UNIVERSITÀ
DEGLI STUDI DELLA
Tuscia

DIBAF
Department for Innovation in Biological, Agro-food and Forest systems

*Ph*D Course in **Forest Ecology**

XXIV Cycle

"BVOC emission responses to acute short-wave monochromatic radiations"

Scientific sector: AGR/05

Coordinator: Prof. Paolo De Angelis

Supervisor: Dr. Mauro Centritto

*Ph*D Student: **Emanuele Pallozzi**

Università degli Studi della Tuscia di Viterbo

Dipartimento per la Innovazione dei sistemi Biologici, Agroalimentari e Forestali (DIBAF)

Via S. Camillo de Lellis, 01100 Viterbo, Italy

Corso di Dottorato di Ricerca in Ecologia Forestale - XXIV ciclo

Coordinatore: Prof. Paolo De Angelis

Tesi di Dottorato di Ricerca in Ecologia Forestale

Tutor: Dr. Mauro Centritto

Autore: Emanuele Pallozzi

Marzo 2012

Acknowledgements

First and foremost I offer my sincerest gratitude to my supervisor Dr. Mauro Centritto who has supported me throughout these three wonderful and intense years of interesting and worthwhile work.

I would like to express my gratitude to Dr. Enrico Brugnoli and Dr. Massimo Zacchini for hosting me at the IBAF institute of CNR, allowing me to perform my experiments.

It was easier to succeed in achieving my objectives with a great team work, it would have been quite impossible without Dr. Alessio Fortunati and Giovanni Marino and their priceless friendship. And only with the huge help of Prof. Tsonko Tzonev we were able to accomplish our results.

It is impossible not to mention Mauro Medori, Arianna Morani, Paola Tassone and Ettore D'Andrea for their friendship and continual stimulation in giving my best.

I also want to thank Dr. Francesco Loreto for his precious collaboration, Dr. Violeta Velikova, Prof. Tariq Mahmood, Dr. Carlo Calfapietra, Isabel Nogues and Fernando Migliaccio for their important collaboration in my work.

Dr. Silvano Fares was vital with his clear lessons and valuable help with PTR-MS, Maria Rita De Paolis was very kind helping me with examinations and statistics, Valentina Iori was my muffin pusher and Daniele Bianconi was unexpectedly calm in sharing our office.

Coffee breaks (and it was a great coffee, made by Sabrina, Daniela and Nicoletta) were an extraordinary relaxing corner while sharing stories with Chiara Rossini.

Technician are the pulsing heart of every institute, their support was determining. Led by captain Giuseppe Santarelli, I want to mention all of them in strictly alphabetical order: Alessandro Tomassetti, Anna Mariani, Cesarino Nicoletti, Ermenegildo (Dino) Magnani, Fabrizio Lelli, Fabrizio Morvillo, Leandro Brunacci, Marco Giorgetti, Pierangelo Bertolotto and Valerio Muzzini.

It was a pleasure for me to work with Giorgio Matteucci and his team (Bruno, Mario, Flavia, Marco M., Marco B., Andrea and Antonia) and I want to thank them for the precious information about forest environment.

I want also to thank Laura Passiatore, Fabrizio Pietrini, Manuela Galli, Daniela Lippi, Daniela Di Baccio and Federico Brilli.

Thanks to the guys of IBBA institute (Giulio, Donato, Silvia, Nadia, Francesco, Giovanni, Elisabetta, Adelaide, Giovanna and Chiara) for the nice time spent with them.

English is easier with Adrian!

Field measurements in Follonica were unforgettable, thanks to Dr. Claudio Cantini, Alessandra and Andrea of IVALSA/CNR institute. Every moment spent there was special.

Each of you left your mark.

Lastly, and most importantly, I wish to thank my father and Daniela who supported me in this incredible journey.

Table of Contents

CHAPTER 1 – Introduction

1.1 BVOCs (Biogenic Volatile Organic Compounds)

All plants produce a vast and diverse assortment of volatile compounds such as nitric oxide, carbon monoxide, and non-methane volatile organic compounds, the so-called biogenic volatile organic compounds (BVOC). The great majority of these substances, traditionally referred to as secondary metabolites, do not appear to participate directly in growth and development, but their functions are being elucidated with increasing frequency. Despite BVOC emissions represents only 1-1.5% of total carbon exchanged between biosphere and atmosphere: i.e. ~1.5 Pg $year^{-1}$ (Welp, 2011) of the total C cycle (circa 120-175 Pg $year^{-1}$), BVOC emissions have received increased scientific attention in the last two decades because BVOCs are highly reactive and, thus, may profoundly influence the chemical and physical properties of the atmosphere. In fact BVOCs can remove hydroxyl radicals (·OH) in the atmosphere quickly, indirectly causing accumulation of greenhouse gases like methane (Brasseur & Chatfield, 1991; Di Carlo *et al.*, 2004). Moreover BVOCs can react with anthropogenic NO_x emissions in presence of UV radiation, causing the formation of photochemical smog, i.e, tropospheric ozone and secondary organic aerosols (Chameides *et al.*, 1988; Kavouras *et al.*, 1998).

Terpenoids perhaps are the most structurally varied class of plant natural products. The smallest terpenes contain a single isoprene unit (the five-carbon units that make up the terpenoids); as a group, they are named hemiterpenes (half-terpenes). C10 terpenoids, although they consist of two isoprene units, are called monoterpenes. The monoterpenes are best known as components of the volatile essences of flowers and of the essential oils of herbs and spices. The terpenoids that derive from three isoprene unit contain 15 carbon atoms and are known as sesquiterpenes (i.e., one and one-half terpenes). Like monoterpenes, many sesquiterpenes are found in essential oils. In addition, numerous sesquiterpenoids act as phytoalexins, antibiotic compounds produced by plants in response to microbial challenge, and as antifeedants that discourage opportunistic herbivory. The abscisic acid is structurally a sesquiterpene. The diterpenes, which contain 20 carbons (four C5 units), include phytol (the hydrophobic side chain of chlorophyll), the gibberellin hormones, the resin acids of conifer and legume species, phytoalexins, etc.

The best known hemiterpene is isoprene (2-methyl-1,3-butadiene), a volatile, highly reactive non-methane hydrocarbon (NMHC) released from photosynthetically active tissues. Isoprene is the most abundant phytogenic VOC and is emitted in copious quantities by many forest species (Kesselmeier

& Staudt, 1999; Fall, 1999; Guenther *et al*., 1999). Isoprene is produced enzymatically, released from photosynthetically active tissues in the light and emitted through stomata to the atmosphere (Sharkey & Yeh, 2001). The enzyme isoprene synthase is present in the leaf plastids of numerous C3 plant species. Isoprene biosynthesis is light dependent and is closely linked with carbon metabolism at ambient CO_2 concentration (Monson & Fall, 1989; Loreto & Sharkey, 1990). By feeding $^{13}CO_2$ to leaves it has been demonstrated that about 72-91% of the carbon in the isoprene molecule originates from recently assimilated photosynthetic intermediates and that the remaining carbon is derived from alternative sources (Sanadze *et al*., 1972; Delwiche & Sharkey, 1993; Karl *et al*., 2002; Affek & Yakir, 2003; Funk *et al*., 2004; Schnitzler *et al*., 2004; Brilli *et al*., 2007). Temperature is a major factor controlling isoprene emission, with two- to four-fold higher rates of emissions for each 10 °C increase in temperature (Sharkey & Yeh, 2001; Fares *et al.*, 2011). Thus, rising temperature will likely outweigh the inhibitory effects of elevated [CO_2] on isoprene emission (Rosenstiel *et al.*, 2003; Centritto *et al.*, 2004; Fares *et al.*, 2011), leading to future increased ozone formation which, in turn, will negatively affect plant photosynthesis and growth.

The principal function of isoprene biosynthesis in plants remains unclear, but isoprene acts as a thermoprotective molecule, potentially stabilizing chloroplast membranes during high temperature events (Singsaas *et al.*, 1997; Singsaas and Sharkey 2000; Behnke et al. 2007). Because isoprene production is enhanced by both high temperatures and light, it has been proposed to maintain photosynthetic capacity during rapid leaf temperature fluctuations caused by sun flecks in the canopy (Sharkey and Singsaas 1995; Behnke et al. 2010). Several experiments showed in fact that isoprene can protect plants against short high-temperature episodes (Singsaas *et al.*, 1999; Singsaas & Sharkey, 1998) and it can interact with the phospholipid bilayer of membranes to maintain membrane stability during high temperature events (Siwko et al. 2007).

Despite isoprene can react with NO_x, producing dangerous hydroperoxides in the atmosphere (Hewitt *et al.*, 1990), *in planta* isoprene seems also to act as an antioxidant protecting plants against ozone (Loreto & Velikova, 2001). When the oxidation potential becomes high, such as under acute or prolonged ozone exposures, isoprene quenches ozone-dependent ROS, attenuating the damage at the membrane level and probably the consequent damage at biochemical and physiological levels (Loreto & Velikova, 2001). This antioxidant function should be even more important during simultaneous light and heat stress, when thermotolerance and reactive oxygen quenching mechanisms are needed (Peñuelas et al. 2005). Many studies have also pointed out the importance of BVOCs in plant-insect-environment interaction, highlighting how these compounds, both

induced or constitutive, can attract specific species and can also be a powerful deterrent against pathogens and herbivores (Gershenzon & Dudareva, 2007; Brilli *et al.* 2009).

Methanol and acetaldehyde emissions are generally associated to changes in the cell wall structure and demethylation of pectin (Galbally & Kirstine, 2002). This can be caused by growth processes, mechanical wounding and other stress occurrence. Considerable but transient emission of methanol in the atmosphere is therefore associated to cell wall damage occurring because of wounding (Karl *et al.*, 2001). Methanol formation in leaves may also be associated with as expansion growth, i.e. cell wall loosening during cell expansion (Nemecek-Marshall *et al.*, 1995), and in senescing leaves, because of irreversible decomposition of cell walls (Fall, 2003). Acetaldehyde is principally 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). Consistently, large fluxes of acetaldehyde have been observed when roots are flooded and are exposed to anoxia (Kreuzwieser *et al.*, 2000). Furthermore, acetaldehyde is also emitted following wounding (Fall *et al.*, 1999) and after ozone stress episodes (Cojocariu *et al.*, 2005).

BVOC emissions are generally closely linked to carbon assimilation in many forest and agriforest species, which are the main BVOC emitters, but they play a contrasting role in the biosphere-atmosphere interaction. In fact, forest and agriforest vegetation is a dominant sink for the atmospheric CO_2 and ties about 90% of the globe's biomass carbon. Thus, it plays, by mean of photosynthesis, a key role in mitigating global change. In contrast, BVOCs have an important, negative impact in atmospheric chemistry, because they play a major role in the production of tropospheric ozone and aerosols. BVOCs rapidly react with anthropogenic and natural compounds and particularly with nitrogen oxides in the atmosphere leading to the formation of tropospheric ozone and photochemical smog. Furthermore, BVOCs affect the residence time of other greenhouse gases (including methane), and may cause the formation of secondary aerosols, a component of PM10 in the atmosphere. This emphasizes the importance of biogenic emissions, and inventories of BVOC emissions are, thus, a key issue in atmospheric sciences. Moreover, given the importance of both BVOC emissions and carbon assimilation for the biosphere-atmosphere interactions, the consequences of climate change (rising [CO_2] and temperature, Fig. 1.1) for the biogeochemical carbon cycle and for the atmospheric chemistry are potentially extremely large. Thus, a thorough understanding of BVOC synthesis mechanisms and plant and ecosystem responses to climate change is required if future emissions are to be reliably predicted.

Climate change has been going on since the earth first existed, but it has been accelerated by human activities starting from the industrial revolution (Donaghy, 2007). The use of fossil fuels to produce

energy has caused an increasing in the atmospheric concentration of carbon dioxide (CO_2). The global atmospheric CO_2 concentration increased from a pre-industrial value of about 280 ppm to 379 ppm in 2005 (IPCC, 2007a). The annual CO_2 concentration growth rate, along the previous 10 years, was 1.9 ppm per year. This increment in CO_2 atmospheric concentration is the most important factor that causes global temperature increase: because of its chemical and physical characteristics CO_2 is able to absorb thermic infrared radiations so that more infrared radiation is reflected back to the Earth by the atmosphere, leading to an increased temperature of the surface of the Earth (Mitchell, 1989).

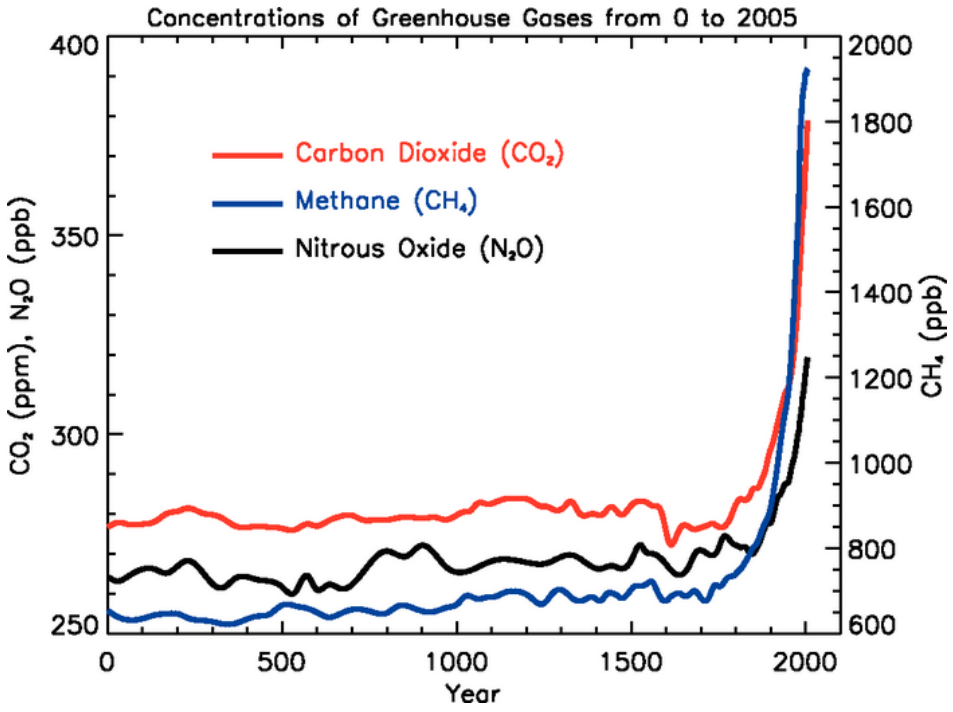

Figure 1.1: atmospheric concentration of important long-lived greenhouse gases over the last 2,000 years. Increases since about 1750 are attributed to human activities in the industrial era (*IPCC 2007: Working Group I: The Physical Science Basis).*

Tropospheric ozone is a highly reactive gas that damages any living tissue with which it comes in contact. It has been increasing at a faster rate than CO_2. Prior to large-scale emissions of pollutants, ozone concentrations were relatively low, however, since the Industrial Revolution concentrations have increased several-fold. While ozone is a naturally occurring constituent of the atmosphere, fossil fuel combustion has increased the emission of the precursor to ozone formation by more than an order of magnitude from pre-industrial concentrations (Fowler *et al.*, 1998). In order for ozone to

form, both volatile organic compounds (VOC) and nitrogen oxides (NO_x) must exist in the presence of sunlight. Both anthropogenic activities and natural processes release VOCs to the atmosphere, including fuel consumption, byproducts of industrial processes, forest fires, natural geologic emissions (Etiope & Ciccioli, 2009) and secondary compounds released from vegetation. The release of NO_x occurs naturally as a result of biological activity in soils, lightning, forest fires, etc, but the release as a result of anthropogenic activities far exceeds that from natural sources (Fowler *et al.*, 1998). Ozone formation is relatively complex and is dependent on the relative concentrations of the precursors. For example, maximum ozone formation will occur with optimal concentrations of NO_x for a given concentration of VOCs (Fig. 1.2) (Bernacchi *et al.*, 2012). Higher or lower concentrations of either of these will result in a decrease in the formation of ozone. Ozone concentrations are predicted to continue increasing over the next 50 years with average concentrations reaching 20% over current, although changes in ozone formation are variable. Some locations are experiencing rapid increases in ozone formation while other locations are experiencing decreases (Forster *et al.*, 2007).

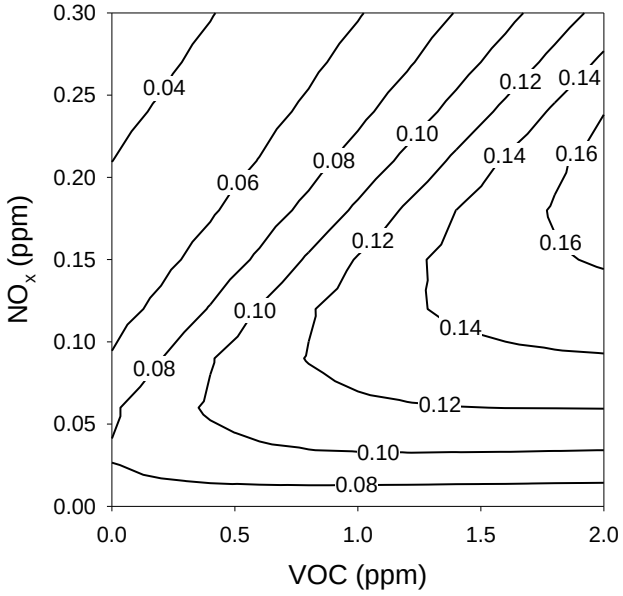

Figure 1.2: an ozone isopleth graph modeled for Champaign, IL USA on August 15, 2008. The lines on the plot show the predicted ozone concentration (ppm) for a given concentration of the two main precursors, nitrogen oxides (NO_x) and volatile oxygenic compounds (VOC). Note that for a given concentration of VOC (from Bernacchi *et al.*, 2012).

1.2 Solar irradiance

Solar irradiance is the radiation emitted by the sun that has been transmitted through the atmosphere of the earth (Fig. 1.3). Radiation can affect plants in various ways. First, the radiant energy absorbed by a plant affects tissue temperature and, consequently, rates of metabolic processes and energy exchanges such as transpiration. Second, the visible fraction of the incident solar radiation can be utilized in the synthesis of reduced carbon compounds (i.e., photosynthesis). Thirdly, energy of specific wavelengths in the solar spectrum can be used by plant as cues for "growth behaviour", e.g., the red:far-red ratio that can influence plant form and dry matter distribution among plant components, and the diurnal duration (photoperiod) of incident radiation, which can influence rate of development.

Short-Wave Radiation (280-3000 nm): short-wave radiation or solar radiation is the energy of wavelengths in the solar spectrum. Approximately 50% of the energy in the solar spectrum is photosynthetically active radiation (PAR). Short-wave radiation includes parts of the ultraviolet (UV) spectrum: UVB = 280-320 nm and UVA = 320-400 nm. Short-wave radiation also includes part of the near infrared (IR) spectrum (700-3000 nm).

Long-Wave Radiation (3000-10000 nm or 3-10 µm): in addition to short-wave radiation from the sun, long-wave radiation contributes to the radiation balance of plants. Long-wave radiation or terrestrial radiation is emitted by atmospheric gasses in the sky (water vapour and CO_2, for instance) and by objects on earth. Long-wave radiation is also called thermal radiation.

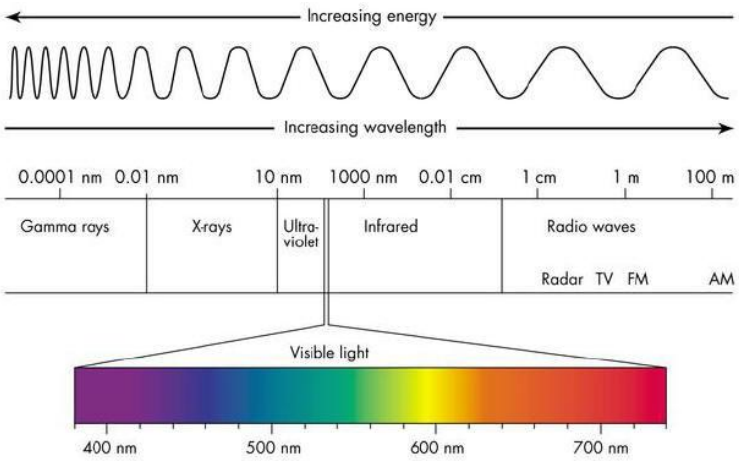

Figure 1.3: electromagnetic spectrum chart.

1.3 Ultraviolet radiation and its effect on plants

7% of the electromagnetic emission from the sun is in the range of UV radiation (200-400 nm). This radiation is modified and reduced during its passage through the atmosphere before reaching the terrestrial surface: shortwave UV-C radiation (100-280 nm) is completely absorbed by atmospheric gases; UV-B radiation (280-320 nm) is absorbed by stratospheric ozone and only a small portion continues its run to the terrestrial surface, whereas UV-A radiation (320-400 nm) is weakly absorbed by ozone (Frohnmeyer & Staiger, 2003) (Fig. 1.4).

