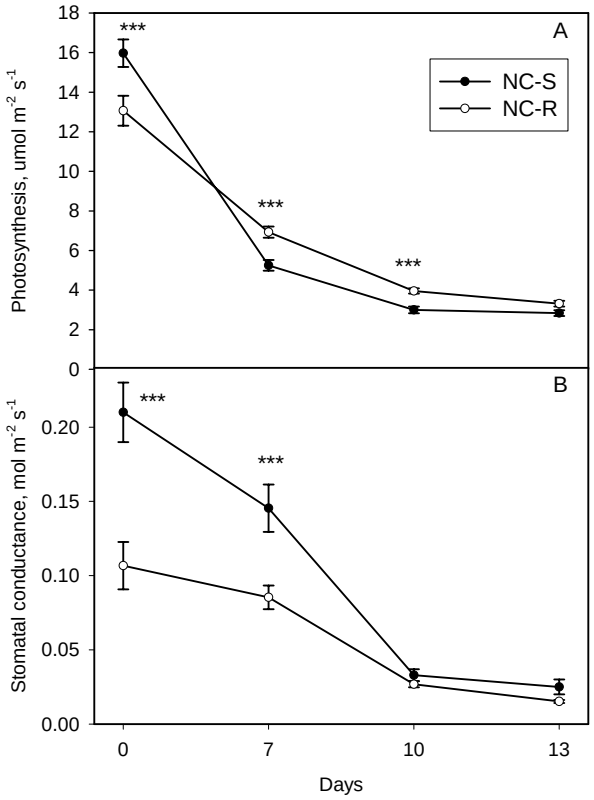

Figure 14: Photosynthesis (panel A) and stomatal conductance (panel B) of a resistant (NC-R) and a sensible (NC-S) clone of *Trifolium repens* cv "Regal". Measures were carried on the day before ozone fumigation at 100 ppb (day = 0), after a 7 and 10 d ozone fumigation, and 3 d after recovering from the ozone treatment (day = 13). Means ± standard errors (n = 16) are shown and means significantly different in the two clones at the same time are identified by asterisks (ANOVA and Tukey test, P<0.001).

2.2.3.2 The feedbacks between ozone uptake and VOC emission

I compared the ozone uptake by leaves of tobacco (*Nicotiana tabacum)* and birch (*Betula pendula*), two species that do not emit isoprenoids, leaves of holm oak (*Quercus ilex*) emitting monoterpenes, and leaves of two species emitting isoprene, common reed (*Phragmites australis*) and white poplar (*Populus alba*) (Figure 15). Isoprenoids emitted by poplar, reed, and oak species have been demonstrated to act as powerful antioxidants, dramatically reducing oxidative damage in leaves (Loreto and Velikova, 2001; Affek and Yakir, 2002). Since ozone uptake depends on the stomatal conductance and environmental parameters, only measurements made at the same stomatal conductance (0.1 mol m^{-2} s^{-1}) and

at the same environmental conditions (light intensity, 1000 mmol m^{-2} s^{-1}; leaf temperature, 30 °C; relative humidity, 40%; and vapour pressure difference between leaf and air, 15 mbar) are shown in Fig 15.

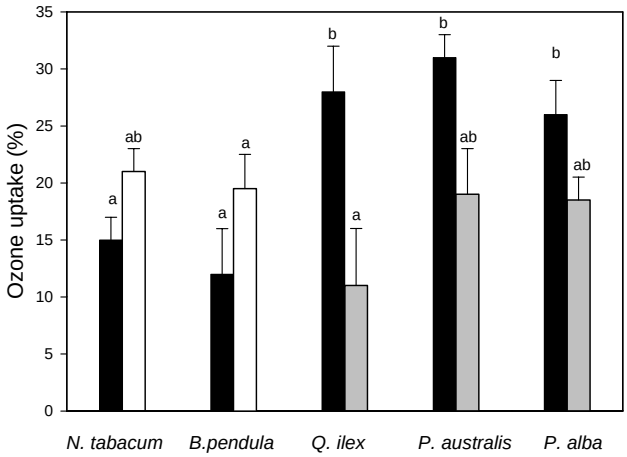

Figure 15: Ozone uptake (black bars) by leaves of two plant species that do not emit isoprenoids (*Nicotiana tabacum* and *Betula pendula*), by a species emitting monoterpenes, *Quercus ilex*, and by two species emitting isoprene, common reed and white poplar. The ozone uptake was also measured in leaves of tobacco and birch fumigated with 3 ppm of exogenous isoprene (white bar), and in leaves of holm oak, reed, and white poplar in which isoprenoid emission was previously inhibited by fosmidomycin (30 mM) feeding for 60 min (gray bars). Measurements were made on leaves showing the same stomatal conductance (0.1 mol m^{-2} s^{-1}) and at the same environmental conditions (light intensity, 1000 mmol m^{-2} s^{-1}; leaf temperature, 30 °C; relative humidity, 40%; and vapour pressure difference between leaf and air, 15 mbar). Measurements were repeated on five different leaves per species. Means and SEs are shown, and means are separated by ANOVA using a multiple range test. Means significantly different are separated by different letters (P = 0.01, single letter; P = 0.05, double letters).

Ozone uptake by leaves of white poplar, common reed, and holm oak was statistically greater than by leaves of tobacco and birch (Fig. 15). Ozone uptake measured in leaves of tobacco and birch fumigated with 3 ppm of exogenous isoprene (white bar) was significantly higher than the uptake by non-treated plants. Moreover, in leaves of holm oak, reed, and white poplar in which isoprenoids emission was previously inhibited by fosmidomycin (30 mM), ozone uptake was lower than in naturally emitting plants. These results confirmed the hypothesis that isoprenoids enhance ozone uptake. Since the retention time of the air in the cuvette is very short (< 5 seconds), a direct reaction between ozone and isoprenoids can be excluded. Table 3 shows the reaction time in air with ozone of the most common isoprenoids

(Atkinson et al. 1997) at different atmospheric ozone levels, and clearly indicate that most of the isoprenoids need more than 10 minutes to react with ozone in the gas phase, which excludes the possibility that gas phase reactions could contribute to the ozone uptake in this experimental set up.

Compound	Rate constants k^{O3} 10^{-18} (cm^3 s^{-1})	RT (min) *O_3 = 50 ppb* (≈$13.4 \cdot 10^{11}$ molec·cm^3)	RT (min) *O_3 = 100 ppb* (≈$26.8 \cdot 10^{11}$ molec·cm^3)	RT (min) *O_3 = 150 ppb* (≈$40.2 \cdot 10^{11}$ molec·cm^3)
Isoprene	0.13	95711	47855	31908
α-Pinene	86.6	144	72	48
Camphene	0.9	13825	6912	4609
Sabinene	86	145	72	48
Myrcene	470	26	13	9
β-Pinene	15	829	414	277
α-Terpinene	21100	1	0.29	0
cis-Ocimene	37	336	168	112
Limonene	200	461	31	154
γ-Terpinene	140	89	44	30
2-Carene	230	54	27	18

Table 3: Main isoprenoids emitted by plants and their rate constants with ozone and retention time in the enclosure at different ozone levels. Rate constants were calculated as reported by Atkinson et al. (1997).

My hypothesis is that ozone captured by leaves through stomatal openings rapidly reacts with cell membranes generating reactive oxygen species (Pell et al. 1997), and these are quenched by isoprenoids through secondary reactions. The reaction time of these reactive species (mainly O'_2, OH′, and H_2O_2) with isoprenoids is not fully studied but is considered to be in the order of few seconds. Moreover, in the intercellular spaces, isoprenoids in naturally emitting species reach the concentration of hundreds to thousands of ppb (Loreto et al. 1998), representing an abundant substrate to form reactive species scavenging ozone. It should be also mentioned that experiments have recently shown that isoprenoid emission can be either positively (Cases study 1 & 3, Loreto et al., 2004; Valkama et al. 2007; Beauchamp et al. 2005), or negatively (Figure 13 A, Velikova et al. 2005; Calfapietra et al. 2007) affected by long-term exposure to ozone, depending on the magnitude of ozone levels in the

atmosphere, on the duration of the exposure, and on the lag-time between measurements and exposure to high ozone. This may change the observed uptake, and the capacity of isoprenoid-emitting leaves to take up ozone, under natural conditions.

The enhancement of ozone uptake in presence of natural antioxidants such as the volatile isoprenoids, suggests that the notion that ozone uptake produces ozone damage may not be always correct. If this were the case, ozone damage should be reduced in the presence of isoprenoids.

The foliar ozone damage was assessed monitoring the photosynthesis, chlorophyll fluorescence, and visible necrosis for holm oak, white poplar, tobacco, birch and reed. As expected, all parameters were negatively affected by ozone, with photosynthesis and maximal quantum yield of fluorescence reaching 50% of the values of intact leaves in tobacco, the most sensitive species (Fig. 16). However, in all plant species, at least one of these parameters was significantly less inhibited by ozone in naturally emitting isoprenoids (holm oak, white poplar, and reed) or when exogenous isoprene was supplied (tobacco and birch), in comparison to the same plants in which isoprenoid emission was inhibited or exogenous isoprene was not supplied (Fig. 16). The effect was very evident when the damage was inherently high, such as in tobacco, as also previously observed (Loreto et al. 2001), and in the monoterpene-emitting species holm oak, in which it may be associated with the more efficient action of monoterpenes as ozone scavengers (also indicated by Fig. 15). Ozone may apparently also induce biosynthesis and emission of volatile isoprenoids, mainly sesquiterpenes, even in plants that do not naturally emit these compounds (Heiden et al. 1999; Beauchamp et al. 2005). Whether any of these compounds might also be responsible for the larger ozone uptake by ozone resistant plant species and cultivars needs to be studied.

These experiments show that ozone flux into leaves may not necessarily be associated with damage. In fact, high ozone fluxes may indicate efficient protective mechanisms of detoxification and resistance to the stressor. This finding calls for a redefinition of the stomatal ozone uptake parameter to include a detoxification component, especially when using stomatal flux to assess ozone risk (UNECE, 2004). The concept that a relevant ozone uptake by plants may be related to protection mechanisms fosters more research to study the biochemistry of ozone reaction inside leaves and to exploit the capacity of vegetation to naturally scavenge ozone.

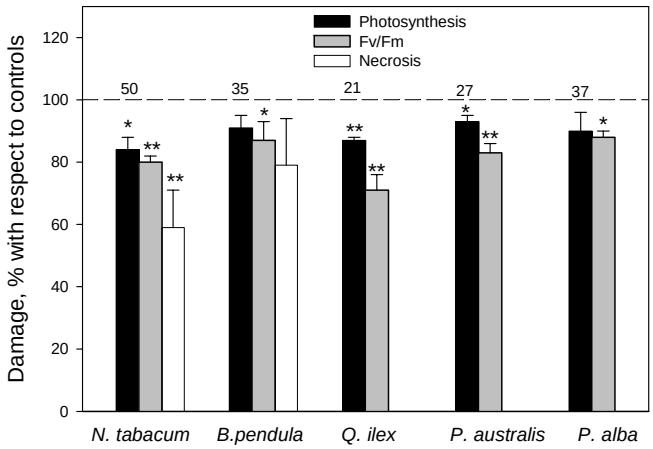

Figure 16: Ozone damage in leaves naturally emitting volatile isoprenoids (*Quercus ilex*, *Phragmites australis*, and *Populus alba*) and in leaves of plants that do not emit isoprenoids but were fumigated with 3 ppm of exogenous isoprene during the ozone treatment (*Betula pendula* and *Nicotiana tabacum*). Ozone (100 ppb) was fumigated consecutively for different times to illuminated leaves, as indicated in the text. Damage indicators were the reduction of net photosynthesis and of maximal quantum yield of chlorophyll fluorescence in dark-adapted leaves (Fv/Fm), measured 12 h after the ozone treatment, and the reduced appearance of visible necrotic areas in the leaf lamina, measured 7 d after the treatment. Visible necrotic damage was assessed only in intact leaves of tobacco and birch, not in those leaves that were cut to feed the isoprenoid inhibitor. All damage indicators are shown as percentage of the level measured in leaves that do not emit naturally isoprene or in which isoprenoid emission was previously inhibited by fosmidomycin, as indicated in the text (dotted horizontal line). The numbers above the dotted line indicate the reduction of photosynthesis (%) caused by the ozone treatment in leaves that do not naturally emit isoprene or in which isoprenoid emission was previously inhibited by fosmidomycin, with respect to the non-fumigated controls. Measurements were repeated on five different leaves per species. Means significantly different from 100% (the damage measured in leaves without endogenous or exogenous volatile isoprenoids) are separated by ANOVA using a multiple range test (P = 0.01, double asterisk; P= 0.05, single asterisk).