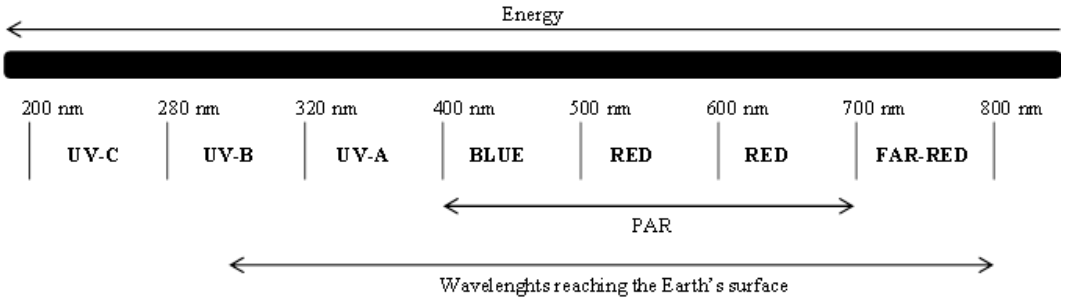

Figure 1.4: the solar spectrum perceived by higher plants. PAR, photosynthetically active radiation. (Modified from Frohnmeyer & Staiger, 2003).

Global climate changes involve continuous reductions in the protective stratospheric ozone layer (Gurney *et al.* 1993). This degradation is a concern because this layer is the primary attenuator of solar ultraviolet (UV) radiation. Moreover, although ozone is a very minor constituent of the atmosphere, it is the only gas of the atmosphere that absorbs appreciably at wavelengths shorter than 300 nm (Caldwell *et al.* 1989). A reduced ozone shield allows more UV radiations to reach the ground, with the largest fractional increase occurring in the shorter-wave UV-B region (Caldwell & Flint, 1997) (Fig. 1.5).

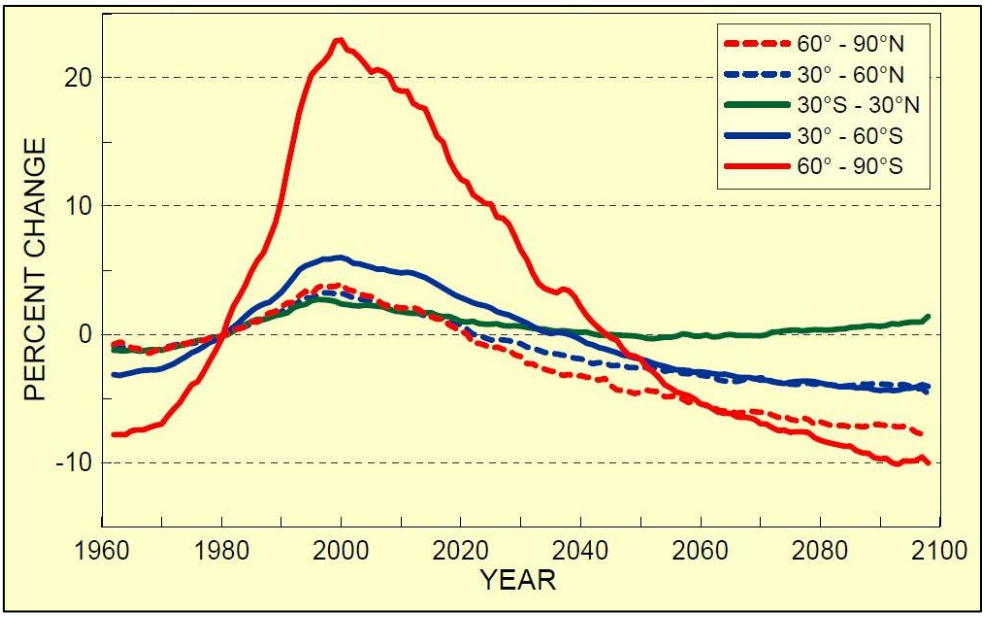

Figure 1.5: Projected changes in sun-burning clear-sky UV radiation (UNEP, 2010).

Although UV radiation is a small fraction of the total solar radiation that reaches the ground, it has the greatest energy per unit wavelength and, consequently, the greatest potential to damage the biosphere. UV radiation has large effects on living organisms because it is absorbed by macromolecules like proteins and nucleic acids (Jansen *et al.* 1998); on plants it includes damage to DNA and membranes, alteration in transpiration and photosynthesis and changes in growth, development and morphology (Teramura & Sullivan, 1994). UV radiation could potentially affect a variety of ecological processes in numerous different ways, involving also nutrient cycling and terrestrial carbon cycle. Several evidence suggests that UV radiation modifies the terrestrial carbon balance through changes in CO_2 capture (photosynthesis), carbon storage (organic carbon pools) and release (respiration and photodegradation) (Zepp et *al.*, 2007). UV radiation has been shown to inhibit photosynthesis because of the sensitivity of photosynthetic machinery (Albert *et al.*, 2008) and by the alteration of gene expression, which is critical to photosynthesis (Ballare *et al.*, 1996; Casati & Walbot, 2004; Izaguirre *et al.*, 2003; Savenstrand *et al.*, 2002).

A variety of indirect effects of UV radiation on the terrestrial carbon balance have also been recently elucidated. It is well documented that the chemical composition of plants is altered under UV exposure due to changes in nutrient content and tissue composition (Rozema *et al.*, 1997). Although UV radiation can affect the terrestrial carbon balance by influencing photosynthesis, organic carbon pools and litter decomposition, the magnitude and the direction and the significance of UV radiation on every part of carbon cycle are still not fully understood. Moreover, the combined effect of UV radiation and other climate change factors like nitrogen deposition, altered precipitation patterns and enriched carbon dioxide in the atmosphere, don't seem to be additive, resulting in complex and possibly synergistic interactions that are difficult to predict.

Considering that solar radiation cannot be accurately and experimentally represented, UV supplementation studies are difficult to relate to the field. Many supplementation studies use square-wave systems (i.e. uniform intensity throughout exposure period), not keeping count of the diurnal cycle of UV radiation. Studies that investigate aspects of plant development and growth should be carefully designed with lamps which can simulate UV levels that peak at solar noon. This may be important for many aspects of plant development, including germination, growth, and biomass allocation (Caldwell *et al.*, 1998; Bjorn *et al.*, 1999). Casati & Walbot (2003) performed a field experiment on maize plants and reported a significant down-regulation of genes associated with photosynthesis under elevated UV. This decreased expression of genes that are involved the in photosynthetic process is consistent with recent studies, both in laboratory and in field, that have shown similar effects of UV radiation in other species (Ballare *et al.*, 1996; Izaguirre *et al.*, 2003;

Savenstrand *et al.*, 2002). Furthermore, Albert *et al.*, (2008) documented UV-B effects on high arctic *Vaccinium uliginosum* exposed to ambient and 60% reduced UV-B radiation: net photosynthesis was 28% higher in treatments with less UV-B radiation. This difference was attributed to an overall reduction in the ability of plants under ambient UV-B to process light energy: photosystem II (PSII) has often been documented as particularly sensitive to UV exposure (Bornman, 1989; Teramura & Sullivan, 1994). These results suggest that photosynthesis may be reduced substantially as a consequence of increasing levels of UV-B radiation which could potentially result in alterations in the plant carbon pool.

Moreover, many studies showed different results in UV-B sensitivity between species: plants at lower latitudes or higher altitude, where UV intensity is higher, have more functional adaptive mechanisms than those from higher latitudes or lower altitude (Sullivan *et al.* 1992). Studies on the impact of either acute or chronic UV radiation on BVOC emission even on a single-species scale are few and show contrasting results. The first published study on the effects of both chronic and acute UV-B radiation on isoprene emission in emitting and non-emitting species, was performed by Harley *et al.*, (1996): they showed that chronic UV-B exposure significantly increased isoprene emission in *Quercus gambelii* but not in *Mucuna pruriens*, both strong isoprene emitting species. Furthermore, when *M. pruriens* and *A. platanoides* were exposed to high acute UV-B exposure, there were no apparent effects on either photosynthesis or isoprene emission. More recently, long-term effects of UV enhancement on BVOC emission were analysed on a pristine, mixed-species subarctic peatland ecosystem. Tiiva *et al.*, (2007) found that enhanced UV-B significantly increased the emission of isoprene during the warm periods of the second growing season, and at the end of the fourth growing season.

On the other hand, the authors did not detect significant UV-B effect on the emissions from subarctic fen ecosystem, during the warm period in the third growing season. Then, in a follow-up study on the same subarctic peatland ecosystem, Faubert *et al.* (2010) showed no overall UV-B effect on the isoprene and monoterpene emissions, apart from toluene and 1-octene. Furthermore, both Tiiva *et al.* (2007) and Faubert *et al.* (2010) found no effects of UV-A radiation (320-400 nm) on BVOC emissions from the subarctic fen ecosystem. In addition, long-term increase in UV-B radiation did not affect terpene concentration in leaf and wood of *Pinus sylvestris* and *Picea abies* seedlings (Turtola *et al.*, 2006), monoterpene and sesquiterpene emissions from *P. abies* (Blande et al., 2009). Similarly, Winter & Rostás (2008) found no differences in qualitative and quantitative composition of induced volatiles in soybean grown under ambient or attenuated UV radiation.

1.4 Blue Light

Plants rely heavily upon the surrounding light environment to direct their growth and development. Apart from the effect of photosynthetic photon flux density (PPFD), quality of light also affects other developmental and biochemical processes such as germination, flowering (Taiz & Zeiger, 2002) and stomatal regulation (Taylor & Assmann, 2001). Several different photoreceptor families mediate the effects of light on plant development. i.e. phytochrome molecule is the photoreceptor for red light responses, absorbing the red (600–700 nm) and far-red (700–750 nm) regions of the solar spectrum, while cryptochromes and the phototropins are involved in absorption of light in the blue (390–500 nm) and UV-A (320–390 nm) regions (Christie & Briggs, 2001), affecting chlorophyll formation, photosynthesis processes, stomata opening and raising the photomorphogenetic response.

The green pigment, chlorophyll, plays a central role in photosynthesis. The fact that it is green means that it absorbs blue and red light and reflects green when it is illuminated by white (all PAR wavelengths) light.

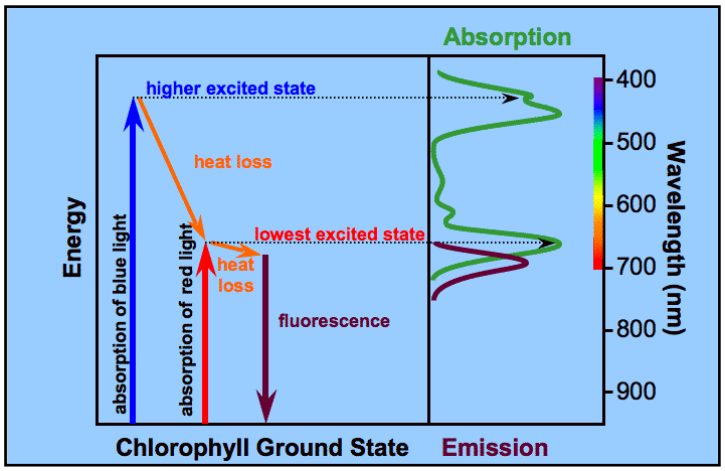

Figure 1.6: The absorption of light relates to electron excitation states (Koning, 1994).

The absorption spectrum in Fig. 1.6 shows two absorption maxima in the blue and red portions in the spectrum. In the same figure, the energy diagram shows how an electron can be elevated to a higher energy level in the electron cloud of chlorophyll by absorbing a high energy photon. Blue is at the high-energy end of the spectrum, so light of this wavelength is responsible for this much excitation and explains the absorption peak in the blue. Red wavelengths are lower in energy and

only boost the electron to a lower energy level than can blue light. This stable excitation state is responsible for the red absorption peak.

Despite the enormous knowledge acquired to date on the photoregulation of plant development under red/far-red, blue (Leicht & Silander Jr, 2006; Christophe *et al.*, 2006), and UV light, very little work has been done on the specific effect light quality.

1.5 Aim of the study and outline of the thesis

The following aims are addressed:

- To investigate the seasonal effects of acute UV-A radiation treatments on *Populus x euroamericana* focusing on the effects on isoprene emission.
- To study the effects of high doses of UV-B radiation on the BVOC emission of wild type and isoprene-emitting transgenic *Nicotiana tabacum* L. plants.
- To determine the effect of blue light on isoprenoid emissions of *Populus* x *euroamericana*, a strong isoprene emitter, *Quercus ilex*, a species with a small storage pool for monoterpenes, and *Citrus reticulata*, a species with a large storage pool for monoterpenes, in experiments carried out in both Estonia and Italy.

CHAPTER 2 – Treatment facilities, plants and methods of analysis

2.1 Plants description

Three tree species, *Populus x euroamericana*, *Quercus ilex* L. and *Citrus reticulata*, and isoprene-emitting transgenic tobacco (*Nicotiana tabacum* L.) were studied. *Populus* x *euroamericana* is a strong isoprene emitter, whereas *Quercus ilex* and *Citrus reticulata* are monoterpene emitters with a small and a large storage pool for monoterpenes, respectively (Schurgers *et al*., 2009). Tobacco plants were transformed with an isoprene synthase gene (IspS) extracted from poplar (*Populus alba*) to obtain plants emitting isoprene at levels comparable to a naturally emitting species (Vickers *et al.*, 2009).

2.1.1 *Populus x euroamericana*

Populus is a genus of woody deciduous flowering plants in the *Salicaceae*. It is a slender plant, which can reach heights of 25-30 m. Poplar trees are native of the Northern Hemisphere. Western balsam poplar (California poplar) was the first tree to have its full DNA coded in the year of 2006. Poplars are large emitters of isoprene with rates often exceeding 5-10% of the carbon fixed photosynthetically. The emission of isoprene is widespread in poplar species, with no reported exceptions (Kesselmeier & Staudt, 1999). We used *populus* x *euroamericana* for our experiments, it is an hybrid born from crosses between black European poplar (*Populus nigra*) and American black poplar (*Populus deltoides*), also known as Canadian poplar which can grow up to 35 cm in diameter in just 10 years, reaching a height of about 30 m.

One-year-old *Populus* x *euroamericana* saplings, propagated from physiologically mature trees growing in a clonal provenance trial in Italy, were transplanted before budburst into 6 dm^3 plastic pots containing standard potting compost (sand:peat:loam mixture 1:5:3) and grown outdoor near Monterotondo (RM), Italy (42.1° N latitude; 123 m above sea level), under natural sunlight conditions. The plants were regularly watered to pot water capacity and fertilized with Hoagland solution once a week in order to supply mineral nutrients at free access rates.

2.1.2 *Quercus ilex* L.

Quercus ilex is an oak (i.e., holm oak) in the *Fagaceae*. The genus *Quercus* is native to the northern hemisphere, and includes deciduous and evergreen species extending from cool temperate to tropical latitudes in Asia and the Americas. *Quercus ilex* is an evergreen species developing in shrub and forest communities. Holm oak is widely distributed in the Mediterranean basin, with the exception of Egypt, particularly in the west, i.e. Italy to the iberian peninsula to Morocco and Algeria. It is a tree of medium size (up to 30 m). It can be found easily in the plane of the evergreen bush and, where conditions permit, up to 700-1000 m above sea level, where it forms pure woods (oak forests) or mixed (thickets) with pine, cork oak, arbutus, mock privet, laurel, mastic, oak, flowering ash, and other plants.

In Italy, holm oak is particularly common in the islands and along the Tyrrhenian and Ionian coasts. On the Adriatic coast populations are more sporadic. Small populations are also present in the Po valley along the shores of lakes, the Euganean hills, in Friuli and in Mesola wood in Ferrara.

Quercus ilex is a strong emitter of monoterpenes despite the absence of storage organs (Staudt *et al.*, 1993). *Quercus ilex* saplings were potted into 10 dm^3 pots containing commercial soil. All saplings were grown outdoor in Monterotondo Scalo (RM), Italy (42°04´N; 12°36´E) under natural sunlight conditions, regularly watered to pot water capacity when needed and fertilized with a supply of mineral nutrients at free access.

2.1.3 *Citrus reticulata*

Citrus reticulata (mandarin) is a citrus in the *Rutaceae*, commonly known as citrus family. Mandarin is a small, sometimes spiny, tree with slender branches and lance shaped shiny evergreen leaves. Mandarin is native to South-East Asia, and grows up to 7 meters in height. The lanceolate to ovate-lanceolate leaves are dark green and will reach up to 4 cm long by half as wide. Spines will reach up to 5 cm long and are very rigid and sharp. Mandarin is a monoterpene emitter with a large storage pool.

Citrus reticulata saplings were potted into 6 dm^3 pots containing commercial soil. All saplings were grown in a Percival AR-95 HIL growth chamber (CLF PlantClimatics) of the University of Tartu (Estonia), under controlled conditions of 600 μmol m^{-2} s^{-1} photon flux density, 25°/20°C of day/night temperature, 65% of relative humidity, and 12 h of photoperiod. Pots were regularly

watered to pot water capacity when needed and fertilized with a supply of mineral nutrients at free access.

2.1.4 *Nicotiana tabacum* L.

Seeds of wild-type and transgenic tobacco plants were provided by Vickers of the Department of Biological Sciences, Essex University, England. Wild-type *Nicotiana tabacum* plants do not naturally emit isoprene as they lack the isoprene synthase gene which converts dimethylallyl diphosphate (DMADP) to isoprene and pyrophosphate (Silver & Fall, 1991; 1995). Vickers *et al.* (2009) engineered *Nicotiana tabacum L.* plants with an isoprene synthase gene extracted from poplar (*Populus alba*), obtaining isoprene-emitting plants at levels comparable to a naturally emitting species. They produced and screened several different lines of emitting plants. After screening three strong isoprene-emitting lines, we finally chose for our experiments the line 6 as it resulted the best emitter.

Tobacco plants (*Nicotiana tabacum* cv. Samson NN and transgenic lines derived from this genotype) were potted in 3.6 dm^3 pots containing standard potting compost (sand:peat:loam mixture 1:5:3) and maintained in a controlled greenhouse at 20-25 °C and 40-50% relative humidity (RH) near Monterotondo (RM). Plants were regularly watered to pot water capacity and fertilized with Hoagland solution once a week in order to supply mineral nutrients at free access rates.

2.2 Gas exchange and chlorophyll fluorescence measurements

Photosynthetic rate (A), leaf intracellular CO_2 concentration (C_i), stomatal (g_s) and mesophyll (g_m) conductance, were measured with an infrared analyser (IRGA) (Licor 6400XT, Lincoln, NE, USA), portable photosynthesis and fluorescence system for *Quercus ilex*, *Populus x euroamericana* and *Nicotiana tabacum*. Full expanded leaves were enclosed into a 2 x 3 cm cuvette with transparent Propafilm® top window with an unfiltered gallium arsenide phosphide PAR (photosynthetic active radiation) sensor placed in the chamber close to the leaf lamina.

Synthetic air, free of contaminants and pollutants, was provided to the leaf in the cuvette. It was controlled through a mass flow controller which allowed us to mix nitrogen, oxygen and carbon dioxide simulating the ambient gaseous conditions (20% O_2, 80% N_2, 380 µmol mol^{-1} CO_2), but not containing isoprenoids. The air flowed in the cuvette at a rate of 0.5 L min^{-1}. Relative humidity (RH)

was controlled by circulation of water from a thermostated water bath and maintained between a range of 45%-60%. Leaf temperature was set at 30°C and kept stable during all measurements. Chlorophyll fluorescence was measured with a pulse-amplitude-modulated fluorometer (MINI-PAM, Heinz Walz GmbH, Effeltrich, Germany) simultaneously to gas exchange measurements by putting the sensor a fibre optic was mounted to the top of the cuvette at an angle of 45°. The optic fibre was placed about 1 cm from the leaf without shading it.

Gas exchange measurements for *Citrus reticulata* were performed in the University of Tartu (Estonia). An open two-channel gas-exchange system described in detail in (Copolovici & Niinemets, 2010) with a thermostatted 1.2 L glass chamber was used. The gas flow rate through the system was 1.4 L min^{-1}, CO_2 and H_2O exchange was monitored with an infrared dual-channel gas analyzer operated in differential mode (CIRAS II, PP-systems, Amesbury, MA,USA). One leaf was enclosed in the chamber. The CO_2 concentration inside the chamber was 385 ± 10 μmol mol^{-1}, leaf temperature was set to 30 °C and relative humidity at 60%. The photosynthetic quantum flux density was varied between 0 and 900 μmol m^{-2} s^{-1}. For the blue light experiment the source from IMAGING-PAM *M-Series* (Heinz Walz GmbH, Effeltrich, Germany) was used.

2.2.1 Mesophyll conductance calculation

Mesophyll conductance to CO_2 was calculated using the *variable J method* (Harley *et al*., 1992) as

$$g_m = \frac{A}{\frac{C_i - \Gamma^* \left(J_f + 8(A + R_d)\right)}{J_f - 4(A + R_d)}}$$

where J_f was calculated as: $J_f = {}^{\Delta F}/_{F_m} \cdot PPFD \cdot \alpha \cdot 0.5$, where ΔF/F'm is the photosystem II operating efficiency, PPFD is the photosynthetic photon flux density, α is the overall leaf absorbance measured with an integrating sphere (LiCor 1800-12S) throughout the experiment and the factor 0.5 was chosen because we assume that light is equally distributed between the two photosystems (Loreto *et al*., 1994). Measurements of dark respiration (R_d) were made after maintaining leaves in darkness for 10 minutes. The CO_2 compensation point to photorespiration (Γ^*), calculated with the Rubisco specific factor estimated by Galmés *et al.* (2005), was used in the gas exchange algorithms for hybrid poplar, tobacco, and holm oak respectively. Because Γ^* is a remarkably conservative parameter (Harley *et al*., 1992) we assumed that the value used in the gas

exchange algorithm did not affect the estimation of g_m. Although diffusion leaks through chamber foam gaskets were taken into account and corrected for according to the manufacturer's suggestions (Li-Cor Inc., 2004), it was impossible to rule out the occurrence of other measurement errors (Flexas *et al*., 2008). However, Centritto *et al*. (2009) have recently calculated g_m by using both the variable *J* method and carbon isotope discrimination in recently synthesized sugars in water-stressed rice genotypes, and found that the two methods yielded congruent estimations of g_m, confirming the reliability of the technique based on simultaneous measurements of gas exchange and fluorescence parameters.

2.3 Proton Transfer Reaction-Mass Spectrometry (PTR-MS)

Biogenic volatile organic compounds (BVOCs) emitted were monitored online by connecting the outflow of a cuvette to a proton transfer reaction-mass spectrometer system (PTR-MS, Ionicon, Innsbruck, Austria). Details on the theory and practice of the PTR-MS technique are reported by Lindinger *et al.* (1998). Briefly, PTR-MS utilises low-energy chemical ionisation to simultaneously monitor a suite of volatile organic compounds in air. Proton transfer from H_3O^+ is a soft ionization method (keeping fragmentation rates rather low as compared to e.g. electron impact ionization often used in GC-MS instruments) thus minimizing coincidences in the mass spectra and improving the identification capability. Samples do not need to be prepared before the measurement thus whole-air samples can be introduced directly into the drift tube allowing for VOC flux measurements. The response time of the instrument is less than 100 ms and the sensitivity is 5-10 ppb.

It is necessary to create ions since these charged particles can be steered and filtered using magnetic and electric fields and radio frequencies (Fig. 2.1). The protons are generated from water. Protonated water H_3O^+ interacts with the gas (*R*) to be analysed. During this interaction a proton transfers from the hydronium to the gas molecule, which leads to a protonated and therefore ionized molecule (RH^+) and a neutral water molecule (H_2O). This process generates a high concentration of H_3O^+ ions as well as protonated water as dimers $(H_2O)_2H^+$ and trimers $(H_2O)_3\ H^+$ that are detected at *m/z* 19, 37 and 55, respectively. The proton transfer reaction is energetically possible for all VOCs with a proton affinity higher than that of water (166,5 kcal/mol) while it doesn't occur to the major components of air (N_2, O_2, CO_2) due to their lower proton affinity.

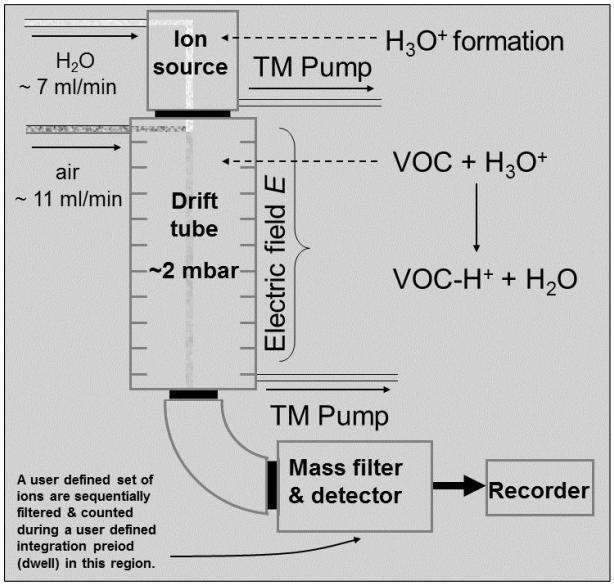

Figure 2.1: Schematic view of the PTR-MS (*PTR-MS tutorial, Lancaster University, 2004)*.

H_3O^+ ions are produced in a hollow cathode discharge ion source from pure water vapour, in such a high purity that no mass filter is needed to preselect the hydronium ions. The ion source includes a small drift region where water molecules collide with other ions (H_3O^+, O_2^+, H^+, etc.) producing more H_3O^+ ions. Most of the water vapour in the ion source is removed by a turbo-molecular pump that also produces the pressure condition needed. The reagent ions are driven by an electric field and they enter the drift tube through a Venturi inlet, through which the sample air is pumped. In the drift tube sample air and H_3O^+ ions are mixed at 2 mbar pressure.

An important factor influencing fragmentation and clustering is given by the *E/N* ratio (*E* is the electric field; *N* is the number density of the gas in the drift tube) and it is expressed in units of Townsend (1 Td = 10-17 $cm^2\ V^{-1}\ s^{-1}$). We set the ionization energy in a range of 120 - 140 Td as it is a good compromise to avoid production of water clusters on the one hand and breaking up of product ions due to collisions with neutral species in the drift tube on the other hand. E/N can be modulated between 80 and 140 Td. Low ionization energy promotes the formation of $H_2O{\cdot}H_3O^+$ ions that become the main source for H^+ transfer. It is necessary in the analysis of some compounds like alcohols, esters and esters from membrane lipids that have more affinity for $H_2O{\cdot}H_3O^+$ than for H_3O^+ ion. Only a small amount of energy is transferred during the ionization process therefore fragmentation is suppressed and the obtained mass spectra are easily interpretable, but high $H_2O{\cdot}H_3O^+$ concentration can affect the relative humidity in the sample and then the analysis sensitivity. High ionization energy (140-150 Td) can be used in the need of analysing different

compounds with the same molecular weight. The higher energy transferred causes an higher fragmentation level and the resulting characteristic fragment ions are detected at a different mass. Protonated molecules are concentrated in the *Collision Dissociation Chamber* (CDC) to avoid the development of hydrate complexes (*clusters*) and then they are selected in a quadrupole (MS) against their characteristic *m/z* ratio. The CDC separates the high pressure in the drift tube (2 mbar) from the high vacuum pressure in the detection region. A second turbo-molecular pump works to keep a pressure of about $2*10^{-5}$ mbar by decreasing the interaction between molecules. The detection efficiency of all ions is not the same, 100% efficiency is reached only for the molecules with a m/z of 80-100. A correction factor, typical for every quadrupole, must be calculated for molecules detection out of this range. The flux of molecules selected by the quadrupole is converted into an electric signal and then amplified by a SEM (*Secondary Electron Multiplier*). Once filtered, ions of a specified mass are counted using an ion counter that integrates the electric signal and gives the result in *count per second* (cps).

The water vapour flow into the ion source was controlled at 8 ml/min. The drift voltage was set at 600V. U2 and U3 voltages in the drift tube were set at 120V and 80V respectively. Mass spectral data were analysed with Balzers Quadstar 422 v.6.02 (Balzers AG) software and then processed utilizing Microsoft Excel (Microsoft Inc.). Several BVOCs were monitored during the experiments: methanol (*m/z* 33), involved in expansion and degradation of the cell wall (Nemecek *et al.*, 1995); acetaldehyde (*m/z* 45) derived from fatty acid oxidation as a consequence of leaf damaging (Brilli *et al.* 2011); isoprene (*m/z* 69), total monoterpenes (main fragment *m/z* 81 and protonated molecular ion of mass *m/z* 137).

BVOC concentration in the sample air can be directly calculated without calibration, starting with the expression below:

$$[BVOC] = \frac{1}{k_t} \cdot \frac{[BVOC \cdot H^+]}{[H_3O^+]}$$

where *k* is the proton transfer reaction rate coefficient and *t* is the reaction time. Alternatively the instrument can be calibrated with a gasses of known concentration. Considering that using the above formula can take to errors caused by the difficult on valuing some parameters, we used certified standard gasses to evaluate quantitatively the PTR-MS signal.

2.4 Gas Chromatography - Mass Spectrometry (GC-MS)

PTR-MS measurements were validated with a gas chromatography-mass spectrometry (GC-MS) system. Compounds were thermally desorbed from tenax tubes (Agilent) at 275°C for 10 min and cryo-focused in a cold trap at -10°C and desorbed worming up to 300°C with max heating rate (°C/sec). Then, desorbed compounds were injected trough a transfer line in a MS-5HP column with an internal diameter (id) of 0.25 mm (J&W Scientific USA, Agilent Technologies). The column temperature was held first at 40°C for 1 min, increasing to 210°C at a rate of 5°C/ min and rising to a final temperature of 250°C at a rate of 20°C/min. The carrier gas was helium with constant pressure.

The samples were analysed by Gas Chromatography–Mass Spectrometry (GCMS-MSD 5975C, Agilent). Terpenes were identified comparing the retention time and the fragmentation spectra with respect to those obtained by the chromatograms of pure standard. Terpenes were then quantified from the corresponding peak area using, for each compound of interest, a calibration curve.

2.5 BVOC quantification in *Citrus reticulata*

Measurements of gas exchange and isoprenoid emissions were carried out on leaves exposed to either actinic or blue light using an open two-channel gas-exchange system described in detail in (Copolovici & Niinemets, 2010). *A* and stomatal conductance (g_s) were measured on whole-leaves of mandarin enclosed in a thermostatted 1.2 dm^3 glass chamber. The gas flow rate through the system was 1.4 L min^{-1}, and CO_2 and H_2O exchange was monitored with an infrared dual-channel gas analyzer operated in differential mode (CIRAS II, PP-systems, Amesbury, MA,USA). The CO_2 concentration inside the chamber was 385 ± 10 μmol mol^{-1}, leaf temperature was set to 30 °C and relative humidity at 60%. The photosynthetic quantum flux density was varied between 0 and 900 μmol m^{-2} s^{-1}. The actinic light was provided by four 50 W halogen lamps, whereas the blue light was provided by from IMAGING-PAM M-Series (Heinz Walz GmbH, Effeltrich, Germany). Leaves were exposed to a synthetic air flux, free of contaminants and pollutants, comprising N_2, O_2 and CO_2 in atmospheric concentrations (80%, 20% and 380 ppmv respectively). To measure isoprenoid emissions, the chamber outflow was diverted into a silcosteel cartridge packed with 200 mg of Tenax (Markes International Limited, UK). A volume of 2 L of air was pumped through the trap at a rate of 200 mL min^{-1}. The measurements were replicated four times.

The silcosteel cartridges were analysed by using an Shimadzu 2010 plus GC-MS instrument (Shimadzu Corporation, Kyoto, Japan) as presented in detail in Toome *et al*. (2010). The GC was supplied with a Shimadzu TD20 automated cartridge desorber. The GC was equipped with a splitless injector and a ZB-624 capillary column (60 m in length, 320 µm i.d. and 1.8 µm film thickness) (Zebron, Phenomenex, Torrance, CA, USA). Helium was used as carrier gas. The concentration of each volatile was calculated by comparison with the peak area of a gaseous standard. The GC-MS was calibrated using authentic standards (GC purity, Sigma-Aldrich, St. Louis, MO, USA). The compound identification was made using the NIST spectral library and based on retention time identity with the authentic standards. GC peak retention time was substantiated by analysis of parent ions and main fragments on the spectra. The absolute concentrations of VOC's were calculated based on an external authentic standard consisting of known amount of VOCs.

2.6 Leaf reflectance measurements

Leaf reflectance was measured by using a portable spectrometer (ASD FieldSpec 3, Analytical Spectral Devices, Inc., USA), operating in the spectral range between 350 to 1025 nm. The instrument scans the visible and near infrared spectrum, measuring in parallel intervals of 1.4 nm and estimating values of irradiance at singular wavelengths through a linear interpolation routine. The spectrometer was equipped with an optic fibre probe with an angular field of view of 25°. The instrument automatically calculates the reflectance value as a ratio between the incident radiation reflected from the surface target and the incident radiation reflected by a reference panel (Spectralon, Labsphere, Inc., USA). This material can be regarded as a Lambertian reflector. In our experiments, reflectance spectra were collected from a distance of 5 cm from the adaxial leaf surface with an angle of 60°, to avoid shading the analysed leaf surface. Each spectral signature was recorded as the average of 100 scans to reduce instrumental noise; a further check of the stability of the reflected signal was performed by measuring the reflectance of the reference panel at the beginning and end of each session. Reflectance spectra were pre-processed by using the ViewSpecPro (ASD) software. Photochemical Reflectance Index (PRI) was calculated as:

$$PRI = \frac{R_{531} - R_{570}}{R_{531} + R_{570}}$$

(Gamon *et al.*, 1990; Gamon *et al.*, 1997), where R531 is the reflectance at 531 nm, that is affected by the xanthophyll cycle status, and R570 indicates reflectance at 570 nm, that is a reference waveband that normalizes the value of the index.

2.7 Light System

Light was provided by a computer controlled light system that combines four lamps with 4 cm^2 arrays of RGBA LEDs (ENFIS Ltd, Swansea, UK), able to generate different monochromatic lights within the visible spectrum as well as actinic light and also allowing to specify an opacity value for a colour. This system is driven by LE Sentinel V2.2 software that allows to set proportions of the three main light compounds (red, green and blue) as well as the opacity and the intensity. To enable measurements of blue light-saturated photosynthetic rates, illumination of the leaf cuvette by RGBA LEDs was supplemented with light provided by an auxiliary blue light led lamp (450-475 nm) (Vinci Fine Instruments, Monterotondo, Italy).

The light system is combined with a lamp with 4 cm^2 arrays UV-A LEDs (100 W, peak wavelength at 365 nm, range 335-395 nm), which intensity can be also controlled by the same software of RGBA LEDs and the UV-A intensity at leaf level was measured with a quantum-photoradiometer and thermometer DO 9721 (Delta Ohm S.r.l., Italy) equipped with a LP 9021 UV-A radiometric probe. An Manhood 31 UV-B lamp (Vinci Fine Instruments, Monterotondo, Italy) was used in the experiments with tobacco. The UV-B lamp has a spectral distribution in range of 250-365 nm, with a peak wavelength at 310 nm, including, therefore, also a small portion of UV-A radiation. Because it was not possible to modulate the UV-B intensity, the amount of the UV-B radiation reaching plants was determined in function of the their distance from the light source. A DO 9721 photoradiometer equipped with a LP 9021 UV-B probe was used to measure the UV-B radiation reaching leaves.

2.8 Biochemical analysis

Leaf samples were immediately plunged into liquid nitrogen in order to stop any biological activity and then stored at -80 °C until used. Biochemical measurements included the determination of reduced ascorbate (ASC), dehydroascorbate (DHA), total glutathione, oxidized glutathione (GSSG). In order to prepare the leaf extracts used in the determination of the antioxidant defence activities, leaf tissue (about 100 mg wet weight) was dissolved in 1,5 ml 3% perchloric acid acid, and the mixture was centrifuged (5000 rpm, for 20 min) at 4°C. The pH was adjusted to 7 by adding 300-400uL of a sodium carbonate solution. ASC/DHA content was determined using the spectrophotometer method described by Takahama & Oniki (1992). For ASC, initial absorbance of a 50 μL aliquot of extract was measured at 265 nm in 100 mM K-phosphate buffer (pH 6.1), then measured again 1 min after the addition of ascorbate oxidase (1 U mL^{-1}). DHA content was determined in another 50 μL aliquot. Initial absorbance was recorded as for ASC, and then the sample was measured again following the addition of 2 mM DL-dithiothreitol (DTT). An extinction coefficient of 14 $mM^{-1}cm^{-1}$ for ASC at 265 nm was used in calculations (Nakano and Asada 1981). Total glutathione was determined enzymatically. A 0.39 mL of 100 mM phosphate buffer (pH 7.4) containing 5 mM EDTA, 0.025 mL of 10 mM DTNB, and 0.08 mL of 5 mM NADPH was added to 0.05 mL of leaf extract solution. After equilibration for 3 minutes at 25°C, the reaction was started by adding 2 units of glutathione reductase. The formation of 2-nitro-5-thiobenzoic acid was continuously recorded at 412 nm with a UV-vis spectrophotometer. The total amount of glutathione in the samples was determined from a standard curve obtained by plotting the known amount of GSH versus the rate of change of absorbance at 412 nm. Samples for GSSG determination were

incubated at room temperature with 20 µl of 4-vinyl pyridine per 1000 µl sample for 1 h. Incubation with 4-vinyl pyridine conjugates any GSH present in the sample so that only GSSG is recycled to GSH without interference by GSH.

The full experimental system is summarized in Fig. 2.2.

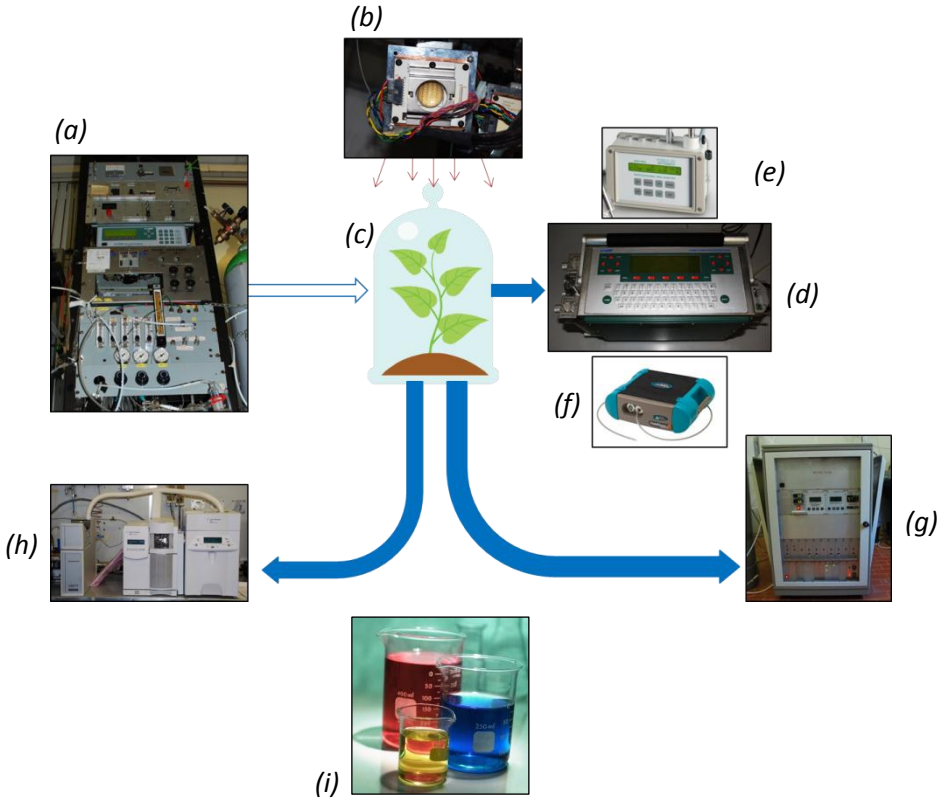

Figure 2.2: Schematic representation of the experimental system used: *(a)* synthetic air generator, *(b)* light system, *(c)* plant cuvette, *(d)* infrared gas analyser, *(e)* fluorometer, *(f)* spectroradiometer, *(g)* PTR-MS, *(h)* GC-MS, *(i)* biochemical analysis.

2.9 *In situ* estimation of a flavonoid and chlorophyll index with a portable fluorimetric sensor

Estimation of flavonoids and chlorophylls was performed by Dr. Giovanni Agati in the Institute of Applied Physics (IFAC) of CNR in Sesto Fiorentino (Italy). Epidermal flavonoids were optically estimated in vivo using the portable fluorimetric sensor Multiplex 3 (Mx) (FORCE-A, Orsay, France) (Ben Ghozlen *et al.*, 2010; Agati *et al.*, 2011). MS measures the chlorophyll (Chl)

fluorescence in both the red, at the 680-690 nm (RF), and far-red, at 730-780 nm (FRF), bands sequentially excited at 4 different excitation wavelengths in the UV-A (385 nm), and in the blue (460 nm), green (525 nm) and red (625 nm) spectral regions. The fluorescence signals were integrated over a 8 cm circular area. For the present experiment, the Chl fluorescence signals FRFR, excited with red (R) light, and FRFUV, excited with ultraviolet (UV) radiation, were used to calculate the flavonoids (FLAV) and chlorophyll indices in the first layers of mesophyll as: FLAV = FRFR/FRFUV, and CHL = FRFR/RFR, where RFR is the red emission excited under red light. The origin of these indices is detailed elsewhere (Bilger *et al.*, 1997; Buschmann, 2007; Ben Ghozlen *et al.*, 2010). The adaxial and abaxial surfaces of newly developed leaves (three individual leaves per plant) were measured once a week from middle of July 2010 to middle of September 2010 on three different plants.