2.2.3.3 An investigation of antioxidant systems alternative to isoprenoids

Trifolium repens is not known as an isoprenoid-emitting species, and no emissions of isoprene or monoterpenes were detected in this experiment. Interestingly, the NC-R clone absorbed more ozone than leaves of the NC-S clone (Fig. 12). The stomatal conductance was higher in NC-S leaves than in NC-R leaves before and during the first 7 d of ozone fumigation (Fig. 14 B), and this might have caused a larger uptake in the first phase of

fumigation, before stomatal closure. This may explain the heavier reduction of photosynthesis observed during the first 7 d of ozone fumigation in the NC-S leaves (Fig. 14 A). However, I observed that even when stomatal conductance of NC-S and NC-R were maximal (between 0.2 and 0.1 mol m^{-2} s^{-1}, Fig.14 B), the best fits of Figure 12 show a lower ozone flux in NC-S leaves. This observation, and the generally lower ozone uptake of NC-S leaves at comparable stomatal conductance shown in Figure 12, again confirms that the ozone sensitivity is not related to a higher stomatal uptake of the pollutant.

A high oxidative effect of ozone in NC-S clones should correspond to a high reactive oxygen species (H_2O_2, singlet O_2, and OH radicals) accumulation, since these compounds are instantaneously formed by ozone inside leaves (Chameides 1989). The same low quantity of H_2O_2 was found in both clones at the beginning of the treatment (Tab. 4). The amount of H_2O_2 surged to a maximum after 7 d of ozone fumigation in the leaves of the NC-R clones. This value was about 4 times higher than the H_2O_2 pre-stress level and did not increase further during the remaining period of treatment and after the 3-d recovery. In contrast, in the NC-S clone the H_2O_2 levels increased continuously throughout the ozone fumigation treatment and even during the recovery. At the end of the recovery period the H_2O_2 amount was 8.3 fold higher than at the beginning of the experiment in the NC-S clone. The accumulation of H_2O_2 indicates either an excess of formation of this dangerous reactive oxygen species, or its inefficient removal by the antioxidant system of ozonated NC-S leaves.

Compound	Day	Plants		p
		NC-R	NC-S	
H_2O_2	*0*	09,52 ± 0,54	07,01 ± 0,46	<0,01
	7	36,70 ± 2,85	22,04 ± 1,14	<0,001
	10	24,44 ± 2,01	36,00 ± 1,00	<0,001
	13	34,60 ± 1,43	58,00 ± 1,40	<0,001
MDA	*0*	0,067 ± 0,001	0,061 ± 0,001	<0,05
	7	0,069 ± 0,016	0,077 ± 0,004	
	10	0,050 ± 0,011	0,064 ± 0,004	
	13	0,083 ± 0,001	0,157 ± 0,003	<0,05
Phenolics	*0*	17,00 ± 1,36	16,60 ± 1,37	
	7	21,41 ± 1,84	32,02 ± 2,30	<0,01
	10	32,77 ± 1,42	31,60 ± 1,00	
	13	12,18 ± 1,10	29,10 ± 0,80	<0,001
Ascorbic acid (oxydized fom)	*0*	0,176 ± 0,023 (69)	0,221 ± 0,016 (60)	<0,01
	7	0,239 ± 0,004 (65)	0,345 ± 0,003 (67)	<0,001
	10	0,138 ± 0,001 (55)	0,135 ± 0,002 (53)	
Ascorbic acid (reduced fom)	*0*	0,078 ± 0,015 (31)	0,146 ± 0,013 (40)	<0,001
	7	0,131 ± 0,009 (35)	0,173 ± 0,004 (33)	<0,001
	10	0,112 ± 0,007 (45)	0,121 ± 0,002 (47)	<0,01

Table 4: H_2O_2 concentration (µmol g^{-1} fresh weight), content of malonyldialdehyde (MDA, mol g^{-1} fresh weight), total phenolics expressed as gallic acid equivalents (mg GAE g^{-1} fresh weight) and ascorbic acid (AA) concentration (µmol g^{-1} fresh weight) of leaves of NC-S and NC-R clones of *Trifolium repens* the day before ozone fumigation (day 0), after 7 and 10 d of ozone fumigation, and 3 d after ozone fumigation (day 13). Enclosed in brackets the percentage value of each form of ascorbic acid with respect to the total amount. Values are means ± standard errors, and significant differences between clones after ANOVA and Tukey test (n=8) are reported for $p \leq 0.05$.

The accumulation of free radicals inside cells of the NC-S clone upon ozone exposure was previously observed by Hippeli and Elstner (1996) and Crous et al. (2006). These authors suggested that the non-photochemical mechanism did not efficiently quench the excess of energy in the NC-S clone as compared to the NC-R clone, which could in turn cause membrane damage.

The second biochemical indicator I used in this study is the level of malonyldialdehyde (MDA, Heath and Parker, 1968) indicating peroxidation of lipids. MDA may strongly increase when membranes are denatured by ozone (Loreto and Velikova, 2001). The level of MDA was slightly but significantly higher in the NC-R leaves before the ozone treatment, but became higher in the NC-S leaves during and after the ozone treatment (Tab. 4).The level of MDA in NC-S leaves was particularly high 3-d after ending the ozone fumigation, when also H_2O_2 highest levels were recorded. This suggests that H_2O_2 accumulation leads to

membrane peroxidation in NC-S leaves. However, as also previously observed by Loreto and Velikova (2001), MDA accumulation may not be strictly connected to the impairment of photosynthetic rates as these were strongly, and equally, reduced in both clones at the end of the treatment.

The emission of oxygenated volatile organic compounds (OVOC) can be also induced by damage to cell walls and cell membranes (Loreto et al. 2006). The emission of OVOC was measured before and after the ozone treatment (Fig. 17). The emission of methanol was significantly higher in the NC-S than in the NC-R leaves. Methanol is formed by the de-methylation of pectins in cell walls (Galbally and Kirstine 2002). However, methanol can be also released by cell wall breaking in rapidly expanding leaves (Nemecek-Marshall et al. 1995). Therefore, the high emission of methanol in NC-S leaves before the ozone treatment, and its reduction after the ozone treatment, is interpreted as an indication that these leaves were growing rapidly (an indication also confirmed by the high photosynthetic rates measured at the beginning of the experiment) and that such a growth was significantly curbed by the ozone treatment.

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). No measurable release of C-6 compounds was detected in leaves of the NC-R and NC-S clones before the ozone treatment, but a clear emission of (E)-2-hexenol was detected only in NC-S leaves after ozone fumigation (Fig. 17).

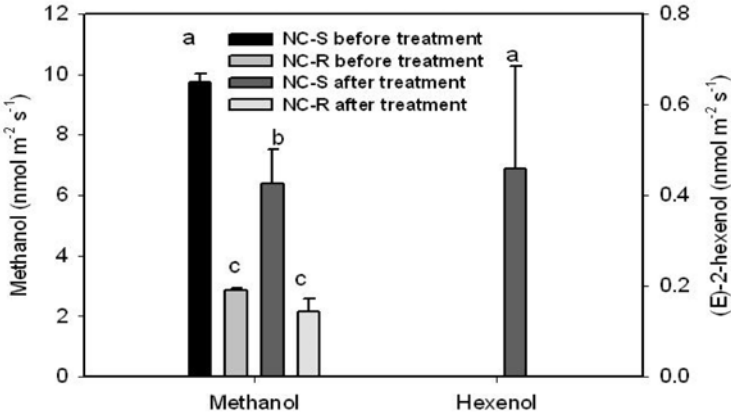

Figure 17: Oxygenated VOC emission (methanol and (E)-2-hexenol) before and at the end of the ozone fumigation (day 10) on NC-S and NC-R clones of *Trifolium repens*. Means ± standard error (n = 5) are shown. A multiple range test (Tukey) was used to compare the means between clones in an analysis of variance scheme, and letters show significant differences for $P < 0.001$.

The emission of (E)-2-hexenol by leaves that also showed the highest concentrations of MDA and H_2O_2 again supports the conclusion that the reactive oxygen species accumulated during the ozone treatment attack and destroy membranes prevalently in the ozone sensitive NC-S leaves.

Ascorbate is considered a major player in apoplastic ozone scavenging processes (Burkey and Eason, 2002), and might have been responsible for the larger ozone uptake of the NC-R clone. Ascorbate may react with H_2O_2 generating monodehydroascorbate that can either directly regenerate ascorbate by using photosynthetic electron transport. In a recent experiment, ascorbate levels of *Catharanthus roseus* explained part of the variation in ozone flux that stomatal conductance did not account for (Eller and Sparks, 2006). Ascorbate was detected at a level 50% to 70% higher in the ozone resistant clone of *T. repens* cv. "Regal" with respect to the sensitive one (Ferreira Severino et al. 2007), although the same results were not found by D'Haese et al. (2005). In particular, the latter report indicated that the constitutive level and the redox status of apoplastic ascorbate were higher in the NC-S than in the NC-R clone. Also in my experiment the ascorbate level of NC-S leaves was significantly higher than in NC-R leaves before and during the ozone fumigation (Tab. 4). Thus, I have no evidence that ascorbate was involved in the mechanisms which make the NC-R clone resistant to ozone.

A last antioxidant defense may involve the production of phenolic compounds. It was found that phenolics act as secondary scavengers of H_2O_2 in reactions tightly associated with guaicol peroxidase (Takahama 1989). Before the treatment both clones presented the same amount of total phenolic compounds (Tab. 4). After starting the ozone fumigation the phenolic compounds in the leaves of the NC-S clone increased much faster than in the NC-R clone, but at the end of the fumigation period the quantities of phenolics accumulated in the two clones were similar. After a 3-d recovery from ozone fumigation, the phenolics of the leaves of the NC-R clone were again as low as before the ozone treatment, while these compounds remained as abundant as during the ozone exposure in NC-S leaves. Interestingly, during the first 7 d of ozone stress, the higher increase of phenolics synthesized in the leaves of the NC-S clone was associated to a lower accumulation of H_2O_2, as compared to NC-R leaves. This may indeed indicate that leaf phenolics are able to quench H_2O_2 levels, at least during the first stage of the ozone stress, before such a defense line becomes overwhelmed by the increasing oxidative potential. A surge of total phenolics was

previously observed in NC-S leaves exposed to ozone, and was in relationship with the increase of total antioxidant activity in these leaves (Ferreira-Severino et al. 2006). However, these authors do not report whether such an increase also characterized ozone-treated leaves of the resistant (NC-R) clone.

2.3 Partitioning of ozone sinks in enclosures containing isoprene and monoterpene emitting plants

2.3.1 Aims of the study

Volatile isoprenoids protect plants against ozone. To understand whether this could be the result of a direct scavenging of ozone by these molecules, the stomatal and non-stomatal uptake of ozone was estimated in plants emitting isoprene or monoterpenes. Ozone uptake by holm oak (*Quercus ilex*, a monoterpene emitter) and black poplar (*Populus nigra*, an isoprene emitter) was studied in this experiment in whole plant cuvettes (continuously stirred tank reactors, CSTR), while experiment 2.2. reported on the same subject studied at single leaf level. I specifically studied: a) the relationship between ozone uptake and stomatal conductance under different environmental conditions; b) whether gas phase reactions of ozone with monoterpenes (more reactive than isoprene) emitted by plants contribute significantly to ozone destruction outside leaves c) wether the total ozone flux is decreased when correcting for intercellular ozone concentration.

2.3.2 Methods

2.3.2.1 The experimental system

Three-year-old saplings of *Populus nigra* and *Quercus ilex*, coming from Italian seed-forests and collected by the National Forestry Division (Circeo National Park, central Italy) were used for these experiments. Plants were grown in 5 L pots filled with organic soil and fertilized with Osmocote® (Scotts Italia S.R.L.).

The experiments were performed during 2006 at the Institute Phytosphere (ICG-III), Research Centre Jülich, Germany. The Institute is equipped with plant cuvettes (Fig. 4E), named Continuously Stirred Tank Reactors (CSTRs) made of glass and Teflon to minimize ozone reactions with chamber surfaces (Neubert et al. 1993; Wildt et al. 1997). Three CSTRs with different volumes (1450, 1100 and 164 L) to accommodate different number of plants, were used for the experiments. Each CSTR was located in a phytotron (ground area 7 m^2, height 3.5 m) that allowed to adjust the air temperature between 10 and 45 °C keeping it

constant within ± 0.5 °C. Custom made temperature and light sensors were introduced into the CSTRs to monitor continuously these two parameters. In order to minimise ozone losses between CSTRs and analyzers also all tubes were either made of Teflon (PTFE or PFA) or glass and the distance between detectors and CSTRs was kept < 2 m. No ozone losses due to tubing were detected under these conditions. Only the canopy of plants (shoots and leaves) was enclosed in the CSTRs while the belowground part of the plants, containing roots and soil in the pots, was separated by PTFE sheets. Holes made to introduce stems in the chambers were sealed by plastic materials (Optosil®, Bayer Dental).