2.10 Statistics

The data presented are means of minimum five measurements made on different plants ± S.E. When needed, means were statistically analysed by ANOVA test, and then they were grouped by significantly homogeneous subsets.

CHAPTER 3 – Effect of UV-B radiation on wild-type and transgenic *Nicotiana tabacum* plants.

3.1 Introduction

The depletion of the stratospheric ozone over the past several decades has resulted in enhanced levels of ultraviolet-B (UV-B) radiation (280-320 nm) reaching the biosphere (Gwynn-Jones *et al.*, 1999; Madronich *et al.*, 1998). Although the production and consumption of ozone-depleting chemicals (e.g. Chlorofluorocarbons (CFCs), halons) has fallen as a result of Montreal Protocol, CFCs can remain in the upper atmosphere with a half-life ranging from 50 to 150 years. Thus, decreased ozone levels could not be recovered to the pre-1970 levels until 2050 (WMO, 1995; UNEP, 2002).

UV-B radiation has many direct and indirect effects on plants. Damage may occur in different ways, including the direct destruction of the genetic material DNA, deactivation of enzymes, disruption of membranes, denaturation of several cell structures and generating highly reactive chemical agents. (Teramura & Sullivan, 1994). Although biological repair mechanisms exist, mutations may remain as errors in the repair processes and, in addition, the repair mechanisms themselves may be deactivated by high UV doses (Mpoloka, 2008). Reductions in photosynthetic rate and capacity because of the sensitivity of photosynthetic machinery have been described for a number of tree species (Albert *et al.*, 2008). Adverse physiological and developmental effects may well be brought about by UV-B indirectly through alterations to the production and concentrations of plant hormones and other growth regulators. Furthermore, UV-B acts as an additional stress exacerbating the detrimental effects caused, for example, by drought, soil contamination or disease (Rozema, 1997).

Because UV-B radiation can damage the photosynthetic apparatus, UV-B can also alter the secondary metabolism of plants (Wellmann, 1983; Caldwell & Flint, 1994) including biogenic volatile organic compound (BVOC) emissions. Studies on the interaction between UV radiation and BVOC emission showed contrasting results. Tiiva *et al.* (2007) showed that isoprene emissions on mixed-species subarctic peatland ecosystem appear to be higher under enhanced UV-B radiation during the warm periods of the second growing season, and at the end of the fourth growing season, but they did not find significant UV-B effect on the emissions during the warm period in the third

growing season. Harley *et al.* (1996) found that isoprene emission on *Mucuna pruriens* was not affected by chronic exposure to UV-B.

There is an increasing body of literature concerning the role of isoprene in relieving environmental stresses (see for a review: Vickers *et al.*, 2009). As a tool for studying the role of isoprene, Vickers *et al.* (2009) transformed tobacco (*Nicotiana tabacum* L.) with isoprene synthase gene extracted from *Populus alba* and obtaining plants emitting isoprene at levels comparable to a naturally emitting species. They demonstrated that isoprene emission plays a protective role against several abiotic stresses like heat, combined heat/light and ozone-induced oxidative stress, allowing plants to respond better to oxidative stress caused by ozone, minimizing potential damage and preventing the over-accumulation of ROS. The mechanisms by which a protective function of isoprene is achieved is still unclear, but the most likely explanation is that because isoprene is embedded in the organelle membranes it can increase their stability by preventing membrane lipid denaturation following oxidative stress (Sharkey & Singsaas, 1995; Loreto & Velikova, 2001; Sharkey, 2005).

In this study, wild-type and transgenic isoprene-emitter plants of *Nicotiana tabacum* were exposed to acute doses of UV-B radiation in order to analyse their effects on photosynthetic parameters and BVOC emissions. Measurements were performed at several different intensities from low values of 3 W m^{-2} to strong dose of 15 W m^{-2}. Here we show only results obtained at 3 W m^{-2}, 9 W m^{-2} and 15 W m^{-2} because they considered more relevant.

3.2 Material and methods

Photosynthesis and BVOC emission were simultaneously measured in laboratory under controlled conditions: steady-state photosynthesis (A) and stomatal conductance (g_s) were measured with a LI-6400 IRGA, by enclosing a portion of a single fully expanded leaf in a 6 cm^2 cuvette with Teflon top window. A stable 300 µmol m^{-2} s^{-1} flow of synthetic air in atmospheric concentrations, free of contaminants and pollutants, was provided to the leaves. Measurements were taken on leaves exposed to saturating photosynthetic photon flux density (PPFD) of 1000 µmol m^{-2} s^{-1} of actinic light with relative humidity in the leaf cuvette ranging between 45-55%. BVOCs were online monitored by a PTR-MS connected to the exhaust line of the leaf cuvette. The PTR-MS was set in a single ion mode to record trace of protonated masses of isoprene (*m/z* 69) and methanol (*m/z* 33).

Plants were irradiated for 90 minutes by positioning the UV-B lamp perpendicularly to the full extended leaf. UV-B intensity was measured with a DO 9721 photoradiometer equipped with a LP

9021 UV-B probe. Gas exchange, BVOCs, leaf reflectance and image fluorescence were analysed before starting and at the end of the UV-B treatments, as well as 24 and 48 hours after the irradiation.

Leaf samples were collected for biochemical analyses. Samples from the fifth node (from the apical meristem) were cut and frozen immediately in liquid nitrogen. Samples (4 plants per treatment) were taken before, immediately after, and 48h after UV-B irradiation, respectively.

3.3 Results and discussion

Stratospheric ozone layer shields the entire Earth from much of the harmful ultraviolet radiation that comes from the sun. While high energy UV-C radiation is completely absorbed and low energy UV-A is weakly absorbed by stratospheric ozone, UV-B is mostly absorbed by stratospheric zone but a substantial part reaches the Earth. Over the past few decades, there has been a depletion of the stratospheric ozone layer due to emissions of halogen-containing compounds of anthropogenic origin. This has resulted in a concomitant increase in solar ultraviolet-B radiation (Mpoloka, 2008). The successful implementation of the Montreal Protocol is having a marked effect on climate change, leading to the decrease of the atmospheric concentration of ozone depleting substances (McKenzie *et al.*, 2011). However, despite the success in limiting the stratospheric ozone depletion, levels of UV irradiances that reach the Earth' surface are expected to be still elevated in the coming decades. The highest UV-B dose rate was found in Lhasa, Tibet, and it is 3.96 W m^{-2} (Norsang *et al.*, 2009). Vickers *et al.* (2009) demonstrated that isoprene emission plays a protective role against several abiotic stresses like, including oxidative stress, minimizing potential damage and preventing the over-accumulation of ROS. To investigate isoprene ipotetical role in protecting the plant against increasing UV-B radiation we performed the experiment on both wild-type tobacco (a non-emitting species) and on transgenic isoprene-emitting tobacco plants.

Tobacco plants were treated with UV-B radiation at 3, 9 and 15 W m^{-2} intensities for 90 minutes to study the UV-B dose-response relationships. Photosynthesis (Fig. 3.1) was lightly affected by exposition at 3 W m^{-2} (Fig. 3.1a), decreasing by about 31% in transgenic plants and ~17% in wild-type plants. *A* recovered partially 24 hours after the end of the treatment and almost completely 48 hours after the treatment. Similarly, reduction in photosynthesis was observed when the plants were treated with 9 W m^{-2} UV-B radiation (Fig. 3.1b), but in this case *A* did not recover 48 hours after the irradiation. Photosynthetic responses in transgenic and wild-type plants were not significantly different in both low (3 W m^{-2}) and intermediate (9 W m^{-2}) intensity treatments. In contrast,

exposition to very high intensity of 15 W m^{-2} (Fig. 3.1c) not only caused higher damages to the photosynthetic apparatus immediately after the stress, but caused a different response in emitting and non-emitting plants. Emitting plants partially recovered from the stress, while non-emitting plants experienced a persistent damage to the photosynthetic apparatus. This significant strong decline may indicate that damages at metabolic level occurred at high intensity of UV-B treatments.

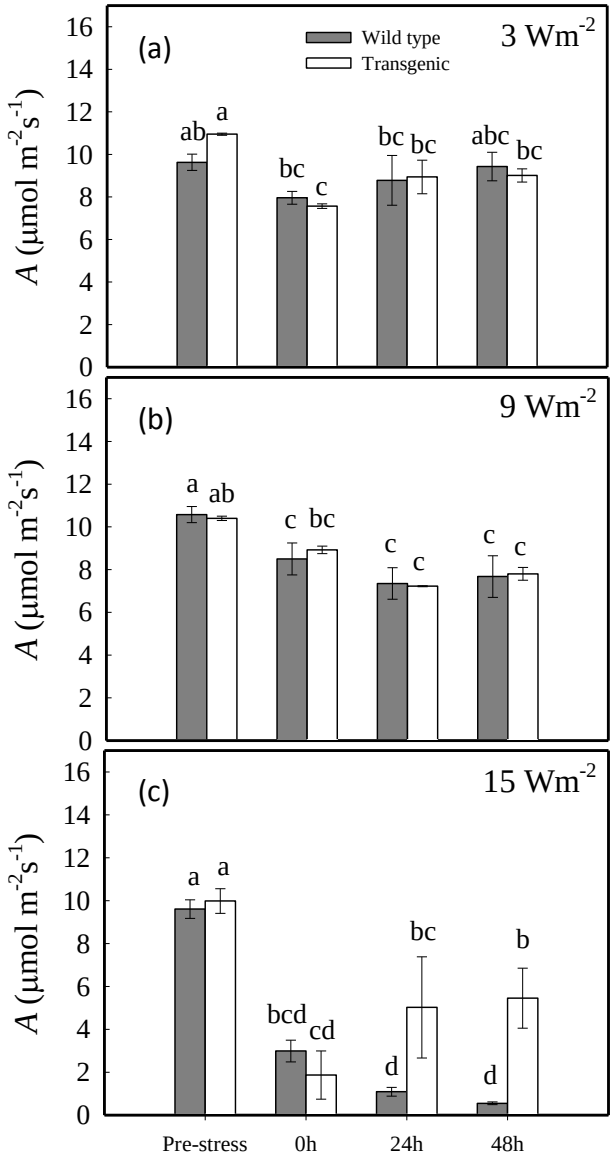

Figure 3.1: Photosynthesis in transgenic isoprene-emitter (grey bars) and wild-type plants (white bars) tobacco plants exposed to 3 (a), 9 (b) and 15 W m^{-2} (c) intensity UV-B radiation for 90 minutes. Measurements were made before, immediately after, 24h and 48h after the UV-B treatments. Results are means of 4 replicates ± 1 SEM. The letters a to d indicate differences at $P < 0.05$ in response to the UV-B treatments.

Transgenic tobacco plants were produced by transformation, inserting the isoprene synthase gene (IspS) extracted from poplar (*Populus alba*), obtaining plants emitting isoprene at levels comparable to a naturally emitting species (Vickers *et al.*, 2009). As expected, isoprene was not detected in non-emitting plants (Fig. 3.2). When treated with low intensity UV-B (3 W m^{-2}) (Fig. 3.2 a), isoprene emission remained stable. Differently, a significant decrease in isoprene emission was detected 48 hours after the treatment, when plants were irradiated with 9 W m^{-2} intensity (Fig. 3.2 b). Similarly, a comparable decrease was observed after treatment at 15 W m^{-2} (Fig. 3.2 c). In both 9 and 15 W m^{-2} treatments, decrease in isoprene emission was detected after 24 hours after the end of the UV-B irradiation.

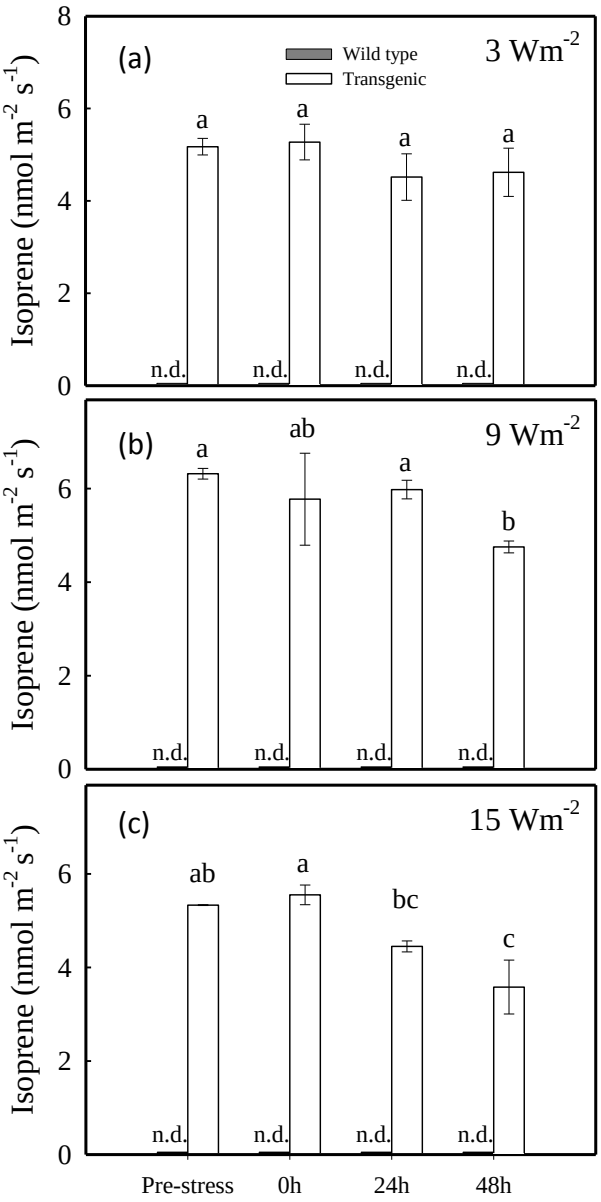

Figure 3.2: isoprene emission in transgenic isoprene-emitter tobacco plants (grey bars) and in wild-type plants (white bars) exposed to 3 (a), 9 (b) and 15 W m^{-2} (c) intensity UV-B radiation for 90 minutes. Results are means of 4 replicates ± 1 SEM. The letters a to c indicate differences at $P < 0.05$ in response to the UV-B treatments.

Methanol emission was also very responsive to increasing UV-B irradiances. Exposure at 3 W m^{-2} did not cause emission bursts from treated tobacco leaves and low basic levels of methanol remained stable during the whole experiment (Fig. 3.3 a). When plants were exposed to 9 W m^{-2} intensity UV-B, we detected a higher quantity of methanol emitted immediately after the irradiation (Fig. 3.3 b). However, the increase in emission was significant in wild-type plants only. Differently, remarkable quantities of methanol were emitted soon after exposure to the strong UV-B intensity of 15 W m^{-2} (Fig. 3.3 c). Values about 6 and 5.5 times higher than pre-stress were detected in wild-

type and in transgenic plants, respectively. Methanol emission is usually detected early in stress development as a consequences of degradation of cell wall pectins (Harley *et al.*, 2007; Loreto & Schnitzler, 2010; Niinemets, 2010). In such a way, the methanol results suggest that the strongest damages of cellular structures occurred only partially after the irradiation at 9 W m^{-2} (about twice than pre-stress level) but it occurred strongly after the maximum UV-B treatment at 15 W m^{-2}, going over about 6.5 times the pre-stress level. Methanol emission returned to basic levels after 24 hours from the treatment in any case.

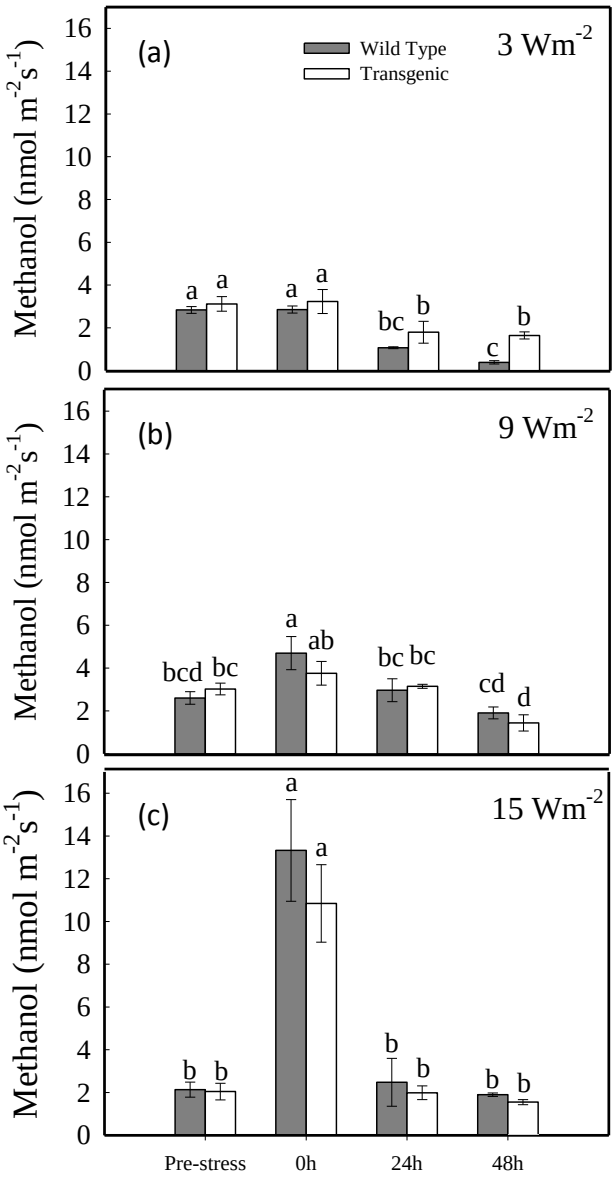

Figure 3.3: methanol emission in transgenic isoprene-emitter tobacco plants (grey bars) and in wild-type plants (white bars) exposed to 3 (a), 9 (b) and 15 W m^{-2} (c) intensity UV-B radiation for 90 minutes. Results are means of 4 replicates ± 1 SEM. The letters a to d indicate differences at $P < 0.05$ in response to the UV-B treatments.

Fluorescence analysis were performed before and after the treatment (Fig. 3.4). We calculated the maximum efficiency of PSII, that is well described by the ratio of variable to the maximum chlorophyll fluorescence (Fv/Fm), and the quantum efficiency of linear electron transport through

PSII (ΔF/Fm') (Genty *et al.*, 1989), that is widely used to examine perturbations of photosynthetic performance (Baker & Rosenqvist, 2004).

The 3 W m^{-2} UV-B treatment did not affect the photosynthetic machinery (Fig. 3.4 a,d): both ΔF/Fm' and Fv/Fm maintained stable values during the treatment, before and after the exposition to UV-B radiation. Similarly, at 9 W m^{-2} UV-B intensity chlorophyll fluorescence parameters did not change significantly during the whole experiment duration (Fig. 3.4 b,e). In contrast, the very high UV-B intensity of 15 W m^{-2} affected leaf photochemistry differently in wild-type and transgenic plants: maximum efficiency of PSII (Fv/Fm) did not change significantly in emitting plants, while it significantly dropped 24 hours after the treatment in wild-type plants. Whereas, PSII operating efficiency (ΔF/Fm') level was reduced soon after the 15 W m^{-2} UV-B exposition in both plant types (Fig. 3.4 c,f) and was significantly inhibited 48 hours after the treatment in wild-type plants, while it recovered to the pre-stress levels in transgenic plants.

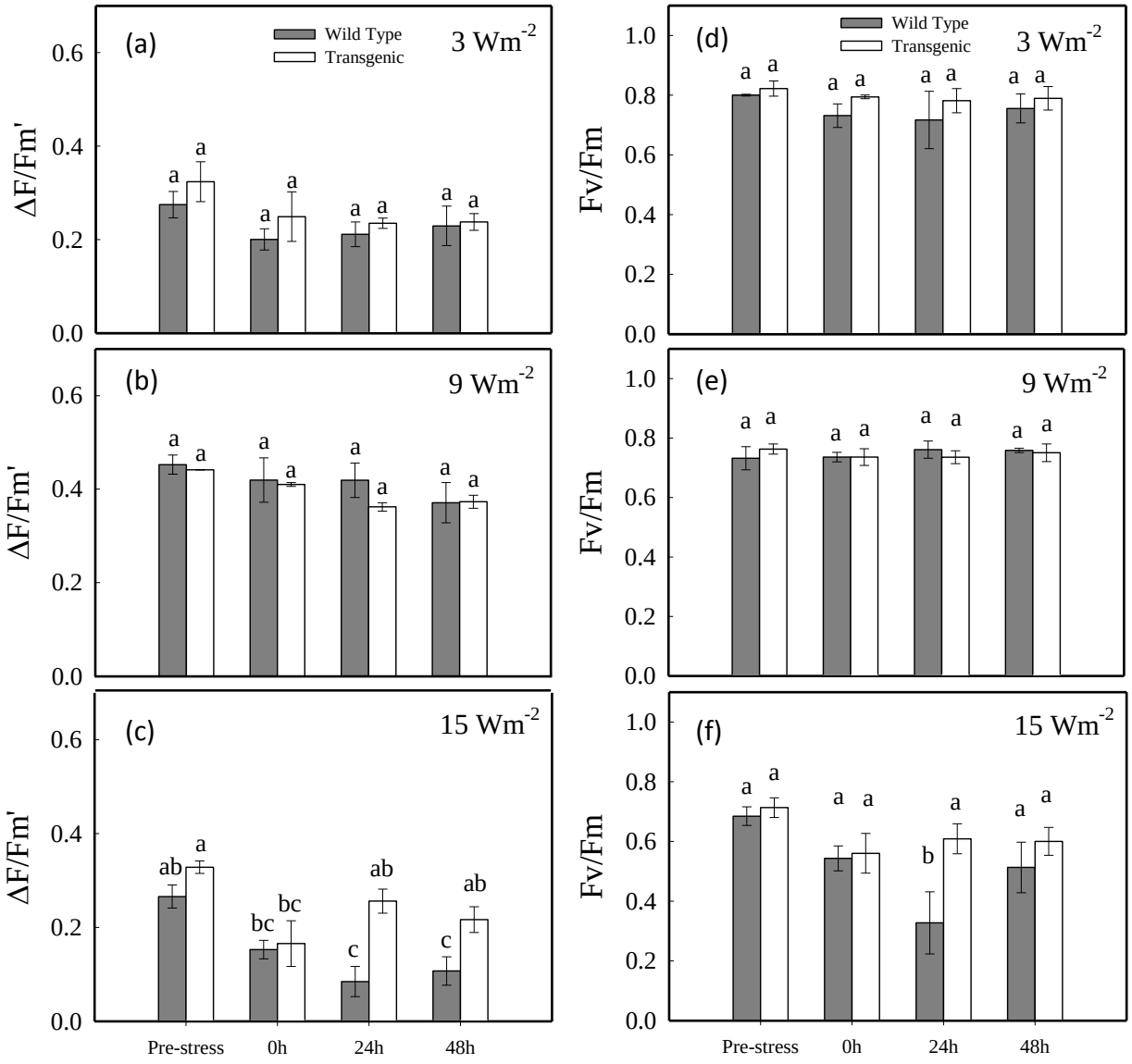

Figure 3.4: Fluorescence measurements in transgenic isoprene-emitter tobacco plants (grey bars) and in wild-type plants (white bars): PSII operating efficiency (ΔF/Fm') and maximum quantum yield (Fv/Fm) in leaves exposed to 3 (a,d), 9 (b,e) and 15 W m^{-2} (c,f) intensity UV-B radiation for 90 minutes. Results are means of 4 replicates ± 1 SEM. The letters a to c indicate differences at $P < 0.05$ in response to the UV-B treatments.

Ultraviolet radiation affects the chemical composition of a plant and it likely induces oxidative damage in consequence of the production of reactive oxygen species (ROS) (Jordan, 1996). Oxidative stress is the consequence of an unbalance between ROS formation and the ROS scavenging capacity of the defence system. In normal and in stress conditions, the redox equilibrium and the capacity of ROS scavenging have a key role for the normal development of a plant and for the perception, signaling and adaptation to stress. To be able to scavenge the

continuously produced ROS, plants possess an efficient cooperative system formed by enzymatic and non-enzymatic antioxidants that include the metabolites from the ascorbate-glutathione cycle, ascorbate (ASC) and glutathione (GSH). ASC, beside reducing H_2O_2 to water via the ascorbate-glutathione cycle can directly scavenge superoxide, hydroxyl radicals, and singlet oxygen (Noctor & Foyer, 1998).

Ascorbate and glutathione contents were determined to test whether the water-soluble antioxidants are involved in the defence system against UV-B-induced oxidative stress in tobacco plants, and if there is any difference in the functioning of the ascorbate-glutathione cycle for ROS scavenging between non-emitting and isoprene emitting plants exposed to UV-B radiation. Isoprene is unlikely to be effective as an antioxidant in the aqueous phase, and is therefore unlikely to be able to directly scavenged H_2O_2. However, aqueous and lipid-phase antioxidant capacities are linked (e.g. through ascorbate-mediated regeneration of tocopherol moieties) (Foyer *et al.*, 2006). Thus, an increase in lipid-phase antioxidant capacity may be expected to impact on aqueous-phase antioxidant capacity (Vickers *et al.*, 2009)

Measurements of ascorbate levels were made at minimum (3 W m^{-2}) and maximum (15 W m^{-2}) UV-B treatments. Samples were collected before, immediately after and 48 hours after the UV-B exposure (Fig. 3.5). Total ASC levels were significantly influenced by UV-B (Fig. 3.5 a,b). Immediately after both treatments at 3 and 15 W m^{-2}, total ASC increased around 1.8 times and 1.5 times in transgenic and non-transgenic plants respectively and decreased to the pre-stressed levels 48 hours after the treatments. However, it is noticeable that 48 hours after the high intensity UV-B treatment total ASC in isoprene-emitting plants was 44% higher than in wild type. On the other hand, the ratio of reduced ASC to total ASC, calculated to explore the regeneration rate of ASC, resulted to be rather stable at both UV-B intensities and during both treatments (Fig. 3.5 c,d). We could observe only an slightly significant increase in non-isoprene emitting plants immediately after both treatments. There were no significant differences, however, in ASC/Total ASC ratios between isoprene emitting and non-emitting plants.

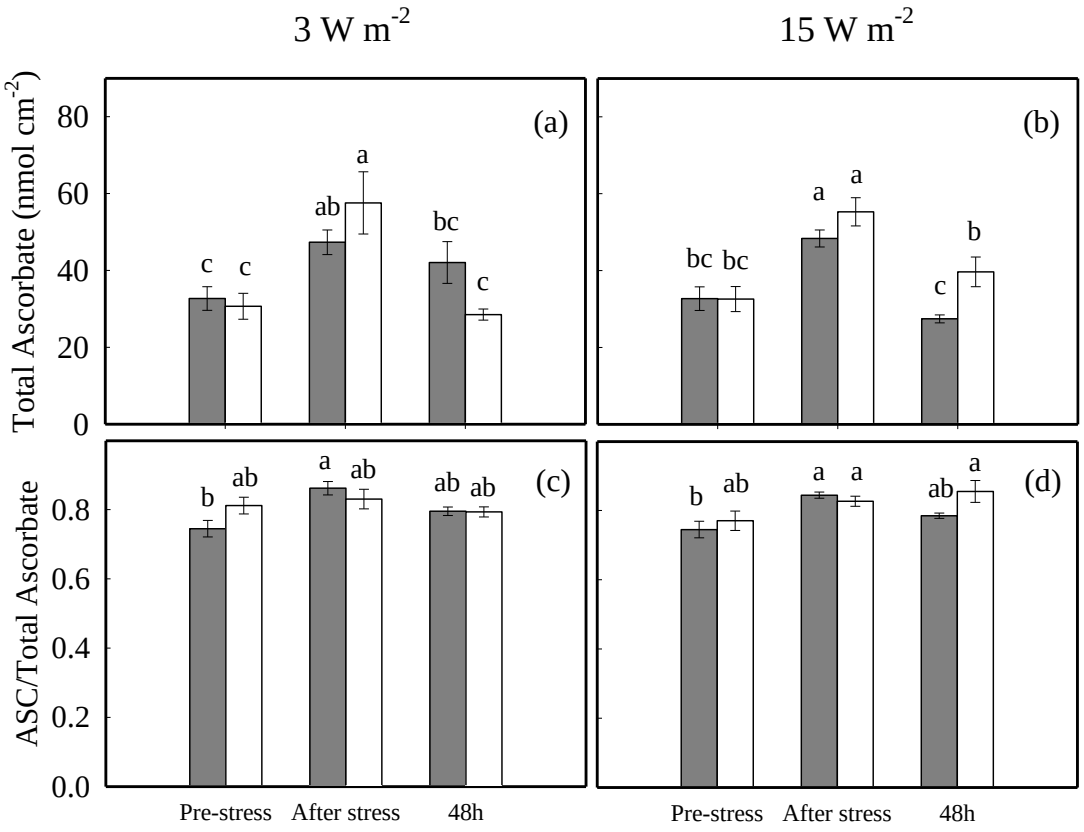

Figure 3.5: Ascorbate (ASC), dehydroascorbic acid (DHA), redox state ascorbate (ASC/ASC+DHA) and total ascorbate content (ASC+DHA) in leaves of transgenic isoprene-emitter tobacco plants (grey bars) and wild-type plants (white bars) treated with 3 W m^{-2} (a,c) and 15 W m^{-2} (b,d) intensity UV-B radiation for 90 minutes. Results are means of 4 replicates ± 1 SEM. The letters a to c indicate differences at $P < 0.05$ in response to the UV-B treatments.

Total glutathione, and reduced glutathione to total glutathione ratios were also affected by UV-B (Fig. 3.6). Total glutathione concentration on leaf area basis increased 1.4 and 1.9 times in non-isoprene emitting plants immediately after the lower and higher UV-B treatments, respectively, and 2 and 2.4 times in isoprene-emitting plants immediately after the lower and higher UV-B treatments, respectively (Fig. 3.6 a,b). However, 48h after the 3 W m^{-2} UV-B treatment values recovered to the pre-stress levels. Whereas 48h after the 15 W m^{-2} treatment, total glutathione concentrations increased further, especially in non-isoprene emitting leaves (Fig. 3.6 b), indicating a strong activation of the antioxidant defences even 48 hours after the exposure to stress. The reduced Glutathione to total glutathione ratio, an indicator of glutathione regeneration rate, responded differently to the UV-B treatments (Fig. 3.6 c,d). At 3 W m^{-2} UV-B, the reduced GSH/total GSH ratio did not change significantly in both plant types. Whereas at 15 W m^{-2} UV-B, the reduced GSH/total GSH ratio decreased of ~20% immediately after the treatment in both plant types, and there was a further ~23% decrease 48 after the treatment in non-isoprene emitting plants and ~16%

in emitting plants. This is because the magnitude of oxidised glutathione increments were higher than those of reduced glutathione increments (data not shown). The decreased in the regeneration rate of GSH 48 hours after the treatment may be an indication of the irreversibility of the oxidative stress caused by the high intensity UV-B treatment in wild type tobacco plants. Our results clearly indicate that ASC and GSH are involved in the defence of tobacco plants against UV-B-induced oxidative stress. Moreover, the induction of these antioxidants as well as their regeneration via the ascorbate-glutahione cycle seems to be independent on the capacity for isoprene emission at low UV-B radiations. At higher UV-B radiations, however, our results suggest that the capacity of isoprene-emitting leaves of maintaining higher ASC levels 48h after the stress occurrence could contribute to counteract better the UV-B-induced oxidative stress. In fact, apart from inducing direct oxidative damage, increased ROS levels trigger programmed cell death (Mittler, 2002). We speculate that the H_2O_2 levels produced during the UV-B stress did not pass the threshold required to initiate cell death processes in transgenic isoprene-emitting plants, whereas this threshold was passed in wild type plants.

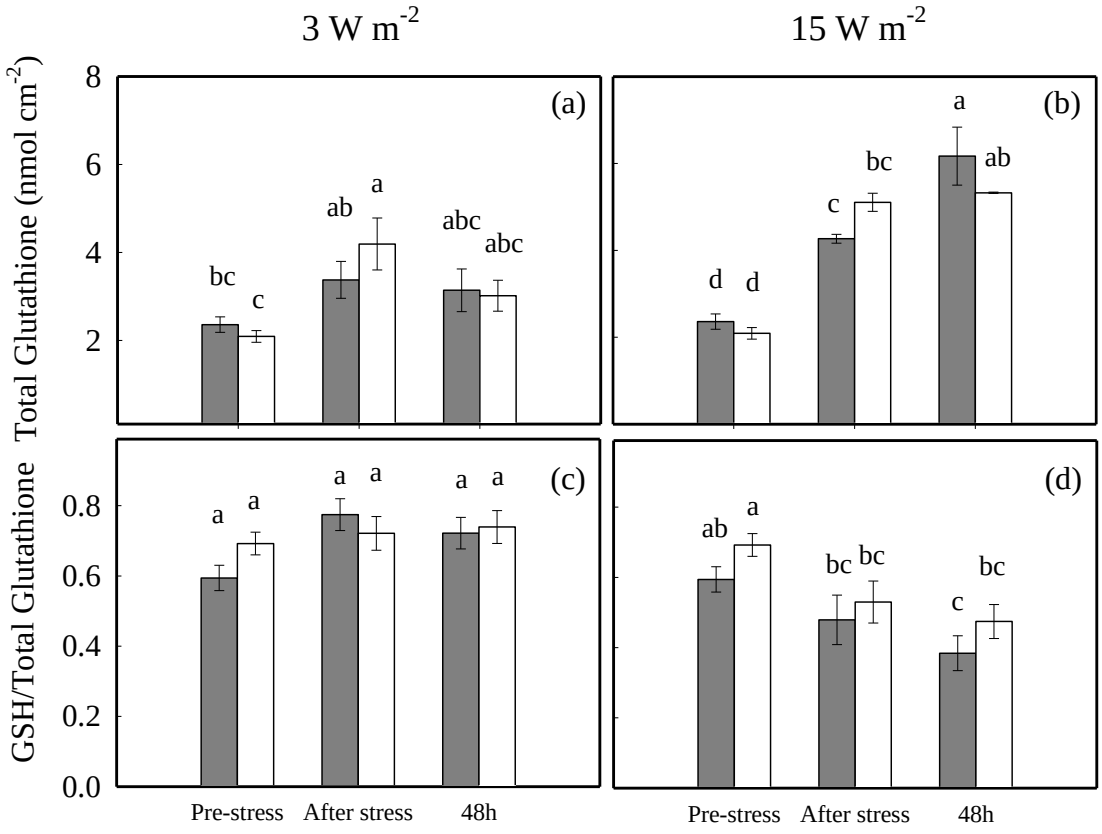

Figure 3.6: Glutathione reduced (GSH), glutathione oxidized (GSSG), redox state glutathione (GSH/GSH+GSSG) and total glutathione (GSH+GSSG) in leaves of transgenic isoprene-emitter tobacco plants (grey bars) and wild-type plants (white bars) treated with 3 W m^{-2} (a,c) and 15 W m^{-2} (b,d) intensity UV-B radiation for 90 minutes. Results are means of 4 replicates ± 1 SEM. The letters a to d indicate differences at $P < 0.05$ in response to the UV-B treatments.

In conclusion, there are evidences showing that the reduced amount of ozone in the stratosphere due to man-made CFCs results in an increase in UV-B radiation reaching the earth's surface (Mpoloka, 2008). The objective of this study was to increase the knowledge about the mechanisms of primary and secondary metabolism processes to UV-B irradiance. Our findings showed that the mechanisms regulating BVOC emissions are not very sensitive to high UV-B, because emission in transgenic plants decreased only at very high UV-B intensities. However, isoprene emission was able to mitigate UV-B damages: isoprene-emitting tobacco plants in fact showed photosynthesis recovery soon after 24 hours, proved also by PSII operating efficiency and maximum quantum yield results. Furthermore, transgenic plants experienced a reduced cell wall damage, as highlighted by a reduced methanol emission at 15 W m^{-2} intensity compared to wild-type plants. The methanol results suggest that the strongest damages of cellular structures occurred soon after the exposition to 9 W m^{-2} and especially after exposition to 15 W m^{-2}, and again transgenic plants showed less damages than wild type plants. Finally, the isoprene-induced protection was combined with an independent higher activation of the ascorbate-glutathione cycle as mechanism for ROS scavenging.

CHAPTER 4 – Response to acute UV-A radiation in *Populus x euroamericana* plants

4.1 Introduction

The amount of ultraviolet (UV) radiation reaching the Earth's surface is steadily increasing over the tropics and southern high latitudes during spring and summer (Hegglin & Shepherd, 2009). Increased UV is likely to have both direct and indirect implications on photosynthesis (Paul & Gwynn-Jones, 2003) and, in turn, on biogenic volatile organic compounds (BVOCs). These primary and secondary metabolisms are now accepted as important components of the biosphere's response to climate change (Centritto *et al.*, 2011b) and consequently there is great interest in determining their sensitivity to different radiation quality. This knowledge is particularly relevant for the biosynthesis of BVOCs, which, besides having an intriguing ecological role in emitting plants (Vickers *et al.*, 2009), are very reactive and influences both the atmospheric chemistry composition and physics.

The biosynthesis of isoprene, the most abundant BVOC released by leaves into the atmosphere (Guenther *et al.*, 1995), occurs in the chloroplast and is closely connected to photosynthesis at ambient CO2 concentration in non-stressed plants (Monson & Fall, 1989). About 90% of the carbon in the isoprene molecule originates from fresh photosynthates which are then the primary substrate for isoprene biosynthesis (Brilli *et al.*, 2007). The literature also shows that BVOC emission is uncoupled from photosynthesis under physical stresses (Brilli *et al.*, 2007; Loreto & Schnitzler, 2010; Centritto *et al.*, 2011a): because BVOCs play a role in relieving environmental stresses (Vickers *et al.*, 2009), their emission is often elicited by stress occurrence (Loreto & Schnitzler, 2010).

Studies on the impact of either acute or chronic UV radiation on BVOC emission even on a single-species scale are few and show contrasting results. The first published study was performed by Harley *et al.* (1996) on isoprene-emitting and non-emitting plant species subjected to both chronic and acute UV-B radiation (280-320 nm). They showed that chronic UV-B exposure significantly increased isoprene emission in *Quercus gambelii* but not in *Mucuna pruriens*, both strong isoprene emitting species, and did not induce isoprene emission in *Acer platanoides*, a non-emitting species. Furthermore, when *M. pruriens* and *A. platanoides* were exposed to high acute UV-B exposure, there were no apparent effects on either photosynthesis or isoprene emission. More recently, long-term effects of UV enhancement on BVOC emission were analysed on a pristine, mixed-species subarctic peatland ecosystem. Tiiva *et al.* (2007) found that enhanced UV-B significantly increased

the emission of isoprene during the warm periods of the second growing season, and at the end of the fourth growing season. On the other hand, the authors did not detect significant UV-B effect on the emissions from subarctic fen ecosystem, during the warm period in the third growing season. Then, in a follow-up study on the same subarctic peatland ecosystem, Faubert *et al.* (2010) showed no overall UV-B effect on isoprene and monoterpene emissions, apart from toluene and 1-octene. Furthermore, both Tiiva *et al.* (2007) and Faubert *et al.* (2010) found no effects of UV-A radiation (320-400 nm) on BVOC emissions from the subarctic fen ecosystem. In addition, long-term increase in UV-B radiation did not affect terpene concentration in leaf and wood of *Pinus sylvestris* and *Picea abies* seedlings (Turtola *et al.*, 2006), monoterpene and sesquiterpene emissions from *P. abies* (Blande *et al.*, 2009). Similarly, Winter & Rostás (2008) found no differences in qualitative and quantitative composition of induced volatiles in soybean grown under ambient or attenuated UV radiation.

The aim of the present study was to assess the direct effects of short-term, acute exposure to UV-A on BVOC emissions from hybrid poplar saplings. Hybrid poplar leaves were exposed to 30 W m^{-2} (a radiation intensity which usually occurs on sunny summer days), 60 W m^{-2} (which represent real-world episodic UV-A radiation stress, i.e. a bad but realistic scenario) (Abukassem & Bero, 2012), 90 and 120 W m^{-2} of UV-A irradiance. We specifically examined the effects of acute UV-A radiation on the emissions of isoprene, methanol and acetaldehyde, on carbon assimilation, photochemical reflectance index (PRI) and chlorophyll fluorescence at the same time. In particular we analysed the seasonality-dependent plasticity of responses to acute UV-A exposure, as seasonal changes in the amount of flavonoids accumulated in the epidermal tissue by determining to what extent UV-A penetrates leaves could contribute to build up protection mechanisms against harmful UV radiation. To our knowledge, there are no earlier data on seasonality and dose-dependent UV-A effects on BVOC emissions from broadleaf trees.