Discharge lamps (Osram HQI 400 W/D) were used for illumination. The photosynthetic photon flux density (PPFD) at mid-canopy height amounted to a maximum of 360 μmol m^{-2} s^{-1} in the 1450 L chamber, 480 μmol m^{-2} s^{-1} in the 1100 L chamber and to a maximum of 800 μmol m^{-2} s^{-1} in 164 L chamber. Light intensity was decreased from the maximum PPFD level (all lamps on) by manually switching off one or more lamps, obtaining the PPFD levels shown in Figure 21. Filters (OptoChem, type IR3) that reflect wavelengths between 750 and 1050 nm were used as heat shields to avoid overheating of the plants by infrared radiation. The short cut off wavelength of the light in the CSTRs was at about 350 nm due to absorption of UV light by the glass walls of the reactors. Plants were illuminated for 12 h every day.

Air was pumped in the CSTRs at a flow rate of 16 ppb. Before reaching the CSTRs, air was purified with an adsorptive drying device (Zander, KEA 70) and a palladium catalyst. The CO_2 was scrubbed passing the air through columns of soda lime, and a 100% CO_2 cylinder was used to supply CO_2 that was mixed with the CO_2-free air to generate a stable 380 ppm CO_2 concentration in the air. The CO_2 source was excluded during measurements in absence of CO_2 (experiment b1). The air flow through the chambers was kept constant by mass flow controllers (Brooks 5850). A Teflon fan inside the chambers was used for homogeneous mixing of the chamber air and to destroy the boundary layer resistance at the surfaces of the plants. Ozone was generated by UV lamps, out of the chambers using a part of the chamber air flow in a bypass mode. The ozone generators allowed to vary ozone concentrations between 0 and 300 nL L^{-1} .

Ozone concentrations were measured by UV absorption (Thermo Environmental Instruments, model 49). H_2O and CO_2 concentrations were measured by IR absorption (Rosemount Binos 100 4P). Absolute H_2O concentrations were also determined with the aid

of dew point mirrors (Walz MTS-MK1). The ozone uptake by plants was estimated measuring ozone concentration at the inlet and outlet of the reactors.

The determination and quantification of isoprene emitted by *Populus nigra* and of monoterpenes emitted by *Quercus ilex* was performed with online gas chromatography mass-spectrometry (GC-MS) analyzing the air flowing out of plant chambers. A detailed description of the GC-MS system used for VOC analysis has been given by Heiden et al. (1999). At the same time, attention was given to the possible presence of products formed by lipoxygenation and decay of cell membranes (LOX products: the aldehydes (Z)-3-hexenal, (E)-3-hexenal , (E)-2-hexenal and the alcohol (E)-2-hexenol) and by the product of isoprene-ozone reaction: methacrolein (MACR) and methyl-vinyl-ketone (MVK).

Calibration of the GC-MS system was performed with a permeation source containing pure chemicals in individual vials in combination with a dynamic dilution system (Heiden et al. 1999).

In the first experiment (a) four plants of *Populus nigra* were enclosed in the 1100 L CSTR and fumigated with synthetic air containing ozone at the concentration of 100 ppb for 5 days, continuously (day and night). While this continuously high ozone concentration may not be often observed currently in nature, it facilitated the control of ozone concentration in the chamber. Ozone uptake, emission of isoprene and isoprene reaction products were measured during fumigation. This experiment was repeated three times, using 3 other plants in the same chamber in each replication (data not shown). Isoprene emission and isoprene reaction products formed during ozone fumigation, methacrolein (MACR) and methyl-vinyl-ketone (MVK), were compared with background levels under ozone-free conditions, and means were statistically separated by ANOVA ($p < 0.01$, $n=3$). Percent changes of ozone uptake in dependence of PPFD levels were also tested for statistical significance (ANOVA followed by Tukey's test, $p < 0.01$, $n = 3$).

In the second experiment (b) a single plant of *Quercus ilex* was enclosed in the 164 L chamber and fumigated with synthetic air containing ozone at a concentration of 100 ppb continuously (day and night) for 4 days. In this experiment, which was replicated three times with different plant specimens, ozone flux and monoterpene emissions were measured at ambient CO_2 (380 ppm) and at low CO_2 (1 to 16 ppm in the three replications). CO_2 was removed for 2.5 h in order to measure ozone uptake while reducing monoterpene emissions

and opening stomata (Loreto et al. 1996). Percent change of ozone uptake in dependence of CO_2 presence was tested for statistical significance by ANOVA ($p < 0.01$, $n = 3$).

In a second phase of experiment b, attention was focused on the possible occurrence of gas phase reactions outside the monoterpene-emitting plants, in addition to ozone uptake by the same plants. The ozone losses exclusively due to gas phase reactions outside leaves were separated from the ozone uptake by the plant by using the set up shown in Figure 18.

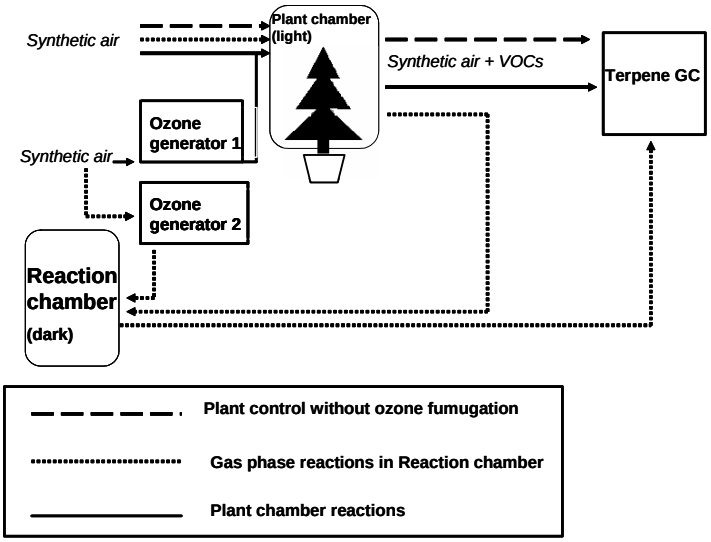

Figure 18: Set up of the experiment using two coupled chambers with *Quercus ilex*. A flow of synthetic air (80 % N_2, 20 % O_2, 380 ppm CO_2) flushed the CSTR where plants were enclosed. The air flow at the outlet of the CSTR, with monoterpenes emitted by plants, was conveyed to the reaction chamber. Ozone was produced at the outlet of the plant chamber (ozone generator 2) when studying the reactions in the reaction chamber, or at the inlet of plant chamber (ozone generator 1) when studying the reactions in the plant chamber.

In this experiment four plants of *Quercus ilex* were introduced into the 1100 L chamber. These plants were allowed to photosynthesize and to emit monoterpenes under ambient air without exposing them to ozone. The air flow leaving the plant chamber was injected into the 1450 L chamber (from here on denominated reaction chamber). A second air flow containing ozone was also injected (at a flux of 6 L min^{-1}) into the reaction chamber to study the reaction between ozone and monoterpenes in absence of biotic ozone uptake and the subsequent ozone losses in the gas-phase. This experiment was repeated three times generating three different ozone concentrations (50, 89, and 180 ppb) in the reaction chamber. The reaction chamber was left in darkness in order to avoid complications by photolytic processes leading to further ozone destruction.

The first experiments (a) with *Populus nigra* were carried on assuming an intercellular concentration $[O_3]_{ci}$ of ozone equal to 0, as agreed by the scientific community. In the last part of this case study, however, I tried to calculate the intercellular concentration of ozone ($[O_3]_{ci}$) in the substomatal cavity of *Populus nigra*.

2.3.2.2 The theory for calculation of ozone flux

The formalism adopted in this study to calculate ozone flux is similar to that described in Neubert et al. (1993). For the determination of ozone consumption in the plant chamber the following reactions were considered:

O_{3air}	$\xrightarrow{g_{O3}}$	O_{3plant}	uptake of O_3 by the plant	(R8)
O_{3air}	$\xrightarrow{g^w_{O_3} \cdot A^w}$	O_{3wall}	O_3 wall losses	(R9)
O_3 + VOC	$\xrightarrow{k}$	Products	Gas phase reactions of O_3 with VOC	(R10)

Reaction 8 considers the uptake of ozone by plants. In reaction 8, g_{O_3} (cm s^{-1}) represents the conductance to ozone from the air to the plants, as explained below. In reaction 9, wall losses of ozone are described by $g^w_{O_3} \cdot A^w$, where $g^w_{O_3}$ is the conductance of ozone to the chamber walls (cm s^{-1}) and A^w is the wall area (cm^2), respectively. Reaction 10 considers the consumption of ozone due to reactions of ozone with the emitted BVOC in the gas phase outside the plants. In reaction 10, k (cm^3 s^{-1}) represents the rate constant for the reaction of an individual VOC with ozone.

According to the principle of gas diffusion shown in equation 3, for steady state conditions ozone conductance (g_{O_3}) is:

$$g_{O_3} = \frac{F}{A^L} \cdot \frac{[O_3] - [O_3]_{in}}{[O_3]_{ci} - [O_3]} - \frac{V}{A^L} \cdot \sum_i [VOC_i] \cdot k^i - \frac{g^w_{O_3} \cdot A^w}{A^L} \qquad (12)$$

where V is the volume of the chamber (cm^3), F is the air flow ($cm^3 \cdot s^{-1}$), and $[O_3]_{in}$ and $[O_3]$ is the ozone concentration measured at chamber inlet and chamber outlet (molecules·cm^{-3}),

respectively, and $[O_3]_{ci}$ is the intercellular concentration of ozone. A^L is the one-sided leaf area of the plants under investigation (cm^2). The sum: $\sum_i [VOC_i] \cdot k^i$ is used in equation 11 because the total loss of ozone in gas phase reactions should be due to the sum of the losses of its reactions with all individual BVOC.

In case of negligible ozone losses due to gas phase reactions (e.g. for low rate constants as for the isoprene-ozone reaction) the term $\frac{V}{A^L} \cdot \sum_i [VOC_i] \cdot k^i$ was also negligible, and g_{O_3} was determined using equation 13:

$$g_{O_3} = \frac{F}{A^L} \cdot \frac{[O_3] - [O_3]_{in}}{[O_3]_{ci} - [O_3]} - \frac{g_{O_3}^w \cdot A^w}{A^L} \tag{13}$$

The same formula can be used to calculate ozone flux (Φ_{O_3}, (mol m^{-2} s^{-1})), under the assumption that $[O_3]_{ci}$ is equal to 0. It results that:

$$\Phi_{O_3} = \frac{F}{A^L} \cdot ([O_3] - [O_3]_{in}) - \frac{V}{A^L} \cdot \sum_i [VOC_i] \cdot k^i - \frac{g_{O_3}^w \cdot A^w}{A^L} \tag{14}$$

Stomatal conductances to ozone, $g_{O_3}^S$, were calculated from stomatal conductances to water vapour, $g_{H_2O}^S$, by multiplying $g_{H_2O}^S$ with the ratio of diffusion coefficients of ozone and water vapour, respectively in air:

$$g_{O_3}^S = g_{H_2O}^S \cdot \frac{D_{O_3}}{D_{H_2O}} \tag{15}$$

The diffusion coefficient for water vapour and ozone are, respectively: D_{H_2O} = 0.25 cm^2 s^{-1} (Marrero and Mason, 1972), and: D_{O_3} = 0.167 cm^2 s^{-1} (Laisk et al. 1989).

In a first approach (equation 13, experiment a) the gas phase losses of ozone was neglected. In cases where reactions of BVOC emitted by the plants are a significant sink for ozone (experiment b) the data determined for g_{O_3} are expected to be higher than those for $g^S_{O_3}$ with a slope higher than 1. In addition, if the amount of BVOC emitted by plants is related to stomatal conductance, a plot of g_{O_3} versus $g^S_{O_3}$ will show a linear behaviour. In this case, the gas phase losses may be estimated from the part of the slope exceeding the 1:1 relationship.

The ozone losses in the reaction chamber (experiment b) are described by the differential equation 16:

$$V \cdot \frac{d[O_3]}{dt} = -F \cdot \left([O_3] - [O_3]_{in}\right) - V \cdot [O_3] \cdot \sum_i [VOC_i] \cdot k^i - g^w_{O_3} \cdot A^w \cdot [O_3] \tag{16}$$

Again, at steady state conditions, from equation 16 it follows that:

$$\sum_i [VOC_i] \cdot k^i = -\frac{F \cdot \left([O_3] - [O_3]_{in}\right) - g^w_{O_3} \cdot A^w \cdot [O_3]}{V \cdot [O_3]} \tag{17}$$

Equation 17 allows to estimate the overall reactivity of VOC emitted by plants with ozone. The intercellular ozone concentration ($[O_3]_{in}$) was calculated solving equation 12 for $g^S_{O_3}$:

$$[O_3]_{ci} = \frac{F}{A^L} \frac{[O_3] - [O_3]_{in}}{\left[\frac{V}{A^L} \cdot \sum_i [VOC_i] \cdot k^i + \frac{g^w_{O_3} \cdot A^w}{A^L} + g^S_{H_2O} \cdot \frac{D_{O_3}}{D_{H_2O}}\right]} + [O_3] \tag{18}$$

In the third part of this case study, ozone flux (equation 19) was re-calculated considering $[O_3]_{ci}$ >0, as calculated from equation 18.

$$\Phi_{O_3} = g^S_{O_3}\left([O_3] - [O_3]_{ci}\right) \tag{19}$$

and the ozone flux was compared with results obtained under the assumption that $[O_3]_{ci} = 0$.