4.2 Material and methods

Photosynthesis and BVOC emission were simultaneously measured during two different experiments carried out in the laboratory under controlled conditions. In both experiments, poplar leaves were exposed to 30 W m^{-2}, i.e. a radiation intensity that often occurs on sunny summer days in the area where the experiment was performed and for this reason it will be referred to as ambient treatment in the remainder of the text, and increasing UV-A radiation (60, 90, and 120 W m^{-2}). A computer controlled light system (ENFIS Ltd, Swansea, UK), that combines four lamps with 4 cm^2

arrays of RGBA LEDs (140 W) generating white light with a lamp with 4 cm^2 arrays UV-A LEDs (100 W, peak wavelength at 365 and range 335-395), was used. To mimic the ambient UV-A radiation level recorded in full sunlight conditions during summertime, leaves were exposed to saturating photosynthetic photon flux density (PPFD) of 1000 µmol m^{-2} s^{-1} of white light supplemented with a fixed dose-rate of 30 W m^{-2} UV-A. The UV-A intensity at leaf level was measured with a quantum-photoradiometer and thermometer DO 9721 (Delta Ohm S.r.l., Italy) equipped with a LP 9021 UVA radiometric probe.

Photosynthesis (*A*) was measured during the UV-A treatments using a portable gas-exchange system (LI-6400, LI-COR, Lincoln, NE, USA) by enclosing a portion of a single fully expanded leaf in a 6 cm^2 gas-exchange cuvette with Teflon top window to allow the use of the external UV-A led lamp. Leaf temperature was set at 30 °C, and relative humidity in the leaf cuvette ranged between 45-55%. Leaves were exposed to a flux of synthetic air, free of contaminants and pollutants, comprising N_2, O_2 and CO_2 in atmospheric concentrations (80%, 20% and 380 µmol mol^{-1}, respectively). Leaf temperature was measured with an array of three thermocouples near to the centre and at two margins of the abaxial leaf side. Measurements were recorded when *A* was steady state.

BVOC emission was detected simultaneously with *A* by connecting the exhaust line from the leaf cuvettes to a proton transfer reaction mass spectrometer (PTR-MS; Ionicon, Innsbruck, Austria). 200 ml min^{-1} of the air flowing out of the cuvette were diverted to the PTR-MS. The PTR-MS was set in a single ion mode to record trace of protonated masses of methanol (*m/z* 33), acetaldehyde (*m/z* 45) and isoprene (*m/z* 69). The instrument was calibrated daily with gaseous standard for each compound (Rivoira, Milano, Italy) and measurements were validated with gas-chromatography analysis (Syntech Spectras BTX Analyzer GC 855, Netherland). Details on the theory and practice of PTR-MS technique are reported in Lindinger *et al.*, (1998).

The first experiment was performed between the end of June and middle of July 2010. Individual leaves of five plants were kept under ambient UV-A radiation for about two hours. Leaves were then exposed for a 20 minute period at the UV-A doses of 60, 90, and 120 W m^{-2}, respectively; each exposition period was followed by a recovery of 20 min at ambient UV-A conditions prior to further increase the UV-A doses (Fig. 4.1).

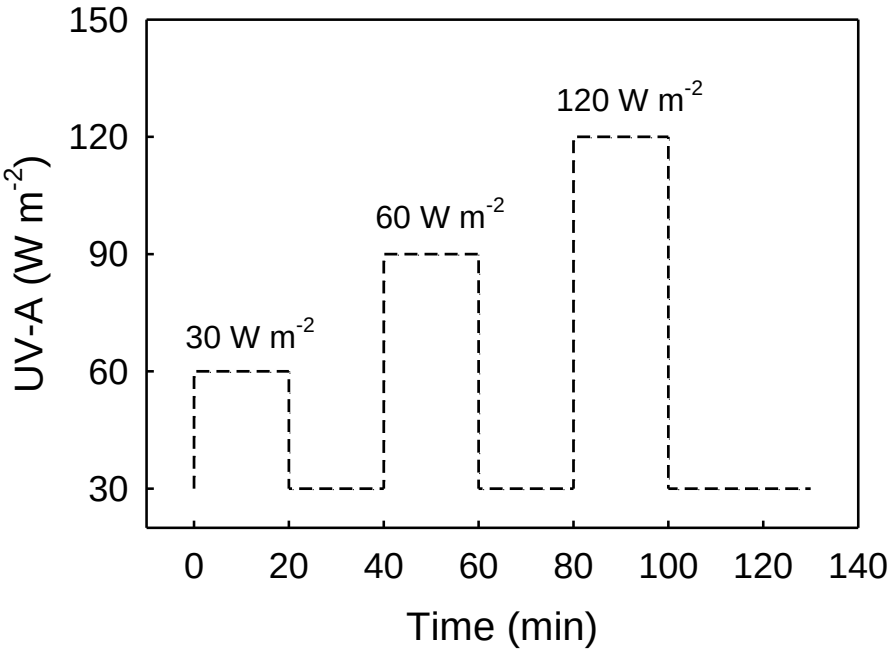

Figure 4.1: time course of acute UV-A treatments.

In parallel with all the aforementioned gas exchange measurements, leaf spectral reflectance and chlorophyll *a* fluorescence were also measured. The fluorescence yield (i.e. the quantum yield of PSII in the light, $\Delta F/F_m'$) was measured by using a saturating pulse (10,000 μmol m^{-2} s^{-1}) of white light with a pulse-amplitude-modulated fluorometer (MINI-PAM, Heinz Walz GmbH, Germany). The electron transport rate was measured by fluorescence as described by Genty *et al*. (1989). Leaf reflectance was measured by using a portable spectrometer (ASD FieldSpec 3, Analytical Spectral Devices, Inc., USA), operating in the spectral range between 350 to 1025 nm. The spectrometer was equipped with an optic fibre probe with an angular field of view of 25°. The instrument automatically calculates the reflectance value as a ratio between the incident radiation reflected from the surface target, and the incident radiation reflected by a reference panel (Spectralon, Labsphere, Inc., USA). Reflectance spectra were collected from a distance of 5 cm from the adaxial leaf surface with an angle of 60°, to avoid shading the analysed leaf surface. Reflectance spectra were pre-processed by using the ViewSpecPro (ASD) software. Photochemical reflectance index (PRI) was calculated as the $(R_{531}-R_{570})/(R_{531}+R_{570})$ ratio (Gamon et al. 1990, Gamon et al. 1997); where R_{531} is the reflectance at 531 nm, which is affected by the xanthophyll cycle status, and R_{570} indicates reflectance at 570 nm, i.e. a reference waveband that normalizes the value of the index.

The second experiment was performed in the middle of July 2010 and then repeated during the first half of the month of September 2010. Plants were exposed to ambient UV-A radiation and treated with the UV-A doses of 60, 90, and 120 W m^{-2} (5 plants per treatment), respectively, for two hours. Measurements of leaf reflectance were performed on leaves before and immediately after the exposition to UV-A radiation using the same method described above.

Epidermal flavonoids were optically estimated *in vivo* using the portable fluorimetric sensor Multiplex 3 (Mx) (FORCE-A, Orsay, France) (Ben Ghozlen *et al.*, 2010, Agati *et al.*, 2011). Mx measures the chlorophyll (Chl) fluorescence in both the red, at the 680-690 nm (RF), and far-red, at 730-780 nm (FRF), bands sequentially excited at 4 different excitation wavelengths in the UV-A (385 nm), and in the blue (460 nm), green (525 nm) and red (625 nm) spectral regions. The fluorescence signals were integrated over a 8-cm circular area. For the present experiment, the Chl fluorescence signals FRF_R, excited with red (R) light, and FRF_{UV}, excited with ultraviolet (UV) radiation, were used to calculate the flavonoid index (FLAV) as: FLAV = FRF_R/FRF_{UV}. Chlorophyll (CHL) in the first layers of mesophyll was evaluated by the CHL index: CHL = FRF_R/RF_R, where RF_R is the red emission excited under red light. The origin of these indices is detailed elsewhere (Bilger *et al.*, 1997; Buschmann 2007; Ben Ghozlen *et al.*, 2010). The adaxial and abaxial surfaces of newly developed leaves (three individual leaves per plant) were measured once a week from middle of July 2010 to middle of September 2010 on three different plants.

All measurements were performed on fully expanded mature leaves of the same age. Data were tested using a simple factorial ANOVA. Where appropriate, the treatment means were compared using Tukey's Post-hoc test.

4.3 Results and discussion

UV radiation reaching the Earth's surface has increased between 1979 and 2008 (Herman, 2010). Despite the successful implementation of the Montreal Protocol in limiting stratospheric ozone depletion, the levels of UV irradiances that reach the Earth' surface are expected to be still elevated in the coming decades (McKenzie *et al.*, 2011). Exposure to UV radiation is known to be harmful to terrestrial plants (Li *et al.*, 2010, Ballaré *et al.*, 2011). UV-B is typically the most destructive form of UV radiation because it has enough energy to cause photochemical damage. Whereas UV-A, which is the most commonly encountered type of UV light because stratospheric ozone layer absorbs very little of this part of the UV spectrum, is thought to have a minor biological effectiveness. However, the UV-B lamps used in supplementation studies have a spectrum that

includes also a substantial fraction of UV-A radiation and, thus, it is impossible to dissect the effects of UV-B exposure alone (Ren *et al.*, 2010). Then, only a few studies have been conducted in which UV-A and UV-B treatments were applied separately. These studies, which were mostly performed by manipulating UV radiation by using filters that either transmitted UV or excluded only UV-B or both UV-B and UV-A, respectively, have shown some degree of negative modifications in the plant physiological and growth responses to UV-A (Häder 1996, Day *et al.*, 1999, Kotilainen *et al.*, 2008, Yang and Yao 2008, Morales *et al.*, 2011). Consequently, there are still major uncertainties regarding the basic mechanisms of plant physiological responses to high UVA levels.

We performed a first supplementation (addition of UV-A radiation using a LED system) study at the beginning of summer. Hybrid poplar saplings were irradiated with increasing acute UV-A doses, ranging from ambient to unrealistically high amounts, for about 20 minutes at each different dose (Fig. 4.2 a), in order to elucidate the plasticity of innate physiological traits to UV-A radiation alone. These irradiances induced significant effects on the physiological status of poplar leaves. After 20 min of exposition at 60, 90 and 120 W m^{-2} of UV-A, *A* resulted significantly reduced by about 17%, 33% and 42%, respectively, compared with the pre-stress value (Fig. 4.2 b). It is noteworthy that *A* recovered only partially when plants were exposed back to the ambient UV-A radiation (30 W m^{-2}) after each acute treatment. Stomatal conductance showed a trend similar to that of photosynthesis, with a strong decrease of about 30% of the pre-stress value at the end of the UV-A treatments (data not shown). The increasing inhibition of the photosynthetic efficiency was also detected by the decreasing trend of both photochemical reflectance index and fluorescence yield during the whole duration of the UV-A treatments (Fig. 4.2 c). PRI was originally developed to estimate rapid changes in the xanthophyll cycle that occurs over a minute time scale (Gamon *et al.*, 1992), but there are increasing evidences that PRI is related to changes in the photosynthetic light use efficiency (Gamon *et al.*, 1997; Sun *et al.*, 2008). Similarly, the fluorescence yield is a measure of the overall efficiency of PSII reaction centres of a leaf in bright light. The significant strong decline in both PRI and ΔF/Fm' may indicate that damages at the metabolic level occurred as the intensity of the UV-A treatments increased. However the dynamics of these two parameters differed from that of photosynthesis, because ΔF/Fm' and especially PRI showed a very little increase during the recovery phases which followed each UV-A acute treatment. This different declining trend of A and of the two parameters describing the efficiency of the photochemical apparatus, may indicate that the partial recovery of photosynthesis during the exposition at ambient UV-A which followed the acute treatments, could have been mostly caused by reduced diffusional limitations (Centritto *et al.*, 2003; Loreto & Centritto, 2008).

Differently from the pattern shown by the photosynthetic parameters, the increasing acute UV-A treatments significantly triggered the emissions of isoprene and methanol (Fig. 4.2 d,e). Isoprene emission resulted to be very sensitive to the UV-A irradiances, because it burst increasingly and significantly as the UV-A dose increased (Fig. 4.2 d), and quickly and significantly declined during the recovery phases (Table 4.1). At the end of the experiment, isoprene emission was still about three times higher than the pre-stress level. This is a further evidence that environmental stresses uncouples photosynthesis from isoprene emission (Brilli *et al.*, 2007; Loreto & Schnitzler 2010; Centritto *et al.*, 2011a). Methanol emission was also very responsive to increasing UV-A irradiances, but contrary to isoprene, methanol showed the highest emission peak after the first applied UV-A intensity (60 W m^{-2}) and two progressively and significantly lower bursts at 90 and 120 W m^{-2} (Fig. 4.2 e). As for isoprene, methanol emission quickly and significantly declined during the recovery phases (Table 4.1), and the emission was still significant higher at the end of the experiment than at the pre-stress phase. Methanol is a short-chained oxygenated compound which emission is usually elicited early in stress development as a consequences of degradation of cell wall pectins (Loreto & Schnitzler, 2010; Niinemets, 2010). Thus, the methanol results suggest that the strongest damages of cellular structures occurred during the first UV-A treatment, although an immediate strong release from a large methanol pool already built inside the leaf mesophyll cannot be ruled out (Filella *et al.*, 2009). As methanol, also acetaldehyde emission can be induced by environmental stresses (Kreuzwieser *et al.*, 2001). However, the apparent effects of increases in UV-A radiation on acetaldehyde emission was much less visible (Fig. 4.2 f), and also in this case the emission peaks declined although non significantly as the applied UV-A intensities increased (Table 4.1). Furthermore, there was a significant decline in the emission during the recovery phases, which resulted in lower acetaldehyde emission at the end of the experiment than at the pre-stress phase. However, this emission pattern may have been probably affected by the degradation of acetaldehyde in the gas phase by the UV-A treatments, which may have likely obscured the overall acetaldehyde emission (Ohko *et al.*, 1998).

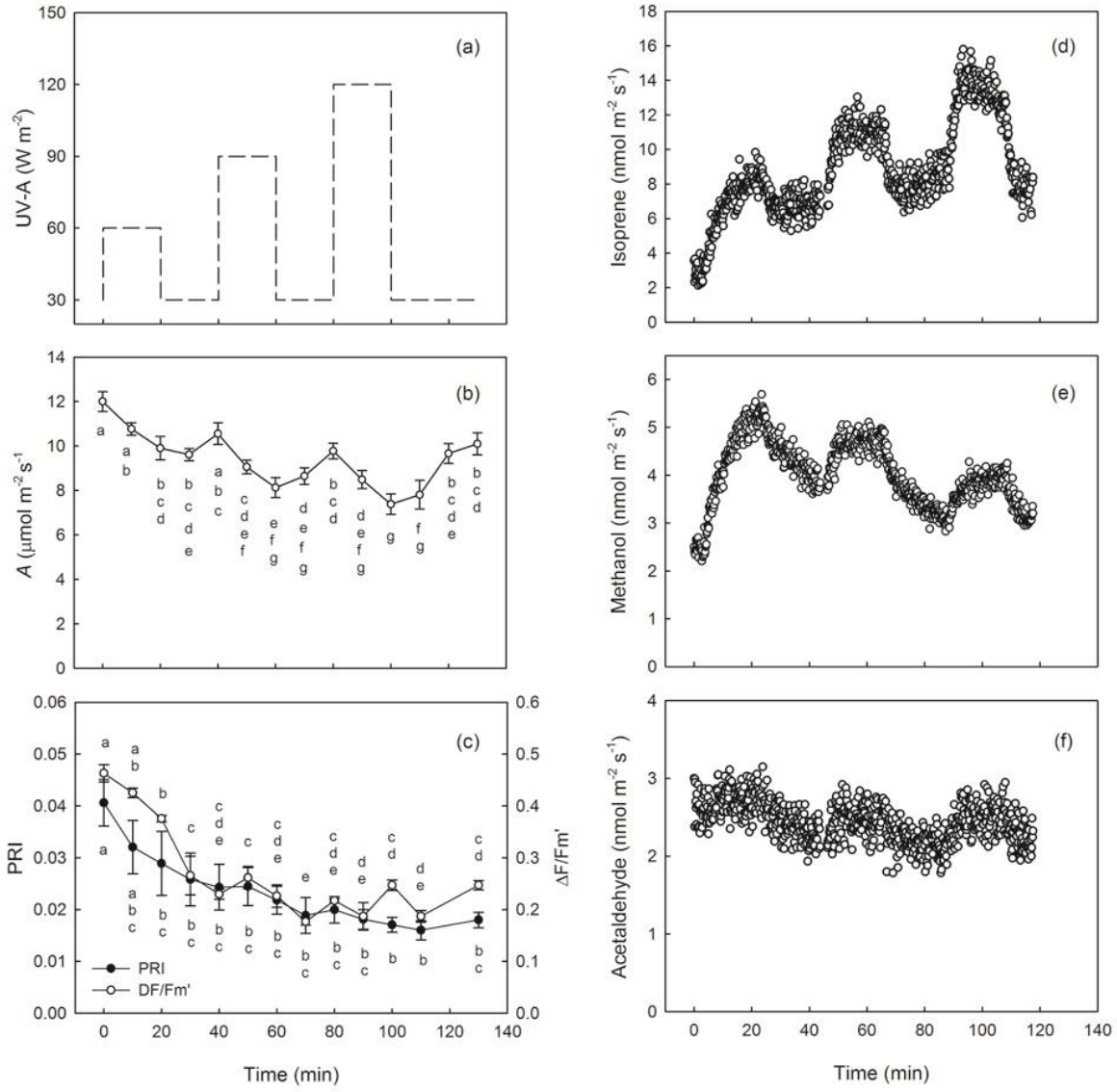

Figure 4.2: Time course of (a) acute UV-A treatments, (b) photosynthesis (*A*), (c) photochemical reflectance index (PRI) and fluorescence yield (ΔF/Fm'), and emissions of (d) isoprene, (e) methanol, and (f) acetaldehyde. Leaves of Populus x euroamericana saplings were first exposed to ambient UV-A radiation (30 W m^{-2}) for about two hours and then subjected for 20 minutes to increasing acute UV-A treatments, which were then followed by a 20 min-recovery at ambient UV-A conditions prior to further increase the UV-A doses, as shown in the panel a. Results shown in the panels b and c are means of five replicates ± 1 SEM, whereas data shown in the panels d-f, measured on line with the PTR-MS, are from a single leaf but are representative of experiments replicated five times on different plants. The letters a to g indicate significant differences at $P < 0.05$.

Time (min)	0	20	40	60	80	100	120
UV-A	30 W m^{-2}	60 W m^{-2}	30 W m^{-2}	90 W m^{-2}	30 W m^{-2}	120 W m^{-2}	30 W m^{-2}
Isoprene	3.00 ± 0.49 f	8.45 ± 0.73 cd	6.97 ± 0.57 e	11.21 ± 0.66 b	8.29 ± 0.65 cd	13.69 ± 0.46 a	7.79 ± 0.80 de
Methanol	2.47 ± 0.11 e	5.16 ± 0.20 a	3.87 ± 0.18 c	4.68 ± 0.15 b	3.26 ± 0.14 d	3.84 ± 0.15 c	3.14 ± 0.09 d
Acetaldehyde	2.68 ± 0.25 a	2.79 ± 0.18 a	2.36 ± 0.49 ab	2.46 ± 0.16 ab	2.22 ± 0.09 b	2.51 ± 0.14 a	2.19 ± 0.14 b

Table 4.1: Average emission values of isoprene, methanol and acetaldehyde (nmol m^{-2} s^{-1}) during the UV-A treatments shown in Fig. 4.1. Results are means of five replicates ± 1 SEM. Letters (a - f) indicate significant differences at $P <$ 0.05 in the same row.

A second supplementation experiment was performed to study the UV-A dose-response relationships. Poplar leaves were exposed for two hours to 30 W m^{-2} of UV-A, a radiation intensity which usually occurs on sunny summer days, 60 W m^{-2} a high intensity that may occur in the Mediterranean Basin (Abukassem & Bero, 2012), 90 W m^{-2}, which represent real-world episodic UV-A radiation stress (Gelsor *et al.*, 2011), and 120 W m^{-2}, which are unrealistically high amounts of UV-A. This experiment was performed in the middle of summer and then was repeated towards the end of the same summer season.

As expected, independently of the seasonality, the ambient treatment did not cause any significant change in the time course of both *A* (Fig. 4.4 a,c) and isoprene emission (Fig. 4.4 b,d). Whereas, the response of these two parameters to high UV-A doses showed a significant temporal evolution. In fact, there was a clear response to the different irradiance levels immediately after the UV-A stress application when the experiment was made in the middle of summer. While 60 W m^{-2} of UV-A radiation affected photosynthesis only transitorily (Table 4.2), *A* decreased rapidly in leaves exposed to higher UV-A doses (Fig. 4.4 a): *A* was already significantly reduced after about 10 min in leaves exposed to 90 and 120 W m^{-2}, and after about 30 min from the onset of the UV-A treatments, *A* was further inhibited by about 17% and 28% at 90 and 120 W m^{-2}, respectively, and remained steadily inhibited to similar levels until the end of the treatments (i.e. -20% and -34% at 90 and 120 W m^{-2}, respectively) (Table 4.2). The spectral reflectance analysis performed before and immediately after the treatments, mirrored the inhibition of *A* (Fig. 4.3).

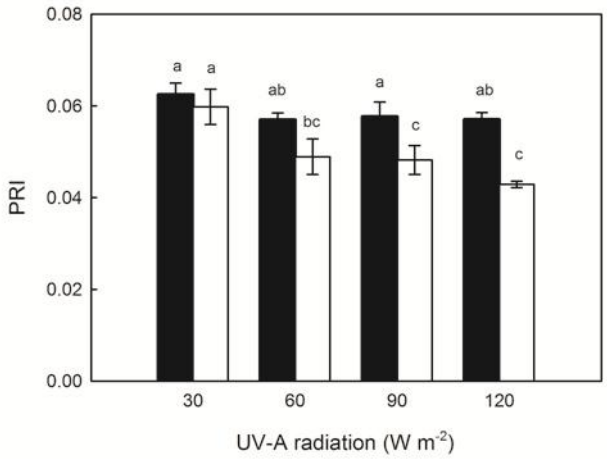

Figure 4.3: Effects of ambient UV-A radiation (30 W m^{-2}), and acute UV-A doses (60, 90, and 120 W m^{-2}) on photochemical reflectance index (PRI) in *Populus x euroamericana* saplings. Measurements (n = 5 ± 1 SEM) were made during the middle of the summer season, before (filled bars) and immediately after (open bars) the UV-A treatments. Results are means of five replicates ± 1 SEM. The letters a to c indicate differences at $P < 0.05$ in response to the UV-A treatments.

In fact, PRI significantly declined by about 15% and 24% in leaves exposed to 90 W m^{-2} and 120 W m^{-2}, respectively, whereas the 60 W m^{-2} treatment did not cause any significant decline in the efficiency of the photochemical apparatus. Isoprene emission resulted rather stable when exposed to ambient level of UV-A (Fig. 4.4 b). Whereas, when leaves were exposed to higher UV-A irradiance doses, the emission of isoprene increased soon after the onset of the treatments: after 10 min there were already significant differences with respect to the ambient control (Table 4.2), and after about 30 min from the beginning of the treatments isoprene emission was stimulated by about 26%, 66% and 86% by the 60, 90 and 120 W m^{-2} treatments, respectively. While at the end of the treatments, isoprene emission was stimulated by about 22%, 82% and 106% by the 60, 90 and 120 W m^{-2} doses, respectively. These results are in keeping with those observed in the first experiment (Fig. 4.2 d).

Photosynthesis (µmol m^{-2} s^{-1})				
UVA intensity	10 min	30 min	60 min	120 min
30 W m^{-2}	16.98 ± 0.41 a	16.63 ± 0.10 a	16.40 ± 0.60 a	16.49 ± 0.49 a
60 W m^{-2}	16.71 ± 0.54 a	15.23 ± 0.54 b	15.84 ± 0.71 a	16.25 ± 0.55 a
90 W m^{-2}	15.12 ± 0.17 b	13.83 ± 0.45 c	13.59 ± 0.79 b	13.21 ± 0.54 b
120 W m^{-2}	14.37 ± 0.87 b	11.97 ± 0.48 d	11.27 ± 0.77 c	10.90 ± 0.48 c
Isoprene (nmol m^{-2} s^{-1})				
UVA intensity	10 min	30 min	60 min	120 min
30 W m^{-2}	9.20 ± 0.24 c	8.92 ± 0.17 d	9.03 ± 0.17 d	8.79 ± 0.17 d
60 W m^{-2}	10.72 ± 0.17 b	11.23 ± 0.19 c	11.22 ± 0.16 c	10.76 ± 0.18 c
90 W m^{-2}	14.21 ± 0.16 a	14.83 ± 0.18 b	15.94 ± 0.21 b	16.01 ± 0.22 b
120 W m^{-2}	14.67 ± 0.17 a	16.57 ± 0.19 a	17.97 ± 0.18 a	18.11 ± 0.18 a

Table 4.2: Average values of photosynthesis and isoprene emission during the UV-A treatments shown in panels (a) and (b) of Fig. 4.4 (i.e., experiment made during the middle of the summer season). Results are means of five replicates ± 1 SEM. Letters (a - d) indicate significant differences at $P < 0.05$ in the same column.

Photosynthesis and isoprene emission were still very high towards the end of the summer season. However, by this time (first half of the month of September), there were no longer significant differences in the time course of A (Fig. 4.4 c), PRI (data not shown) and isoprene emission (Fig. 4.4 d) in response to the different UV-A radiation treatments.

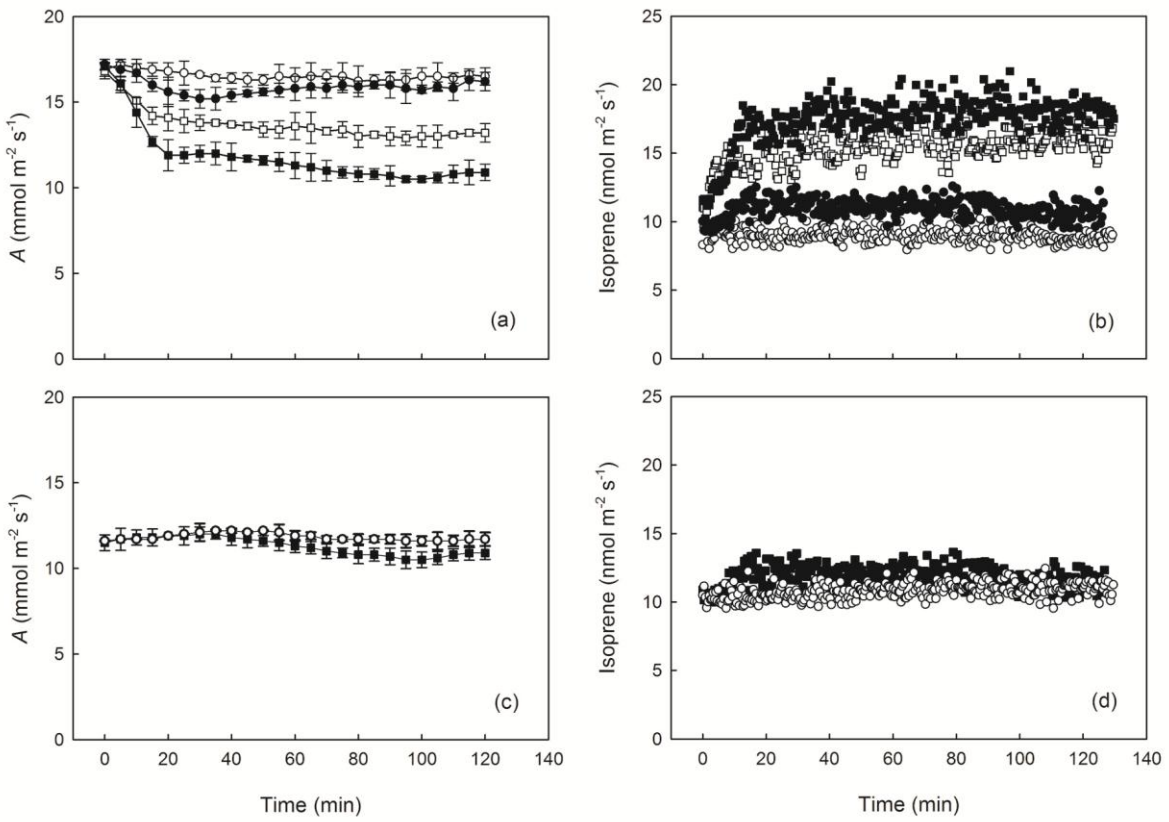

Figure 4.4: Time course of (a,c) photosynthesis (*A*) and (b,d) isoprene emissions in *Populus x euroamericana* saplings exposed to ambient UV-A radiation (30 W m^{-2}, ○) and to acute UV-A doses of 60 (●), 90 (□), and 120 (■) W m^{-2}. The measurements were made during the middle (a,b) and towards the end (c,d) of the summer season, respectively. For clarity reasons, only the results obtained with the ambient and 120 W m^{-2} UV-A treatments are shown in the panels (c) and (d). Results in the panels (a) and (b) are presented as means of five replicates ± 1 SEM, whereas in the panels (b) and (d) data, measured on line with the PTR-MS, are shown from a single leaf but are representative of experiments replicated five times on different leaves.

Much early studies of the effects of UV radiation on plants have shown that these produce an array of self-protective responses that include UV absorbing compounds which are primarily located in the vacuoles of epidermal cells and that are able to screen plants against harmful UV radiation (Schnabl *et al.*, 1986; Schmelzer *et al.*, 1988; Agati & Tattini, 2010; Li *et al.*, 2010). Close *et al.* (2007) have characterized phenolic compounds in leaf of *Eucalyptus nitens* seedlings in a study that either included or excluded UV-A radiation. They found that flavonoids dominated the absorption of UV-A, despite their concentration in leaves was not affected by ambient UV-A radiation. In contrast, Reifenrath & Muller (2007) in a study on the effects of ambient UV-A and UV-B radiations on *Sinapis alba* and *Nasturtium officinale* found that both UV components elicited the accumulations of flavonoids in the exposed leaves. Furthermore, Morales *et al.* (2010) recently

observed, in an attenuation study that selectively excluded UV-A, UV-B or both UV bands, that both UV radiations regulated, although differentially, gene expression and epidermal flavonoid concentration in birch. To follow the evolution of pigments that could have been affected by natural UV radiation, a fluorescence excitation technique was used as a non-destructive method for assessing levels of flavonoids and chlorophylls (Agati *et al.*, 2011), in both the upper (adaxial) and lower (abaxial) leaf surfaces, from the middle to the end of the summer season. The amount of chlorophylls in both leaf surfaces and that of flavonoids in the abaxial surface did not change over the period monitored (Table 4.3). Whereas, the flavonoid level in adaxial surface increased significantly over the period monitored (data not shown), and towards the end of the summer the flavonoid level was about 85% higher than the level measured in the middle of July (Table 4.3). Despite a temporal increase in epidermal flavonoids caused by ambient UV radiations has been frequently showed (Kotilainen *et al.*, 2010; Morales *et al.*, 2011; Klem *et al.*, 2012), we are not able to state whether this increase in the epidermal flavonoids was caused by the summer UV radiation load or by the leaf ontogeny, or even by a combination of both factors. However, the dramatic increase in the adaxial flavonoids may have played a protective role against UV-A radiation (Li *et al.*, 1993; Burchard *et al.*, 2000; Agati *et al.*, 2011) by shielding leaves and then nullifying the impact on photosynthesis (Fig. 4.4 c) and isoprene emission (Fig. 4.4 d).

	Flavonoids		**Chlorophyll**	
	Adaxial surface	*Abaxial surface*	*Adaxial surface*	*Abaxial surface*
Middle of July	0.73 ± 0.03 a	0.55 ± 0.01	4.75 ± 0.08	2.94 ± 0.09
Middle of September	1.35 ± 0.09 b	0.53 ± 0.01	4.40 ± 0.07	2.79 ± 0.08

Table 4.3: Flavonoid index, FLAV (Multiplex units), and Chlorophyll index, CHL (Multiplex units), in the upper (adaxial) and lower (abaxial) leaf surfaces in *Populus x euroamericana* saplings grown under natural sunlight conditions. The data, averaged across three individual different leaves per plant, refers to measurements made in three different saplings (n = 3 ± 1 SEM) during the middle and towards the end of the summer season, respectively. Letters (a, b) indicate significant differences at $P < 0.05$ in the same column.

In previous experiments performed on subarctic fen ecosystem exposed either to ambient (Faubert *et al.*, 2010) or to enhanced UV-A radiation (Tiiva *et al.*, 2007) no effects were found on BVOC emissions. While Faubert *et al.*'s (2010) findings are in keeping with the results obtained in our study on poplar saplings exposed to ambient UV-A doses (i.e., 30 W m^{-2}), Tiiva *et al.*'s (2007) results are in contrast with our findings. This is because Tiiva *et al.*, (2007) did not found any stimulation in isoprene emitted by the subarctic fen ecosystem during three different growing season under enhanced UV-A exposure, despite this ecosystem emitted high amount of isoprene, during the warm periods of the second growing season and at the end of the fourth growing season, when exposed to enhanced UV-B radiation. Unfortunately, no information is provided by Tiiva *et al.* (2007) about the UV-A doses used in their study. Consequently, it is impossible to understand whether these contrasting findings resulted from different intensity of UV-A radiations used or from specie-specific responses.

In conclusion, there is mounting evidence that the atmospheric physical and chemical properties are changing. A major change is an increase in the levels of UV irradiance reaching the earth's surface. The objective of this supplementation study was to increase knowledge of the mechanisms of primary and secondary metabolism processes to UV-A irradiance. The UV-A computer controlled LED system employed in this study allowed estimates of dose-response functions. Our findings showed that both the mechanisms regulating photosynthesis and BVOC emissions are very sensitive to high UV-A. However, the responsiveness of both processes is reduced over the summer season likely because of the temporal increase in epidermal flavonoids in the adaxial surface which shield the leaf interior against UV. Taken collectively, our findings add to the understanding of the basic physiology of plant response to UV radiation and provide an important notion in the discussion of the effects of UV radiations on the emissions BVOCs which are major components in many global physicochemical processes as well as ecological process.

CHAPTER 5 – Study on BVOC emission, photosynthesis and mesophyll conductance in response to blue light.

5.1 Introduction

A wide range of plant processes such as phototropism, photomorphogenesis, stomatal opening are influenced by blue light (Whitelam & Halliday, 2007). Positional movements of chloroplasts are also induced by blue light in vascular terrestrial plants (Banaś *et al.*, 2012). In the mesophyll of these plants, chloroplasts align perpendicularly to the light direction along cell walls (periclinal walls) in weakly illuminated part of the cell. Whereas, when exposed to highly energetic radiation, chloroplasts move away from blue light appressing themselves to the cell wall parallel to the light beams (anticlinal walls) (Kasahara *et al.*, 2002; Wada *et al.*, 2003; Tsuboi & Wada, 2011; Banaś *et al.*, 2012).

Chloroplasts redistribution in response to blue light influences photosynthesis (*A*) because the avoidance response, in regions exposed to strong radiation rates, may reduce the absorbance of the incident photosynthetic photon flux density (PPFD) reducing its efficiency. Furthermore, McCree (1972) and Evans (1987) demonstrated a strong reduction in both action spectrum and quantum yield of leaf photosynthesis (i.e., spectral quantum yield) under blue light compared to red light regardless of the directional movement of chloroplasts. These blue light-driven effects were related to inefficient energy transfer from carotenoids to chlorophylls (Duysens, 1952) by increasing absorption by epidermal cells (Inada, 1976) and because of unbalanced excitation of the two photosystems (Evans, 1987). Furthermore, strong light leads to inactivation of photosystem II (PSII) of oxygenic photosynthetic organisms, a phenomenon called photoinhibition (Ohnishi *et al.*, 2005). Oguchi *et al.* (2009) showed that blue light has a larger impact on photoinactivation than white, green and red lights.

The well-known influence on stomatal conductance (g_s) (Sharkey & Raschke, 1981) and the reduced surface of chloroplasts exposed to intercellular airspaces (when positioned along anticlinal walls) (Tholen *et al.*, 2008) affect the CO_2 diffusion path to the Rubisco carboxylation sites. Loreto *et al.* (2009) performed a study on the effects of blue light on the diffusional limitations to *A* in leaves of *Nicotiana tabacum* and *Platanus orientalis*. They showed that when leaves were exposed to blue light fractions between 0 and 80% of incident light intensity (fixed at 300 µmol photons m^{-2} s^{-1}), *A* was quickly inhibited despite stomatal conductance (g_s) being increased. *A* inhibition was only partially explained by chloroplast movements, because there was a concomitant but

independent fast decrease in mesophyll conductance to CO_2 (g_m) to the order of 50%: the decline in g_m, upon exposure to blue light, was completed in ~2 min and was faster than any possible chloroplast movement from periclinal to anticlinal walls (Wada *et al.*, 2003; Tholen *et al.*, 2008). Kaldenhoff (2012) has recently hypothesizes that this very fast decline in g_m may have been related to the involvement of aquaporins, which are significant components facilitating CO_2 diffusion in the mesophyll.

Because many early studies have demonstrated a link between photosynthesis and the instantaneous emission of isoprene (Sanadze, 1969; Rasmussen, 1972), it is reasonable to hypothesize a negative effect of highly energetic light wavelengths (e.g. blue light) on isoprene and other volatile isoprenoids. Isoprenoids are produced enzymatically in the light and emitted through stomata to the atmosphere (Loreto & Schnitzler, 2010). The biosynthesis of isoprene and monoterpenes, the most abundant biogenic volatile organic compounds released by leaves, occurs in the chloroplast from photosynthetic intermediates (Delwiche & Sharkey, 1993; Loreto *et al.*, 1996). Brilli *et al.* (2007) demonstrated that in poplar about 90% of the carbon in the isoprene molecule originates from recently assimilated photosynthetic intermediates which are then the primary substrate for isoprene biosynthesis. Thus, reduced photosynthesis is expected to lead to reduced carbon and energy supply for the synthesis of these volatile isoprenoids (Niinemets *et al.*, 1999), at least at ambient CO_2 concentration (Loreto & Schnitzler, 2010).

Comparison of light dependences of isoprenoid emissions and photosynthesis could contribute to further clarification of their dependence. It was shown by Sharkey & Loreto (1993) that isoprene emission from kudzu raises without saturating with PPFD up to 3000 µmol m^{-2} s^{-1} while A saturates at much lower PPFD. The light dependence of isoprene emission has been attributed to control by photosynthetic electron transport (Niinemets *et al.*, 1999; Zimmer *et al.*, 2000), as well as to direct light activation of isoprene synthase and/or light-dependent supply of the substrate dimethylallyldiphosphate (Wildermuth & Fall, 1996; Fall & Wildermuth, 1998). Monoterpene emission is controlled in a more controversial way, as both physiological and physico-chemical factors play a role. In plants with only a small storage capacity monoterpene emission is partly controlled by stomatal conductance (Guenther *et al.*, 1993; Staudt *et al.*, 2003; Grote & Niinemets, 2008; Niinemets *et al.*, 2010). Consequently, in these latter plants the synthesis and emission rates of monoterpene are strongly light-dependent.

Despite the substantial information accumulated about the effect of light intensity on photosynthesis and isoprenoid emissions, the influence of light spectral composition on these processes is not understood. In order to understand whether under blue light the quantity of emitted isoprenoids will

change and how these changes will be related to photosynthetic characteristics, we performed a study on three tree species, *Populus x euroamericana* (hybrid poplar), *Quercus ilex* (holm oak) and *Citrus reticulate* (mandarin). *Populus x euroamericana* is a strong isoprene emitter, whereas *Quercus ilex* and *Citrus reticulata* are monoterpene emitters with small and large storage pools for monoterpenes, respectively (Schurgers *et al.*, 2009).

5.2 Material and methods

The experiments were carried out both in Estonia and Italy. Gas exchange measurements were performed in laboratory conditions on single leaves exposed to either actinic or blue light. To measure photosynthesis (*A*)/actinic light response curves, leaves were first exposed to saturating photosynthetic photon flux density (PPFD) and then to progressively reduced intensities. Before performing the *A*/blue light response curves, leaves were exposed to saturating PPFD of actinic light until steady-state values of the photosynthetic parameters were obtained. Afterward leaves were exposed to equivalent PPFD values of blue light, by quickly switching from actinic to blue wavelengths, and then to progressively reduced intensities.

Light was provided by a computer controlled light system that combines four lamps with 4 cm^2 arrays of RGBA LEDs (ENFIS Ltd, Swansea, UK), able to generate different monochromatic lights within the visible spectrum as well as actinic light. The blue LEDs (425-495 nm) had a peak emission at 460 nm. To enable measurements of blue light-saturated photosynthetic rates, illumination of the leaf cuvette by RGBA LEDs was supplemented with light provided by an auxiliary blue light led lamp (450-475 nm) (Vinci Fine Instruments, Monterotondo, Italy). Then, gas exchange parameters and chlorophyll fluorescence yield were measured simultaneously. The PPFD values used to generate photosynthesis-light response curves were 2000, 1800, 1600, 1400, 1200, 1000, 800, 600, 400, 300, 200, 100, 80, 60, 0 µmol m^{-2} s^{-1} with both actinic and blue lights.

Experiments were replicated on five different leaves of different plants. Results are presented as means ± SEM, unless otherwise specified. All statistical analyses were conducted using SigmaStat 4.0 software (SPSS; http:// www.spss.com/). Asterisks (*) indicate significant differences (Tukey's test, $P < 0.05$) between treatment means measured at the same fractions of incident PPFD. Best fits and regression coefficients of the relationships of Figure 4 were generated by the SigmaPlot 11.0 software (Systat Inc., San Jose, CA, USA).