2.3.3 Results

2.3.3.1 Wall losses

Ozone losses due to chamber effects (i.e. deposition to the chamber surfaces) were measured without plants in the CSTRs, to investigate if there were significant losses not attributable to the plants. I found losses which were positively dependent on temperature and on water vapour concentration in the chamber. Depending on the size of the plants, these losses contributed between 2 and 15% to the total losses (data not shown). The losses were subtracted from the total ozone removal measured with plants in all experiments.

2.3.3.2 Populus nigra (exp. a)

Stomatal conductance and ozone uptake of *Populus nigra* plants were broadly dependent on light, being reduced to zero when plants were darkened while increased to above 0.1 mol m^{-2} s^{-1} in illuminated leaves (Fig. 19 B).

When 100 ppb of ozone was added to the air flux in the chamber, an increase of isoprene emission by plants was observed (Fig. 19 A). As expected, isoprene reaction with ozone also yielded measurable amounts of oxidation products, namely methacrolein (MACR) and methyl-vinyl-ketone (MVK) (Atkinson et al. 1997) (Fig. 19 A). However, only a minimal fraction of the emitted isoprene was oxidized (less than 2%) confirming that, at the given concentrations of the two compounds, the residence time in the chamber was too short for isoprene and ozone to effectively react. The ozone losses by gas phase reactions with isoprene were estimated by calculating the term $\sum_i [VOC_i] \cdot k^i$ for isoprene. Isoprene was the only VOC emitted from the poplar in high amounts. Therefore, $\sum_i [VOC_i] \cdot k^i$ can be replaced by: [isoprene]·k where [isoprene] is the concentration of isoprene in the chamber air (maximum about $1.7 \cdot 10^{12}$ molecules·cm^{-3} ≈70 ppb) and k is the rate constant for this

reaction (1.3 10^{-17} cm^3 s^{-1} (Atkinson et al. 1995, 1997)). This resulted in an ozone loss rate of about $2.2 \cdot 10^{-5}$ s^{-1} which yielded a maximum contribution of gas phase reactions to g_{O_3} (= $\frac{V}{A^L} \cdot \sum_i [VOC_i] \cdot k^i$ i.e. the difference between g_{O_3} calculated according to equations 12 and 13, respectively) of $4.9 \cdot 10^{-3}$ cm s^{-1}. This contribution is about 5 % of the measured ozone conductance and thus negligible to a good approximation.

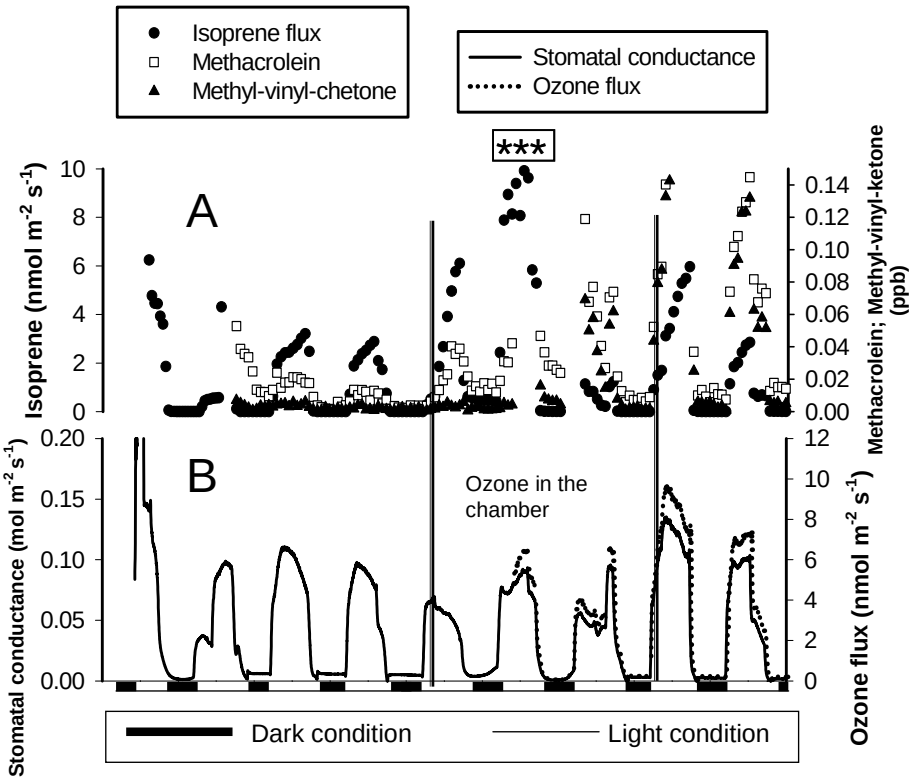

Figure 19: Emission of isoprene and of isoprene reaction products, methyl-vinyl-ketone and methacrolein, (A) during different light conditions before and during ozone fumigation of poplar plants in a typical experiment in CSTRs. Ozone fumigation started at the place identified by the vertical line. The stomatal conductance and the ozone flux are shown in panel (B). The light and dark periods were statistically analysed by ANOVA test. Significant differences of isoprene and isoprene reaction product emissions due to ozone treatment are identified by *** ($P < 0.01$, n=3) in panel (A).

The low reaction rate constant of ozone with isoprene in the gas phase (k) also suggested that ozone losses in the gas phase outside leaves are low in isoprene-emitting species. The ozone

conductance calculated according to equation 13, i.e. neglecting gas phase reactions, and the stomatal conductance to ozone, calculated according to equation 15 (g_{O_3} and $g^{S}_{O_3}$, respectively) showed a clear relationship which extrapolates to 0 and has a slope near to 1 (Fig. 20; n = 3, $r^2 = 0.97$).

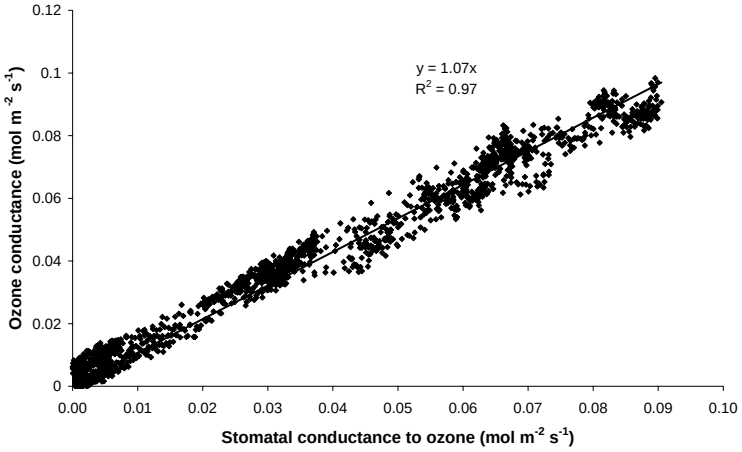

Figure 20: Relationship between ozone conductance and stomatal conductance to ozone in plant cuvettes experiments with poplar plants (n = 3).

No visible symptoms of ozone injuries and negligible emissions of LOX products were observed during and after the ozone fumigations of poplar (data not shown). This implies that ozone injury of cellular structures was not heavy at the ozone concentrations used to fumigate plants.

2.3.3.3 Quercus ilex (exp. b)

Since rate constants for ozone-monoterpene reactions are much higher than those of ozone-isoprene reactions, ozone losses due to gas phase reactions must be considered when determining the ozone uptake by *Quercus ilex*. However, it is not easy to calculate gas phase losses. Therefore these losses were determined experimentally. In a first phase of the experiment b, I repeated the same experiment as conducted with poplars. The maximal monoterpene concentration emitted by an individual *Quercus ilex* plant in the chamber was observed at PPFD of 800 µmol m^{-2} s^{-1} and a leaf temperature of 28°C. The relative

monoterpene abundance in the CSTR air, and the rate constants for monoterpene reactions with ozone are reported in Table 5.

Compound	Rel. Ab. (%)	$k^{O3}\ 10^{-18}$ ($cm^3\ s^{-1}$)
α-Pinene	27	86,6
Camphene	3.5	0,9
Sabinene	2.4	86
Myrcene	7.9	470
β-Pinene	11	15
α-Terpinene	2.4	21100
cis-Ocimene	10.3	37
Limonene	18.4	200
trans-β-Ocimene	4.4	Unknown
γ-Terpinene	3.5	140
2-Carene	1.9	230
Neo-Allo-Ocimene	1.7	Unknown
Total	95	

Table 5: Most abundant monoterpenes emitted from *Quercus ilex.* Relative abundance and rate constants for their reactions with ozone (after Atkinson et al. 1997) are shown.

The emissions of all monoterpenes were dependent on light intensity (Loreto et al. 2004) and were very low in the dark (data not shown). As for poplar, ozone uptake and stomatal conductance were strongly dependent on PPFD also in *Quercus ilex* (Fig. 21), and no visible symptoms of ozone injuries and negligible emissions of LOX products were observed (data not shown).

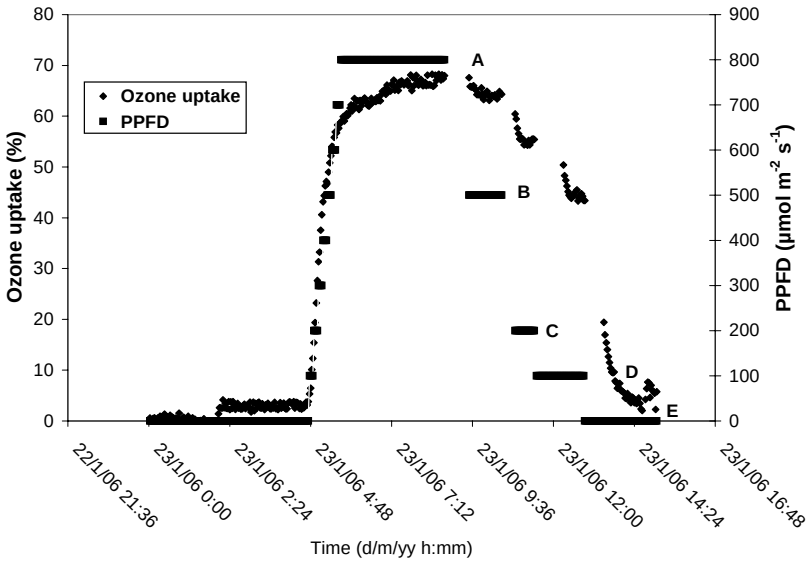

Figure 21: Percent ozone uptake in response to photosynthetic photon flux density (PPFD) during different days in *Quercus ilex* plants. Significant differences of ozone uptake at the different PPFDs were statistically analyzed by ANOVA with Tukey's test and identified by letters ($P < 0.01$, $n = 3$).

In *Quercus ilex,* the relationship between the ozone conductance (g_{O_3}) as calculated according to equation 13 (that is, without accounting for VOC gas-phase reactions), versus the stomatal conductance to ozone ($g^S_{O_3}$) yielded a straight line and a negligible intercept at $g^S_{O_3} = 0$. However, the value of the slope was about 1.9 (Fig. 22). The negligible intercept clearly shows that losses of ozone at stem and cuticle (the non-stomatal uptake of the pollutant) are also irrelevant for *Quercus ilex*, as already observed for the poplar. The slope of the relationship between conductances higher than 1 indicates that more ozone is lost in the chamber than it is explainable by the stomatal conductance to ozone.

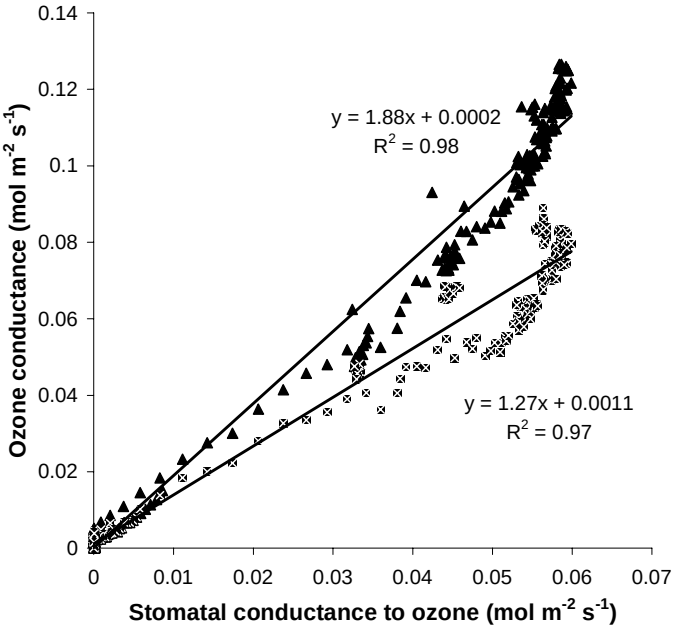

Figure 22: Relationship between ozone conductance and stomatal conductance in plant cuvettes experiment with *Quercus ilex* plants. Black triangles show the ozone conductance data without considering ozone losses in the gas phase. Squares show the ozone conductance data obtained after considering gas-phase ozone losses. These gas-phase losses were estimated from the low CO_2 experiment shown in Fig. 23.

The reduction of CO_2 concentration led to a significant increase of stomatal conductance (Fig. 23), while decreasing the monoterpene emissions by 80% (not shown), and the ozone losses in the chamber by 20% (Fig. 23). When the ozone conductance calculated from equation 12 was corrected for the amount of ozone removed by monoterpene-dependent gas phase reactions as estimated from the ozone uptake reduction under low CO_2 (Fig. 23), the slope of the relationship between ozone conductance and stomatal conductance to ozone decreased to about 1.3 (Fig. 22).

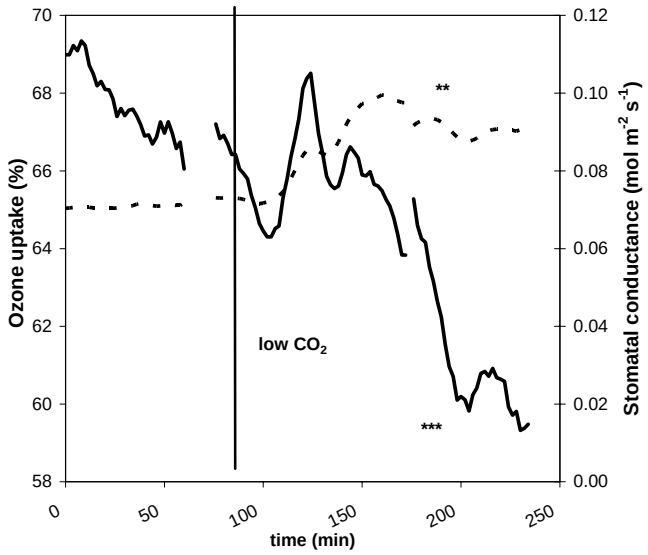

Figure 23: Percent ozone uptake (solid line, left scale) and stomatal conductance to ozone, (dotted line, right scale) in *Quercus ilex* plants exposed to ambient or low CO_2. Significant differences of ozone uptake and stomatal conductance to ozone at the different CO_2 levels are identified by *** ($P < 0.01$, n=3) and ** ($P < 0.05$, n=3), respectively.

In a second phase of the experiment b, ozone and plant volatiles reacted in a reaction chamber different than that in which plants were enclosed and allowed to emit monoterpenes (Fig. 18). This experiment was conducted with three different ozone concentrations in the reaction chamber and $\sum_i [VOC_i] \cdot k^i$ was determined according to equation 17 at each ozone concentration (Tab. 6). The experiment was first done by keeping the plant material in the dark (a condition in which monoterpene emission was inhibited, Loreto et al. 1996) and yielded negligible ozone losses (data not shown). However, switching on the light led to an increasing monoterpene emission by plants and in the plant chamber. An ozone loss variable between 46 and 25 % was observed as consequence of the reactions between monoterpenes emitted by plants (50±11 ppb) and 50, 89, or 180 ppb of ozone into the reaction chamber (Tab. 6). Moreover, as shown by Figure 24, ozone losses in the reaction chamber reached a steady state several hours after the onset of illumination in the plant chamber. Only the data points near to the steady state (at the end of the light and dark phases in the plant chamber) were used to estimate the ozone losses due to gas phase reactions.

O_3 in dark period (ppb)	O_3 in light period (ppb)	O_3 losses (%)	O_3 losses ($\Delta[O_3]/[O_3]$)	$\sum_i [VOC_i] \cdot k^i$ [s^{-1}]
50	27	46	0.85	$2.2 \cdot 10^{-4}$
89	56	37	0.56	$1.5 \cdot 10^{-4}$
180	135	25	0.33	$8.7 \cdot 10^{-51}$

Table 6: Ozone concentrations at the inlet of the reaction chamber (i.e. after considering plant uptake during dark and light periods), absolute (%) and relative ($\Delta[O_3]/[O_3]$) ozone losses due to gas-phase reactions in the reaction chamber between isoprenoids and ozone, and reaction rates ($\sum_i [VOC_i] \cdot k^i$ s^{-1}). The ozone uptake by plants in the dark (column 1) was not different from the ozone concentration fumigated to plants.

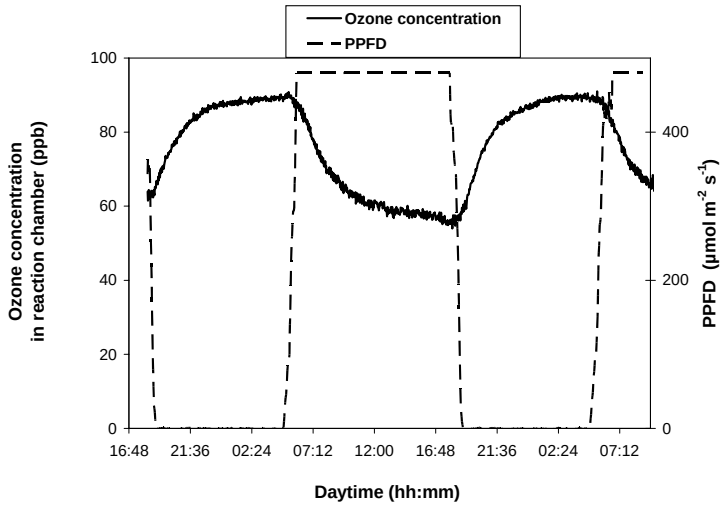

Figure 24: Time course of ozone concentrations exiting the reaction chamber when ozone concentration at the inlet of the reaction chamber was 89 ppb and monoterpene concentration generated in the plant chamber by four Q*uercus ilex* illuminated plants was 50±11 ppb. The dashed line indicates PPFD levels in the reaction chamber. The difference of ozone concentration when the reaction chamber was illuminated was attributed to gas-phase reactions with monoterpenes emitted in the plant chamber.

2.3.3.4 The intercellular ozone concentration in Populus nigra

In the third part of this study, the data about ozone fluxes of *Populus nigra* were re-processed also considering an intercellular ozone concentration higher than 0, as calculated according to equation 18. I considered only poplar in this experiment, since the gas phase reactivity was less relevant in this isoprene-emitting plant than in monoterpene-emitters, as shown in table 3. The intercellular concentration of ozone was plotted versus ozone flux (Fig. 25). In one case the ozone flux was calculated according to equation 19 assuming $[O_3]_{ci}$ = 0, and in another case it was calculated according equation 19 with the additional $[O_3]_{ci}$ calculated according to equation 18. Figure 25 shows that $[O_3]_{ci}$ effect on ozone flux is particularly relevant after 30 ppb of $[O_3]_{ci}$, corresponding to stomatal conductance exceeding 0.1 mol m^{-2} s^{-1}. When $[O_3]_{ci}$ was considered, the calculated ozone flux decreased up to 50 % respect to the total flux calculated without considering $[O_3]_{ci}$.

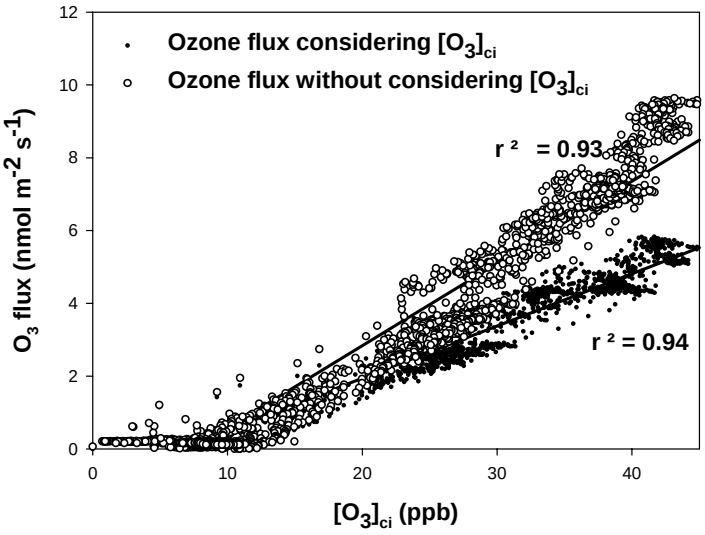

Figure 25: relationship between $[O_3]_{ci}$ and ozone flux for *Populus nigra*. The white circles do not include the value of $[O_3]_{ci}$. The linear best fits for and the Pearson regression coefficients of are reported in the graphs.

2.3.4 Discussion

2.3.4.1. Isoprene-ozone interaction

Volatile isoprenoids (isoprene and monoterpenes) are reactive molecules and may play a role in scavenging ozone (Loreto et al. 2001a), in conjunction with, or in alternative to other detoxification mechanisms such as the enzymatic Halliwell-Asada cycle of detoxification of reactive oxygen species (Asada, 1992). My goal was to assess whether ozone scavenging by isoprene and monoterpenes was measurable at canopy level and was caused by reaction outside leaves or in the mesophyll.

Populus nigra is a strong isoprene emitter, while it does not emit significant amounts of monoterpenes. The well-known dependency of isoprene emission from light and temperature (Guenther et al. 1995) was observed. Isoprene emission showed a clear diurnal trend with peak emission in the light periods (Fig. 19). It was also observed that isoprene emission was stimulated after adding ozone in the plant chamber (Fig. 19 A, dotted line). This confirms results from other studies suggesting that oxidative stresses may activate isoprene biosynthesis as well as formation of other antioxidants (Velikova et al. 2005). Interestingly, also products of isoprene oxidation were stimulated by the ozone treatment (Fig. 19 A) thus indirectly confirming an overall activation of isoprene biosynthesis. The reason of such an activation of the metabolic pathway of isoprene formation is still unknown but it has been hypothesized that isoprene (and other volatile isoprenoids) can play a role in protecting membranes against oxidative stresses (Loreto and Velikova, 2001).

For poplar, it was also found that non-stomatal losses were negligible compared to stomatal uptake confirming once more (see case study 2), but at whole plant level, that volatile isoprene is of minor importance as ozone sink, and that stems and cuticles do not adsorb relevant amounts of ozone. This is also in agreement with reports showing that non-stomatal ozone uptake is negligible unless occurring under conditions of very high humidity (Altimir et al. 2006).

Large non-stomatal fluxes are reported from measurements at ecosystem level but these include components such as reactions in the atmosphere (Kurpius and Goldstein, 2003) or soil adsorption (Fowler et al. 2001). While adsorption at the soil was excluded in our experiment, the results obtained with *Quercus ilex* show that gas phase reactions may

contribute substantially to ozone losses in monoterpene-emitting plants. Both experimental approaches used here for checking the role of gas phase ozone losses (using an empty reaction chamber or manipulating stomatal conductance and monoterpene emission by removing CO_2) led to consistent results. These results showed that a significant fraction of the measured ozone losses was not directly associated to stomatal uptake and suggested that gas phase losses accounted for the major part of non-stomatal ozone destruction.

It is not surprising that ozone losses induced by gas phase reactions could be observed in *Quercus ilex* since this species is a well known strong monoterpene emitter (Staudt and Bertin, 1998; Loreto et al. 2004) and the rate constants for ozone-monoterpene reactions are much higher than those of ozone-isoprene reactions (Atkinsons et al. 1997, Tab. 3). Whether or not these gas phase reactions play an important role in the ambient cannot be concluded from our simple laboratory experiment alone. Beside strong monoterpene emitters, also other plant species contribute biogenic volatiles to the composition of the air, micrometeorological processes determine transport and dilution of BVOC, and, in addition to the ozone reactions as determined in this study, BVOC reaction with OH radicals should be considered (Atkinson et al. 1995; Orlando et al. 2000). However, our experiment shows that, when assessing ozone losses in ecosystems, such gas phase reactions cannot be neglected *a priori*. Ozone destruction can occur by gas phase reactions with the monoterpenes emitted by the plants and probably with other reactive BVOCs such as sesquiterpenes and oxygenated compounds (Schade and Goldstein, 2006; Bonn and Moortgat, 2003). The efficient removal of ozone by monoterpenes outside the leaves also does not exclude a possible role of isoprenoids in removing ozone inside the leaves, either in reactions in the gas phase of the mesophyll or in the liquid phase of the apoplast (Loreto and Velikova, 2001; Loreto et al. 2004).

2.3.4.2 A hypothesis about the intercellular concentration of ozone

Stomatal conductance was the main process driving ozone uptake. The relationship shown in Fig. 20 implies that the uptake of ozone by *Populus nigra* is limited by stomatal aperture and suggests that ozone removal by reactions inside leaves occurs much faster than the delivery of ozone through the stomata. If this is true, plant internal ozone concentrations should be

near to zero, as also shown before (Laisk et al. 1989). However, as stated in case study 2, the relationship between stomatal conductance and ozone uptake is described through an hyperbolic trend (Fig. 12). Looking at this trend, a linear relationship is hypothesized at low stomatal conductances, thus suggesting that a) ozone entering the stomata is rapidly consumed by reactions with cell walls or plasmalemma (Laisk et al. 1989), and b) ozone is efficiently and totally removed by antioxidants (e.g. isoprene) in the intercellular spaces. With increasing stomatal conductance, however, the relationship becomes non linear. This observation suggests that the gradient of ozone from outside to inside the leaf is inversely proportional to stomatal conductance and that ozone can accumulate in the apoplast when freely entering leaves because of low stomatal resistance. The significant values of $[O_3]_{ci}$ increasing with increasing stomatal conductance, as observed in this case-study, seems to confirm this hypothesis. It may be possible that at increasing rates of entry for ozone through the stomata, isoprene and other antioxidants are less and less efficient in scavenging ozone, and ozone consequently accumulates.

One criticism to the present approach to calculate $[O_3]_{ci}$ is that if the calculation of isoprenoids reaction with ozone in equation 12 is not accurate, the estimation of $[O_3]_{ci}$ would be faulty. This problem could be solved using plants which do not emit isoprene, e.g. *Trifolium repens* or *Nicotiana tabacum*. In these cases, the errors could be reduced by applying equation 13 to calculate ozone conductance, instead than equation 12. This will be the object of future research.

2.4 Characterization of a dune vegetation Ecosystem: BVOC emission and possible involvement in ozone-forming reactions

2.4.1 Aims of the study

A field campaign was performed in May-June 2007 in a coastal Mediterranean vegetation in the presidential estate of Castelporziano, Rome. This area is of particular interest because since 1951 it is object of investigation concerning the climatic and biological parameters. Moreover, this well preserved coastal ecosystem is close to the roman urban area subject to a high anthropic impact and to a typical air circulation in which polluted air masses are transported until the coast. The dune ecosystems are characterized by pioneer plant species, able to colonize extreme environment. The importance of these species also rests on their role in stabilizing sand dunes and contributing to soil formation. This vegetation is also important for cleaning the air masses from salty particles transported by the sea breeze, thus protecting the more sensible downwind vegetation. The response of the dune vegetation to biotic and abiotic stresses still have to be fully investigated. Most of the plants living in the dune ecosystems emit BVOCs (Biogenic Volatiles Organic Compounds), and laboratory studies in controlled environments have shown that climate change may dramatically impact on the emission of BVOCs (e.g. Rosenstiel et al. 2003). This may influence the capacity of plants to withstand environmental stresses (Sharkey and Singsaas, 1995; Loreto et al. 2001), especially in regions environmentally fragile and exposed to simultaneous climate warming and urban pollution, such as the Mediterranean area.

The main scientific objectives of the field campaign in Castelporziano were:

A) to characterize physiology and BVOCs emission, for a still not well studied dunal ecosystem in a moment in which environmental constrains were not strongly affecting pant performances.

B) to assess whether and to which extent BVOCs emitted by Mediterranean dune vegetation contribute to the chemical reactions in the atmosphere, and in particular to the formation of ozone.

2.4.2 Methods

2.4.2.1 Site specifications

The experimental site is located in the Presidential Estate of Castelporziano, N 41° 40' 49.3", E 12° 23' 30.6", about 6000 ha wide and is distant 25 Km from the center of Rome, Italy in the SE direction (Fig. 26). The Mediterranean ecosystems are all represented and preserved inside the Presidential Estate, which contains more than 1000 plant species. The part of the Estate facing the Tyrrhenian sea was selected for the field campaign. The specific location of the experimental site is 100 m from the coast line, between a first and a second dune. An area of 1000 m^2 was used for a physiognomic study of the woody species.

The climate of the site is typically Mediterranean with a dry summer period, monthly temperatures ranging between a minimum of 0.8 °C (December) and a maximum of 27.6 °C (August), a pronounced summer drought and rain events concentrated in autumn and spring. The mean annual precipitation over the last three years is 713 mm (480 mm in 2007).

Meteorological data related to the measurement period (May-June 2007), including hourly data about wind speed/direction, rain, temperature, humidity and solar radiation were supplied by the stations of the Presidential Estate.

2.4.2.2 Vegetation

The estate presents high richness in biodiversity due to the meeting point of the Italian Mediterranean dune vegetation with plant species coming from Spain, France, Africa. This area is characterized by sand dunes with "garigue" vegetation mixed to a humid retrodunal area with "maquis" vegetation. The most representative species are reported in table 7. According to Pignatti (2001), I named "garigues" the *Erico-Rosmarinetum* formation, characterized by the abundant presence of low-shrubs species as *Rosmarinus officinalis* and *Erica multiflora*; *Arbutus unedo*, *Phillyrea latifolia and Cistus spp.* The latter are typically located over regosols with richness in $CaCO_3$ due to the deposition of bioclasts.

The Mediterranean "maquis" is a variant of the formation *Quercetum-ilicis* (Pignatti, 2001), which is dominated by *Quercus ilex* already from the early stages of its evolution. The actual evolution of this type is at the early stages, with canopy level at 5 m, formed by a mixture of

Quercus ilex and other co-dominant species: *Erica arborea*, *Phillirea latifolia* and *Arbutus unedo*.

Isoprenoids *(ug(C) g^{-1} DW h^{-1})*	- Quercus ilex (adult leaves)	- Quercus ilex (young leaves)	- Cistus spp. (control)	- Cistus spp. (irrigated)	- Arbutus unedo	- Phyllirea latifolia	- Rosmarinus officinalis	- Erica multiflora	- Juniperus oxycedrus
Isoprene	**0.33**	**0.31**	**0.05**	**0.38**	**0.02**	**0.05**	**0.02**	**1.48**	**0.02**
Tricyclene	0.01	0.02	-	-	-	-	0.12	-	0.02
alpha-Thujene	0.05	0.07	-	-	-	-	0.12	-	0.01
alpha-Pinene	2.39	3.58	0.18	0.31	0.04	0.23	2.44	0.29	5.54
Camphene	0.09	0.14	0.01	0.02	-	0.01	2.81	0.14	0.08
6-metil-5 metil Eptane	0.39	0.36	0.36	0.31	0.14	0.32	0.56	0.54	0.37
Sabinene	1.66	2.29	0.14	0.12	0.02	0.09	0.06	0.01	0.10
beta-Pinene	1.65	2.43	0.12	0.17	0.03	0.11	4.09	0.26	0.19
Myrcene	0.39	0.65	0.03	0.06	0.01	0.06	0.54	0.05	0.58
alpha-Phellandrene	0.01	0.01	-	-	-	-	0.02	-	0.38
D-3-Carene	-	-	-	-	-	-	-	-	-
alpha-Terpinolene	0.01	0.02	-	-	-	-	0.04	-	0.01
para-Cymene	0.02	0.02	-	0.01	-	0.01	0.07	0.01	0.04
1,8-Cineol	0.10	0.34	0.01	0.02	-	0.02	4.94	0.58	0.15
beta-Phellandrene	0.09	0.08	0.01	0.04	0.01	0.01	0.03	-	1.50
Limonene	0.22	0.26	0.09	0.28	0.05	0.09	1.41	0.18	0.19
cis-b-Ocimene	1.77	0.03	0.04	0.23	0.00	0.03	0.25	0.03	0.02
trans-b-Ocimene	0.52	0.02	0.02	0.07	0.01	0.02	0.06	0.16	0.09
gamma-Terpinolene	0.02	0.04	-	-	-	-	0.10	0.01	0.01
Terpinolene	0.01	0.02	-	0.01	-	-	0.04	0.01	0.11
Linalool	-	0.01	-	-	-	-	0.07	0.01	0.00
4-Terpineol	-	-	-	-	-	-	0.18	0.03	0.00
a+g-Terpineol	-	0.02	-	-	-	-	0.47	0.09	0.13
trans-b-Caryophyllene	-	-	-	-	-	-	0.46	-	-
Total of Monoterpenes	**9.39**	**10.41**	**1.02**	**1.67**	**0.29**	**0.99**	**18.89**	**2.40**	**9.56**
Photosynthesis *(umol m^{-2} s^{-1})*	**5.28**	**3.23**	**10.41**	**14.35**	**9.22**	**5.35**	**8.58**	**0.48**	**6.73**
Stomatal conductance *(mol m^{-2} s^{-1})*	**0.04**	**0.04**	**0.12**	**0.20**	**0.10**	**0.07**	**0.11**	**0.01**	**0.06**

Table 7: Blend of isoprenoids (isoprene and all species of monoterpenes) emitted by the plant species in the experimental area of Castelporziano, Rome, central Italy. Photosynthesis and stomatal conductance are also reported. All values indicates means (n = 3). – = non-detected compounds.

2.4.2.3 Experimental set up and measurements

A scaffold tower (7 m high) was mounted inside the macchia, and equipped with a tridimensional sonic anemometer (M81000, Young, Michigan, USA). The outputs of the analyser was also collected by using a CR3000 datalogger (Campbell Scientific, Utah, USA). Air was sampled through a Teflon tube from the top of the scaffold, and convoyed to

a PTR-MS (Proton-Transfer Reaction Mass Spectrometer) enclosed into a cabin, which also furnished power supply for all the instruments (Fig. 26).

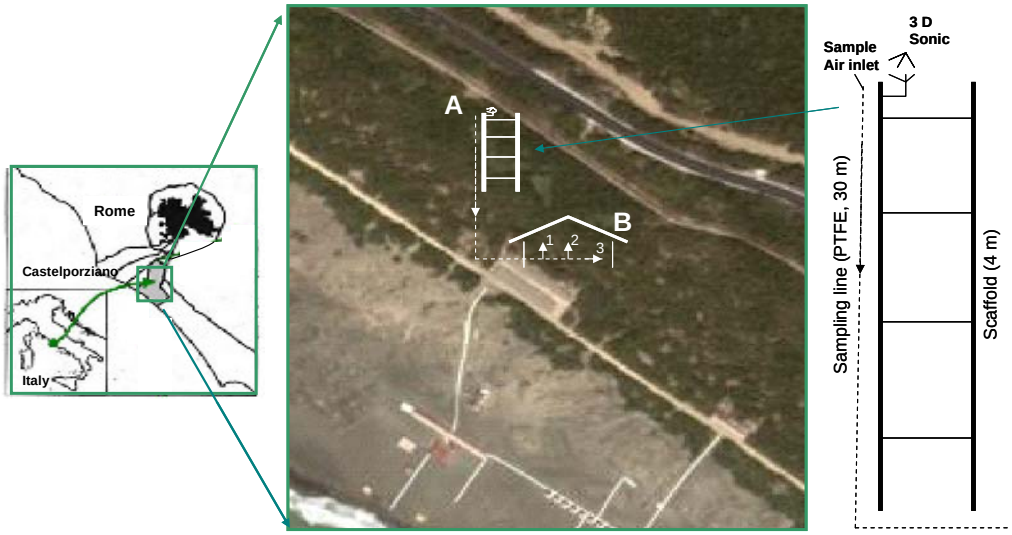

Figure 26: Overview of the experimental site inside Castelporziano Estate, central Italy. A scaffold tower (A) was installed in the middle of the dune ecosystem. A Teflon line (dashed) was sampling air from the top of the scaffold with a vacuum pump (3) enclosed in a cabin (B). A PTR-MS (1) and an ozone detector (2) were sampling from the main sampling line.

The PTR-MS was set to measure oxygenated compounds (Methanol-m/z33, Acetone-m/z59, Acetaldehyde-m/z45), isoprenoids (Isoprene - m/z 69, Σ Monoterpenes - m/z 81+137), and a reaction product between isoprene and ozone (Methyl-Vinyl Ketone-m/z71). Details on PTR-MS functioning are give in section 1.3.1. For flux calculation, the technique of Disjunct Eddy Covariance was adopted, integrating the data from sonic anemometer with the concentration values recorded with the PTR-MS, as described in section 1.3.3. The post-processing of data were performed with Matlab software (The MathWorks Inc., Apple Hill Drive Natick, Ma., USA).

In parallel, the gas exchange of all species was measured with a Li-6400-40 gas exchange system (LI-COR, Lincoln, Nebraska, USA). In this open portable system, a leaf was enclosed in a 6 cm^2 cuvette and an air flow was pumped to the cuvette after being filtered with an active carbon cartridge to avoid isoprenoids contamination. The instrument software allowed to control the light and temperature conditions of the leaf, respectively 1000 µmol m^{-2} s^{-1} of PAR (Photosynthetic Active Radiation) and 30 °C of temperature. These are the basal conditions at which the BVOCs emission is commonly measured. The outlet from the

cuvette was simultaneously convoyed to the Li-6400-40 to measure photosynthesis, transpiration, stomatal conductance to CO_2 and H_2O, and to custom made Tenax traps for BVOC analysis. The adsorption through the traps was controlled by a pump (Pocket pump, SKC, PA, USA) at a flow rate of 200 ml/min to reach a total aspired volume of 10L. The *ex situ* analysis of the traps was performed by technicians of the Institute of Chemical Methodologies (National Research Council) with a gas chromatograph mass spectrometer (GC 5890, Hewelett Packard, Palo Alto, CA, USA) coupled with a a thermal desorption unit (MSD 5970, Hewelett Packard, Palo Alto, CA, USA) and with a Purge and Trap Injector (Chrompack, Middelburg, The Netherlands). The desorption was performed at a temperature of 250°C for 5 min and Helium as a carrier gas at a flow rate of 20ml/min. The desorbed isoprenoids were concentrated in a lyner kept at -190°C with liquid Nitrogen end successively heated (200 °C) and injected in backflash in 5 seconds. The column used for the chromatographic analysis (Chrompack, Fused silica CPsil5, 50m x 0.4 x 0.32, Middelburg, The Netherlands) was set with the following program of temperatures: Temp1=5°C x 3min. Rate1 =3°C/min to 50°C, rate2 = 5°C/min to 250°C x 2min. After exiting the column, the isoprenoids were detected by the Mass Spectrometer for the qualitative and quantitative analysis. These values represented the basal emission factors which I used to model isoprenoids emission with the G95 algorithm (Guenther et al. 1995) described in section 1.3.4.

Ozone concentration was measured with an UV analyzer (Model 1008, Dasibi Environmental Corp., Glendale, California, USA) connected to the main sampling line and enclosed in a cabin (Fig. 26). The output signals were collected with the CR-3000 datalogger. For a comparison, data from the EMEP station of Montelibretti, Rome, central Italy, were used with the courtesy of Dr. Cinzia Perrino.

2.4.3 Results and Discussion

Data collected with the sonic anemometers showed that during the measuring period in spring, the climate was dominated by typical breeze with a light wind regime coming from the land (N-NE) during the night hours and a sea breeze during the day (SW) (Fig. 27 A). These data were coupled with ozone concentration, and showed that ozone levels are never

reduced to zero, but see breeze contains some ozone which is transported over the land during light hours (Fig. 27 B). This ozone formed over the sea is probably produced daily in the air column over the sea through photochemical reactions (Prinn, 2003; Monks, 2005).

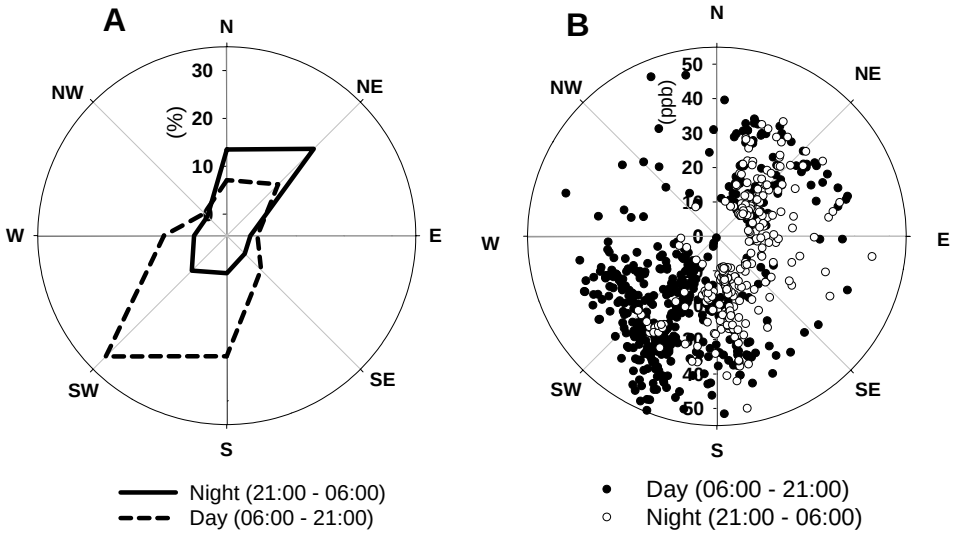

Figure 27: Polar plots showing A) percentage of wind distribution during night (continued line) and day hours (dotted line); B) ozone concentration (ppb) during day (black circles) and night hours (white circles) according to the wind directions in Castelporziano Estate, central Italy.

Figure 28 shows the values for ozone concentration at Castelporziano and Montelibretti (100 Km downwind during the day) in the months of May and June. Day time ozone levels are lower in Castelporziano than in Montelibretti, probably because high amount of ozone is produced in the city. Higher night levels in Castelporziano could be explained by land breeze-driven transport of the pollutant from the city.

The measured ozone concentrations exceeded 40 ppb during the day hours, a threshold after which plants can be damaged (UNECE 2004). However, most of the schlerophyllous species present in the experimental site (*Quercus ilex*, *Arbutus unedo*, *Pistacia lentiscus*) are known to be resistant to chronic ozone exposures (Vitale et al. 2007; Nali et al. 2004). This is due to A) thick mesophyll and waxes which give resistance to ozone entry through stomata and B) the stomatal closure itself, since ozone damages plants mainly after entering stomata, as described in detail previously in the introduction of the case-studies of this work.

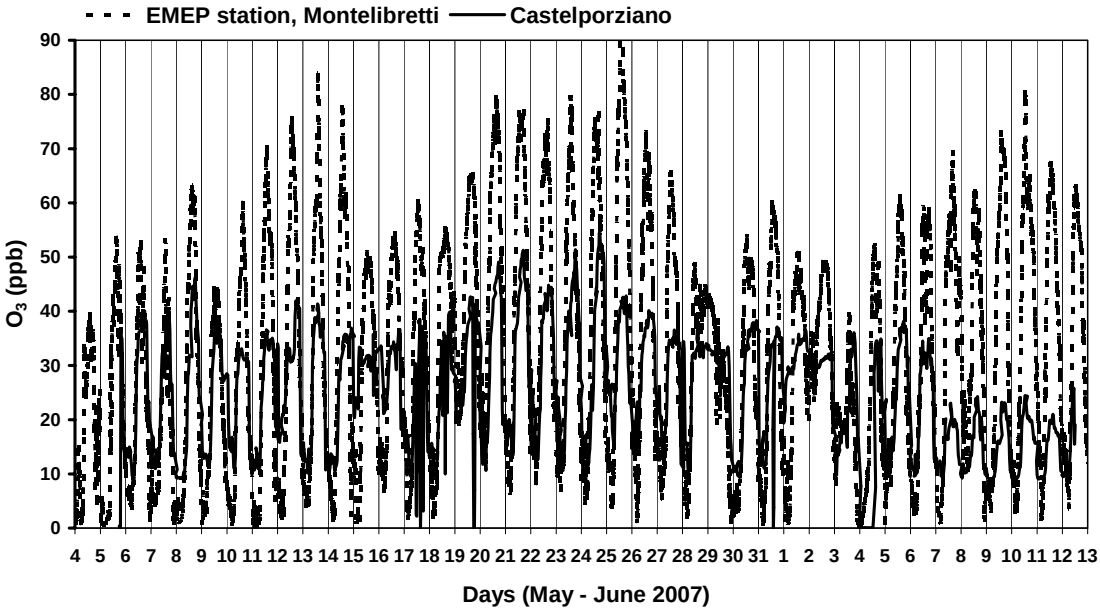

Figure 28: Ozone concentration in Castelporziano Estate, coastal area of central Italy (continued line) and in Montelibretti (dashed line), EMEP station, 25 Km north from Rome city center, central Italy (dotted line).

Plant showed an excellent physiological status during the measurements (Table 7), with high rates of photosynthesis and stomatal conductance under basal conditions, in comparison with rates of photosynthesis and stomatal conductance from literature (Manes et al. 1997). Plants were clearly growing during the measuring season. As a consequence, and considering the dependence of isoprenoids emission from photosynthesis (Loreto and Sharkey 1990), isoprenoids emission was considerably high. On the other hand, this high isoprenoids emission was unexpected considering the unfavourable measuring period. Indeed, isoprenoids basal emission is controlled by the seasonality, and in May there is a predominance of young leaves which produce and emit less amount of isoprenoids, due to a lower production of isoprenoids byosinthesis-related genes (Mayrhofer et al. 2005) and to a lower production of isoprene and monoterpenes precursors, respectively DMADP (dimethylallyl diphosphate) and GDP (geranyl diphosphate) (Noguès et al. 2006).

Between isoprenoids, monoterpenes and not isoprene were emitted in higher amount. This is clearly shown in the measured fluxes in figure 29 (A, B) and in the fluxes measured with traps and reported in table 7. These isoprenoids were emitted mostly during the central hours

of the day (Figure 30). This was expected, considering the dependence of BVOC emission from light and Temperature (Monson et al. 1992; Tingey et al. 1991).

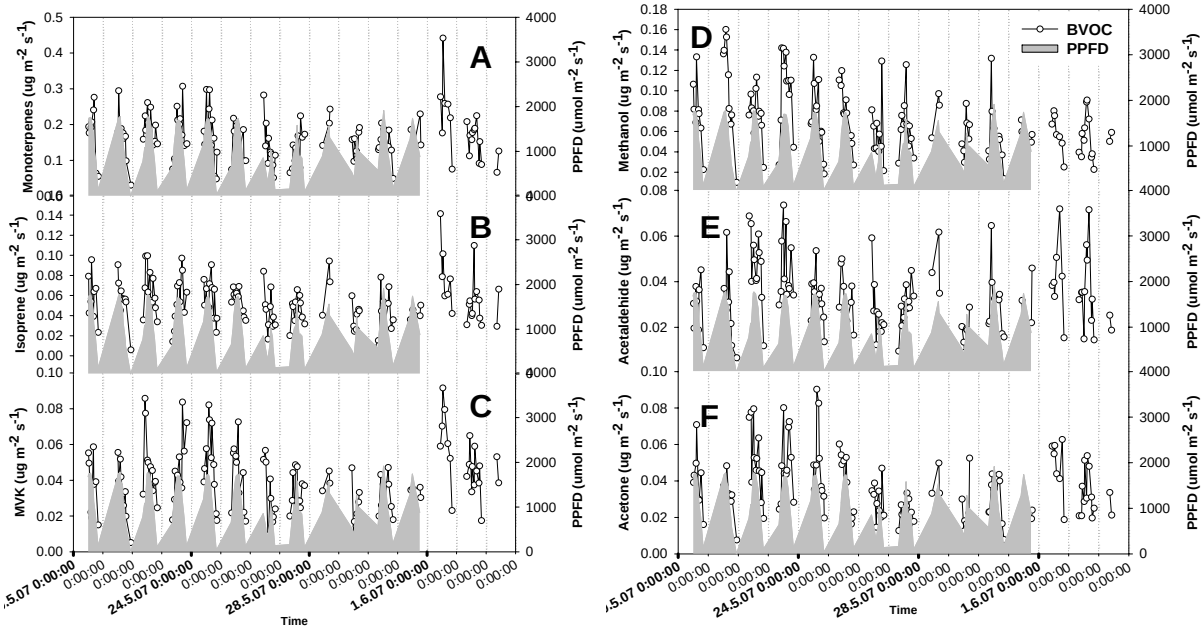

Figure 29: Fluxes of Oxygenated compounds compounds (Methanol-m/z33, Acetone-m/z59, Acetaldehyde-m/z45), and isoprenoids (Isoprene-M/z69, Σ Monoterpenes-m/z81+137) measured with a PTR-MS in the Estate of Castelporziano, central Italy. The low night fluxes were not reported because values were below the instrumental noise and the turbulence was too low.

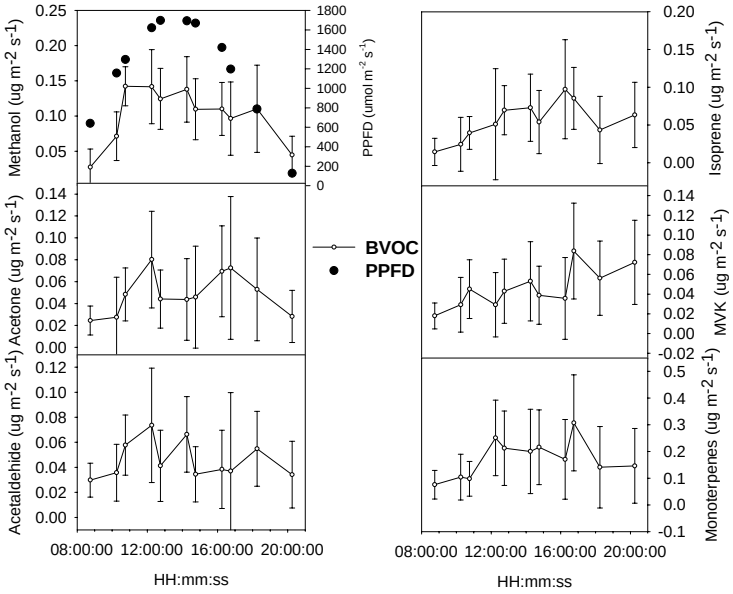

Figure 30: Daily corse of BVOC emission in Castelporziano Estate, central Italy. The example day was May 23rd, hourly means with ± standard error (95% confidence interval) are reported.

Knowing the basal emission of each species (table 7), and after performing an average weighted on the species representativeness on the experimental site (species-specific leaf biomass calculated on site, data not shown), I calculated the stand basal isoprene emission. The basal emission value was 0.31 μg(C) g^{-1}DW h^{-1} , lower than that suggested in 1995 by Guenther et al. (16 μg(C) g^{-1}DW h^{-1}) and in 1997 by Owen et al. (14.88 μg(C) g^{-1}DW h^{-1}). It should be mentioned that these two studies were concentrated in a Mediterranean ecosystem more abundant in isoprene emitting species (*Myrtus communis* and *Citisus* spp.) than the dunal ecosystem investigated here. The same averaging procedure was adopted for monoterpenes, and yielded a basal emission rate of 4.7 μg(C) g^{-1}DW h^{-1}. For monoterpenes, Guenther et al. (1995) suggested a basal emission value of 1.2 μg(C) g^{-1}DW h^{-1}, and a similar indication comes from Owen et al. (1997), with a value of 2.2 μg(C) g^{-1}DW h^{-1}. These findings underline that the Mediterranean dune vegetation is a more important source of monoterpene emission than other Mediterranean ecosystems. This could have strong implications for atmospheric chemistry, since monoterpenes contribute more than isoprene to the formation of SOA (Secondary Organic Aerosol) and atmospheric particles (Di Carlo et al. 2004; Kavouras et al. 1998). Moreover, high levels of NOx and anthropogenic VOC (unpublished data) were recorded during the experimental campaign and these compounds may contribute to particle formation. The SOA and particle formed over this dune ecosystem are daily transported to the city (Fig. 27A) thus contributing to the urban pollution.

From a comparison between measured and modelled fluxes (Fig. 31), it seems that the flux modelled according to Guenther's algorithm (see section 1.3.4) overestimates the actual flux. This could be due to A) an effective overestimation of the emission factors or a dependency of isoprenoids emission from the environmental parameters unpredicted by the model; B) the measurement of isoprenoids did not account for the totality of the flux. The latter hypothesis could hold true if the fetch (effective surface contributing to the measured flux) of the experimental area also included an area located too close to the beach, and which did not contribute isoprenoids emission. Future studies on modelling the fetch location will help to make clear this aspect.

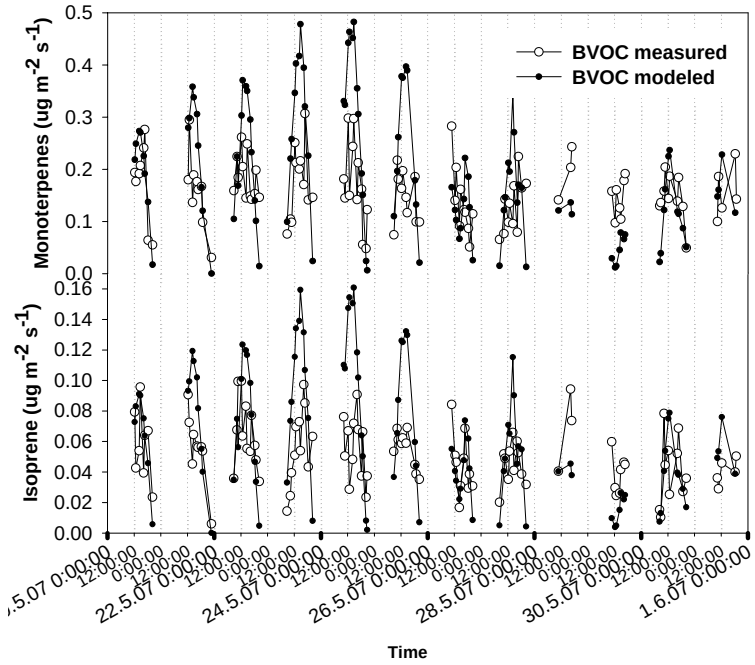

Figure 31: Measured (white circles) and modeled (black circles) emission of isoprenoids at Castelporziano Estate, central Italy.

Methanol was the most significant OVOC (Oxygenated Volatile Organic Compound) emitted by the Mediterranean vegetation (Fig. 29 D,E,F). This considerable emission could be explained by the measurement period (May) characterized by high rates of leaf expansion, of which methanol is a good indicator (Nemecek-Marshall et al. 1995). The low emission rates of acetaldehyde and acetone suggest that the plants were not stressed by biotic and abiotic factors (Kreuzwieser et al. 1999).

An emission, or more precisely a formation of Methyl-Vynil Ketone (MVK) was observed (Fig. 29 C). MVK is a reaction product between isoprene and ozone. However, the initial ozone loss due to reactions with isoprene to form MVK, is a first step of a complex series of photochemical reactions in which OH radicals and NOx take part, leading to ozone formation. The contribution of isoprene to ozone formation through its reaction products (MVK and methacrolein) was previously observed from Lee and Wang (2006) and Dreyfuss et al. (2002). The positive correlation between isoprene emission/MVK formation, and ozone concentration shown in figure 32 seems to confirm the theory that isoprene contribute to ozone formation but, overall, there are not enough evidences that ozone levels were influenced by on-site formation or removal due to isoprenoid emissions.

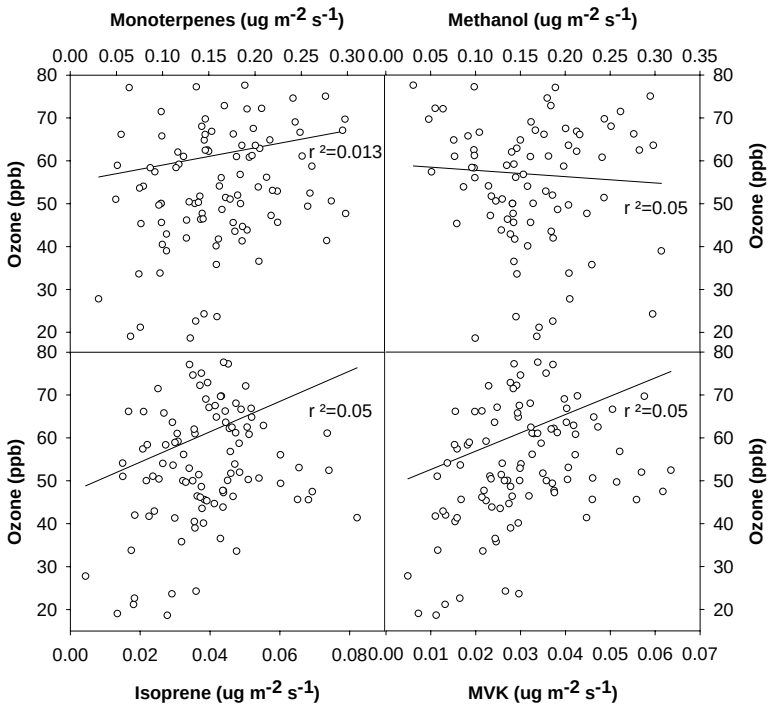

Figure 32: Relationship between BVOCs fluxes (Isoprene, Monoterpenes, Methanol and Methil- Vynil Ketone) and ozone concentration at Castelporziano Estate, central Italy.

The relationship between monoterpenes and methanol with ozone concentration is unclear for this dune ecosystem in the period of experiments (Fig. 32), supporting the conclusion that these compounds are not involved in reactions forming ozone. Previous studies (Kurpius and Goldstein 2003; Goldstein et al. 2004) hypothesized that isoprenoids contribute to the non-stomatal ozone fluxes. In the experimental site however, the abundance of NOx from anthropogenic sources might have enhanced a NOx/VOC ratio suitable to form ozone. The latter hypothesis has to be confirmed with further measurements on anthropogenic VOC and NOx fluxes.

3. Conclusions

Plants are sink for ozone, and VOC emission enhance their scavenging capacity

The case studies described in this thesis are aimed at unravelling the elusive relationships between ozone and isoprenoids at plant level. Early work hypothesized, based on the classic cycle of reactions that lead to isoprene-catalyzed ozone formation in the atmosphere, that isoprene could make worse ozone damage to leaf (Hewitt et al. 1990). Loreto and Velikova (2001), however, first demonstrated that isoprene-emitting leaves are better protected against ozone damage, and the same group demonstrated that isoprene biosynthesis is activated following exposure to ozone, another indication of the possible protective role of these compounds under oxidative environment (Loreto et al. 2004). Stepping from the latter result, my first case-study has focussed on understanding whether isoprene biosynthesis can be stimulated in a systemic way, that is, in also leaves of the same plant that are not exposed to ozone, the metabolic shifts in isoprenoid biosynthesis that characterize leaves directly exposed to the pollutant.

Case-study 1 confirmed that ozone has detrimental, direct effects on leaves. More importantly, however, this experiment in which I used a novel fumigation apparatus, showed indirect effects of ozone in leaves that developed during and after the oxidative treatment but were not in contact with the stressor. This set up made possible a number of original observations. First, and most relevant for the objectives of this thesis, leaves showed the expected stimulation of isoprene emission when developing in plants that were ozonated, in comparison with non-ozonated plants. This confirms that the stimulation of isoprenoid biosynthesis is associated to the oxidative stress, and bring to the important and novel conclusion that the effect is not limited to the parts that are directly exposed to ozone. Interestingly, the stimulation of isoprene biosynthesis was not associated to an increase of photosynthesis, which indicates that a larger fraction of the fixed carbon budget is allocated to form isoprene as an indirect consequence of ozone stress.

A number of other original observations were also made during this work, e.g. changes in leaf anatomy and histology, and starch metabolism whose relationship with the observed changes in isoprene biosynthesis are still unclear. And, it was also observed that leaves developing ex novo under high ozone also possess a series of adaptive (anatomical and

physiological) mechanisms that do not seem to involve enhancement of isoprenoid biosynthesis.

Stepping from the conclusion of the work by Loreto and Velikova (2001), I reasoned that if isoprenoids protect leaves against ozone by removing the pollutant inside the leaf mesophyll, then a higher uptake of ozone should have been observed in leaves that emit more isoprenoids. To test this hypothesis, that again goes beyond the established dogma that the more ozone is taken up by leaves, the more injury occurs (Reich 1987), I planned the experiments that have been shown in case-study 2 of this thesis. First, I established that non stomatal uptake is a minor component of the total uptake under well stirred atmosphere, while stomatal conductance is the prominent way ozone is acquired by leaves. Then I showed that a part of the stomatal flux is associated to isoprenoid biosynthesis and emission, and that ozone stomatal flux is not related to actual damage experienced by the leaves. This suggests that isoprenoids remove and detoxify ozone, constituting an intercellular line of defense against this powerful pollutant. The exact location where isoprenoids exert this action remained unknown. Importantly, plant resistance to ozone is independent on ozone stomatal flux also in species that do not emit isoprenoids, indicating that, in general, the detoxification of ozone by antioxidants drives the flux of the pollutant inside leaves. Thus, the dogma that more ozone flux causes more ozone damage does not rely on a tenable assumption, and stomatal flux of ozone cannot be recommended as a useful indicator of ozone damage (Emberson et al. 2000, 2007).

The attempt to generalize and upscale these conclusions is at the basis of case-study 3 that I have presented here. By using large-scale facilities that allowed to fumigate with ozone entire plants and to enhance the residence time of air in the chambers, I was able to confirm that stomatal uptake is the dominant process regulating ozone removal, even at whole plant level, with the exclusion of the soil component. However, this study also revealed that, while isoprene only reacts with ozone inside leaves, more reactive isoprenoids, e.g. monoterpenes, may also scavenge ozone outside the leaf, which may be interpreted as a contribution to the non stomatal ozone uptake through reactions in the gas phase.

One final point could be tested with the results obtained in case-study 2 and 3. The nowadays widely used models to predict ozone flux, and to assess ozone risk for vegetation

(Emberson et al. 2000, 2007), assume the intercellular concentration of ozone to be zero, as ozone is immediately scavenged upon contact with leaf mesophyll structures (Laisk et al. 1989). My results contradict this dogma as well. Especially when ozone freely diffuses inside leaves with high stomatal conductance, ozone may accumulate considerably inside leaves. If above-zero $[O_3]_{ci}$ are entered in the model, then the predicted values of ozone flux will be lower, thus reducing the estimated stomatal uptake of ozone by leaves and whole plants.

A final consideration has to be made about the importance of turbulent air movements in determining stomatal and non stomatal components of the ozone uptake, and, in particular, the possibility that ozone disappears due to gas-phase reactions with biogenic VOC (monoterpenes, sesquiterpenes) or anthropogenic emissions (e.g. NOx, OH radicals). These reactions occur actually outside leaves, and should not be accounted for plant-driven reactions. During the last year of my studentship I had the chance to help coordinating the ACCENT/BIAFLUX field campaign in Castelporziano, Rome, whose main objective was to establish whether isoprenoid fluxes match fluxes of pollutants by vegetation and in parallel contribute to ozone uptake by plants, or to ozone formation being involved in photochemical reactions in which anthropogenic pollutants (e.g. NOx) take part. Preliminary results of this exercise reported in case-study 4, showed that biogenic VOC emitted by Mediterranean dunal vegetation do not play a decisive role in determining a removal rate of the pollutant through gas-phase reactions, but a positive correlation between isoprene emission and tropospheric ozone concentration was observed. Future studies will be necessary to validate the theory that biogenic isoprenoids contribute to ozone formation in this periurban area.

4. References

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