5.3 Results and discussion

The light response curves performed on *C. reticulata*, a sclerophyllous species with a large storage pool for monoterpenes, showed significant differences in maximum quantum yields, as indicated by the linear relationships between *A* and PPFD in the light-limited portion of the curves (i.e., electron transport rate limitations to photosynthesis) (Fig. 5.1 a). Then as PPFD increased towards saturation, the effects of light quality on *A* tended to become non significant. Whereas, there were no significant effects on g_s between the treatments (Fig. 5.1 b); g_s scaled linearly as PPFD increased. Total monoterpene emission was in general decreased under blue light, but statistical differences were evident only at intermediate blue light intensities (i.e., between 150 and 450 μmol m^{-2} s^{-1}) (Fig. 5.1 c). Furthermore, total monoterpene emission was clearly saturated only under actinic light. Light quality did not affect the blend of monoterpenes emitted by mandarin leaves: this blend was composed mainly by α-pinene (~63%), camphene (~23%), carene (~5%), limonene (~5%) and β-pinene (~4%).

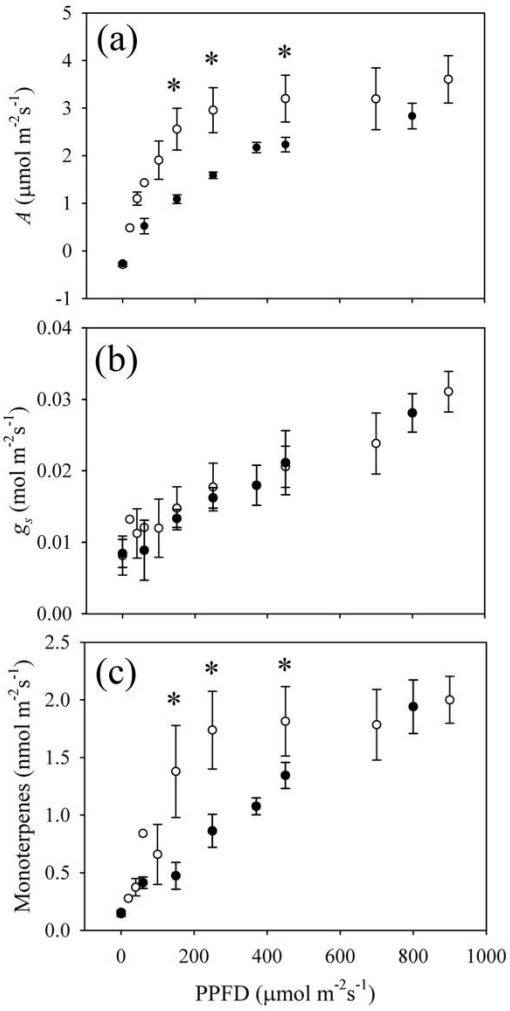

Figure 5.1: average responses of (a) photosynthesis (*A*), (b) stomatal conductance (g_s) and (c) total monoterpene emission to photosynthetic photon flux density (PPFD) in *Citrus reticulata* saplings. The measurements were made in actinic (○) and blue (●) lights. Values are means of four plants ± 1 SEM: the significance level (* = $P < 0.05$) indicate differences between treatments in response to the same fractions of PPFD.

The light response curves performed on holm oak saplings, a sclerophyllous plant, showed a different response in *A* (Fig. 5.2 a) and g_s (Fig. 5.2 b) with respect to mandarin. The analysis of the *A*/PPFD curves in holm oak indicated that there were no significant differences in the light compensation points and in the maximum quantum yields (i.e., initial slope of the *A*/PPFD curves) between the two light quality treatments (Fig. 5.2 a). As PPFD increased, the shape of the CO_2-limited part of the curves (i.e., rubisco carboxylation capacity or triose phosphate metabolism limitations to photosynthesis) changed dramatically with light quality, with blue light strongly reducing PPFD-saturating *A*. We observed significant differences in *A* starting from about 850 μmol

$m^{-2}s^{-1}$ of PPFD. Similarly, also g_s (Fig. 5.2 b) and g_m (Fig. 5.2 c), which were not significantly affected in the light-limited portion of the curves, became significantly reduced as the blue light intensity increased. G_m was the most affected parameter as it started to be inhibited at lower intensity (i.e., ~600 µmol $m^{-2}s^{-1}$) than A and g_s. The C_i (intercellular CO_2 concentration)/light response curves were not affected by light quality (Fig. 5.2 d). This indicates that the relative effects of blue light on A and g_s were similar. *Q. ilex* is a strong monoterpene emitter with a small storage pool for monoterpenes (Staudt & Seufert, 1995). Total monoterpene emission trend was similar to that of *C. reticulata* in response to blue light: Monoterpene emission was in general increased under actinic light, but statistical differences were evident only at blue light intensities of 850 and 900 µmol $m^{-2}s^{-1}$ (Fig. 5.2 e).

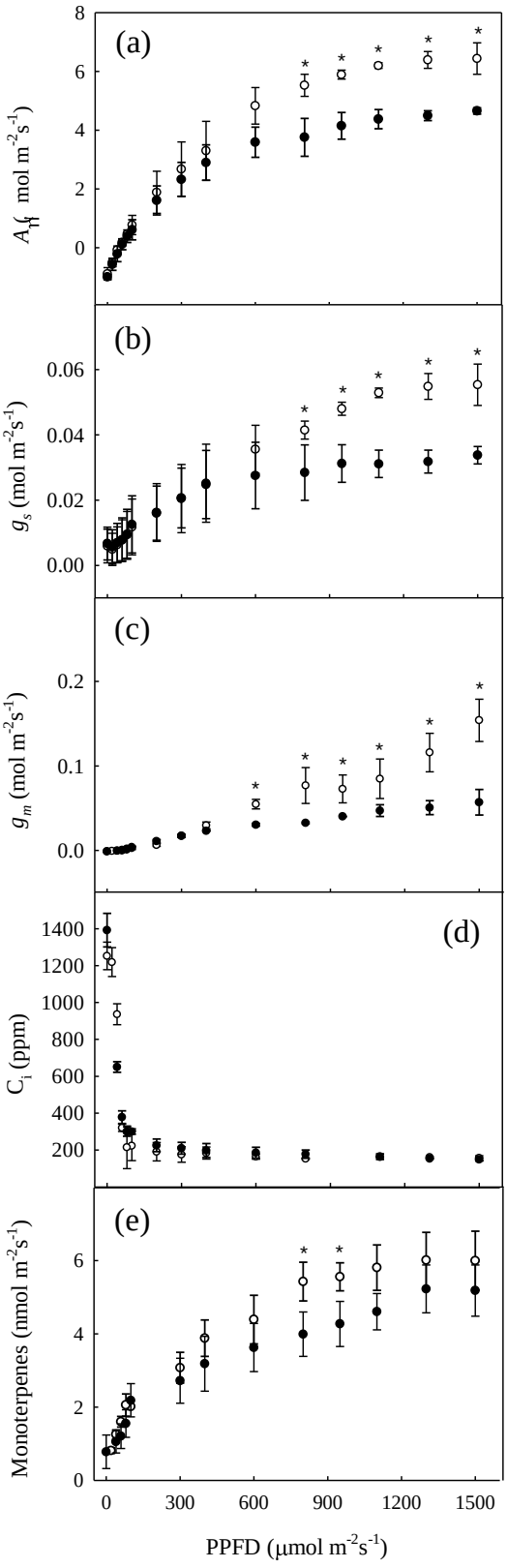

Figure 5.2: average responses of (a) photosynthesis (*A*), (b) stomatal conductance (g_s), (c) mesophyll conductance (g_m), (d) leaf intercellular CO_2 concentration (C_i) and (e) total monoterpene emission to photosynthetic photon flux density (PPFD) in *Quercus ilex* saplings. The measurements were made in actinic (○) and blue (●) lights. Values are means of five plants ± 1 SEM: the significance level (* = $P < 0.05$) indicate differences between treatments in response to the same fractions of PPFD.

The responses of *A* (Fig. 5.3 a), g_s (Fig. 5.3 b) and g_m (Fig. 5.3 c) to blue light intensity in hybrid poplar saplings were similar to those observed in holm oak: these parameters were significantly decreased by blue light only in the CO_2-limited portion of the curves. We observed significant differences in *A* starting from about 600 µmol $m^{-2}s^{-1}$ of PPFD. Similarly, also g_s (Fig. 5.3 b) and g_m (Fig. 5.3 c) became significantly reduced as the blue light intensity increased. As seen in *Q. ilex*, g_m resulted to be more affected than g_s under blue light, since g_m started to be significantly inhibited at lower intensity (i.e., 600 µmol $m^{-2}s^{-1}$) than g_s (i.e., 800 µmol $m^{-2}s^{-1}$). The dynamical responses of *A* and g_s to blue light were reflected by an increase in C_i which became significantly enhanced than under actinic light at intensities higher than 1400 µmol $m^{-2}s^{-1}$ (Fig. 5.3 d), indicating that *A* was relatively more inhibited than g_s at these intensities. Isoprene emission was highly correlated ($P < 0.001$) with photosynthesis under both light quality treatments (data not shown). Consequently, the response of isoprene emission to blue light mirrored that of *A*, and resulted not affected at lower light intensities, whereas as the blue light intensity increased isoprene emission decreased significantly with respect to that of leaves under actinic light (Fig. 5.3 e).

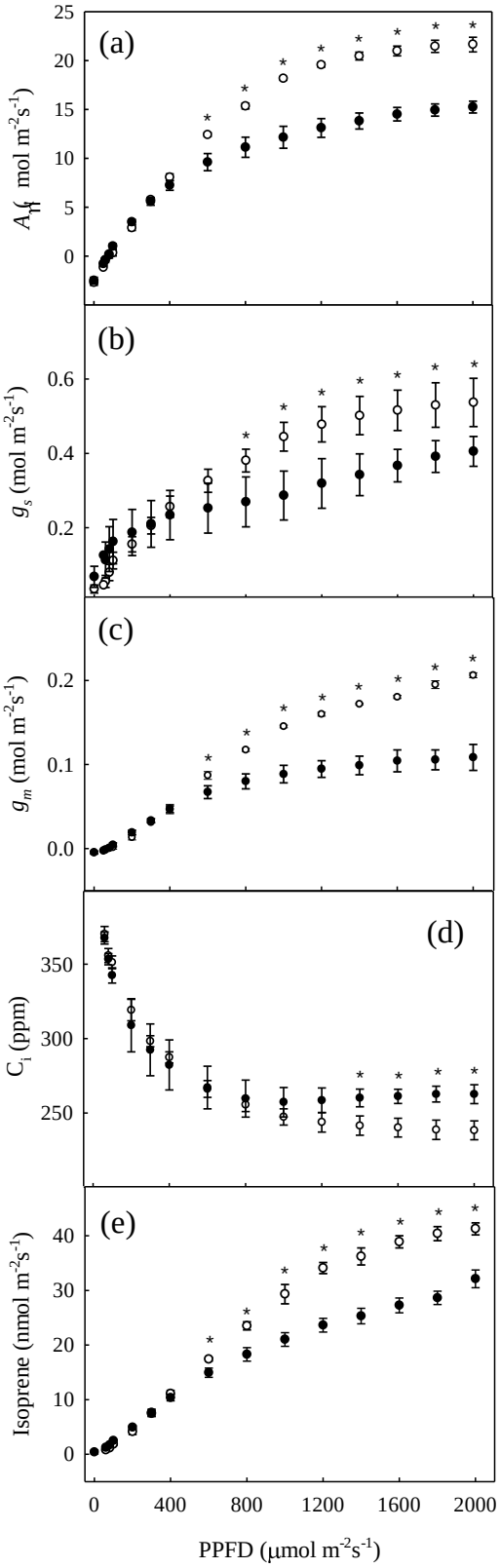

Figure 5.3: average responses of (a) photosynthesis (A), (b) stomatal conductance (g_s), (c) mesophyll conductance (g_m), (d) leaf intercellular CO_2 concentration (C_i) and (e) isoprene emission to photosynthetic photon flux density (PPFD) in *Populus x euroamericana* saplings. The measurements were made in actinic (○) and blue (●) lights. Values are means of five plants ± 1 SEM: the significance level (* = $P < 0.05$) indicate differences between treatments in response to the same fractions of PPFD.

In our study, we applied the methodology used by Loreto *et al*. (2009) to estimate g_m. Thus, J_f was estimated taking into account the possible occurrence of alternative electron transport (Laisk and Loreto, 1996) and the strong adsorption of blue light by carotenoids which may reduce the efficiency of energy transfer to chlorophylls (Duysens, 1952). We found that the reduction of g_m under blue light was particularly strong. It is noteworthy that the reduction in the photosynthetic parameters in hybrid poplar upon exposure to 100% of blue light was extremely fast and occurred within 3 min. In fact, after switching from saturating PPFD of actinic light to equal PPFD values of blue light, *A* was inhibited by ~30%, whereas g_s and g_m declined by ~25% and ~47%, respectively. Similarly, also in holm oak the response to blue light was extremely fast, and resulted in a significant decline in *A*, g_s and g_m of about 28%, 25% and 63%, respectively, under PPFD-saturating light. These results confirm the earlier findings of Loreto *et al*. (2009) who showed that *A* and g_m declined within 2 min in tobacco and plane tree leaves upon exposure to blue light. As in Loreto *et al*.'s (2009), it is unlikely that the g_m inhibition may have been caused by chloroplast movements from periclinal to anticlinal walls as found by Tholen *et al.* (2008), whereas it may have been elicited by yet unknown factors affecting aquaporin-facilitated CO_2 diffusion in the mesophyll (Kaldenhoff, 2012).

Blue light is known to induce stomatal opening (Sharkey & Raschke, 1981). However, in our experiment g_s was inhibited by blue light, but was never as negatively affected as gm. Furthermore, under PPFD-saturating actinic light g_s was ~2.8 times higher than g_m in hybrid poplar, whereas in holm oak g_s was much smaller (~0.4) than g_m. In general, these data confirm that the photosynthetic limitation by g_m is in the same order of magnitude as that by g_s (Centritto *et al*., 2003; Flexas *et al*., 2008; Terashima *et al*., 2011). There was a hyperbolic relationship between *A* and g_m in both species, after pooling together data from both light treatments, indicating that *A* was strongly influenced by g_m (Fig. 5.4). This is also a clear indication that g_m was the main limitation of photosynthesis under blue light, as found by Loreto *et al*. (2009). These findings confirm the importance of the internal diffusion of CO_2, from intercellular spaces to the active sites of Rubisco inside chloroplasts, in limiting photosynthesis for plants with different growth form and leaf structure, across different biomes and phylogenetically distant groups, and in plants exposed to abiotic stress (Shi *et al*., 2006; Galmés *et al*., 2007; Flexas *et al*., 2008; Meyer *et al*., 2008; Shi *et al*., 2008; Aganchich *et al*., 2009; Centritto *et al*., 2009; Niinemets *et al*. 2009; Centritto *et al*., 2011b; Terashima *et al*., 2011; Velikova *et al*., 2011).

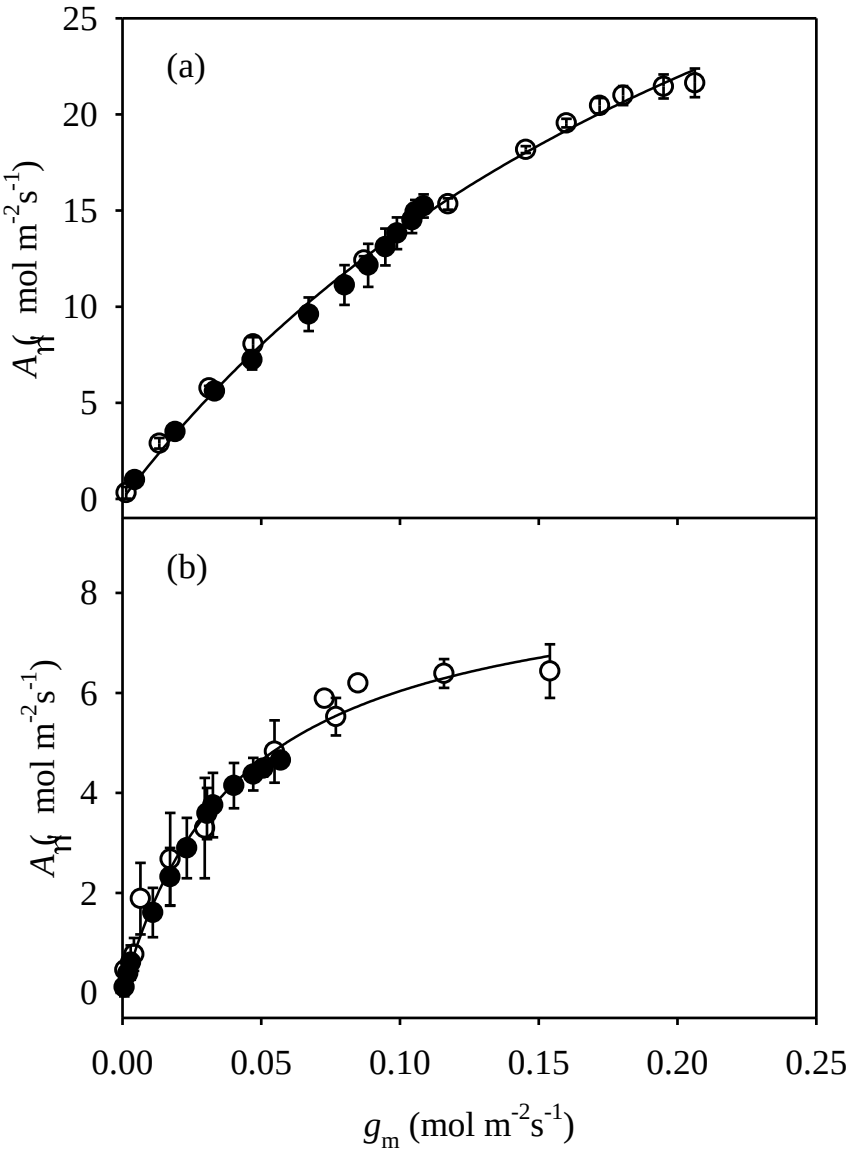

Figure 5.4: effects actinic (○) and blue (●) lights on the relationships between photosynthesis (*A*) and mesophyll conductance (g_m) in (a) *Populus x euroamericana* (r^2 = 0.997, P < 0.01) and (b) *Quercus ilex* (r^2 = 0.984 - P < 0.01). Data are means of 5 plants per treatment ± SEM.

The reduction in the emissions of both isoprene, in hybrid poplar, and of total monoterpenes, in holm oak, upon exposure to 100% of blue light followed that of photosynthesis, and resulted in a significant inhibition in isoprene emission (-22%) and in a non significant decrease of ~14% in monoterpene emission under PPFD-saturating light. The emissions of isoprene and total monoterpenes were differently influenced by g_m and C_i (Fig. 5.5). There was a linear relationship between g_m and isoprene emission in poplar, and the slope of this relationship was significantly (P < 0.05) increased by blue light (Fig. 5.5 a). Whereas in holm oak, there was a typical hyperbolic

relationship between g_m and isoprene emission which was not affected by blue light (Fig. 5.5 b). In contrast, the emissions of both isoprene by single leaves of hybrid poplar (Fig. 5.5 c) and holm oak (Fig. 5.5 d), respectively, were negatively related to C_i, and these significant relationships were not affected by blue light. However, there was no significant relationship between total monoterpene emissions and C_i in mandarin irrespective of light treatments (data not shown). An inverse relationship between isoprene emission and C_i was already observed in *Populus alba* (Loreto *et al*., 2007) and poplar genotypes of different species (Guidolotti *et al*., 2011). The inverse sensitivity of isoprene emission to increasing [CO_2] was already showed in many studies (Rosenstiel *et al*., 2003; Centritto *et al*., 2004). Rosenstiel *et al*. (2004) pointed out that isoprene emission is in competition with light respiration for cytosolic phospho-enolpyruvate (PEP), and because light respiration is stimulated by rising [CO_2] this metabolic competition for PEP results in an inhibition of isoprene emission as [CO_2] increases. We show, for the first time to our knowledge, that C_i may also determine monoterpene emissions in species with small storage capacity such as *Q. ilex*, whereas C_i results irrelevant in species like *C. reticulate* with a storage capacity within leaves where emission is more controlled by physico-chemical components (Grote & Niinemets, 2008; Niinemets *et al*., 2010). Consequently, C_i may therefore be a valuable proxy of isoprene emission in poplar and monoterpene emissions in holm oak. Rising [CO_2] is known to increase C_i whereas many environmental stresses are known to operate in an opposite way as a consequence of increased transport limitations to CO_2 (Brilli *et al*., 2007; Centritto *et al*., 2009; Niinemets *et al*., 2009; Centritto *et al*., 2011a). In addition, we have shown that g_m may strongly limit both isoprene and total monoterpene emissions (Fig. 5.5 a,b). Then, it may be surmised that climate change may have contrasting effects on the emission of isoprene and monoterpenes (in species with a with small storage capacity) depending of prevailing factors operating in the interactions between elevated [CO_2] and stresses (e.g. high temperatures, water stress and salinity) and on the resulting effects on C_i and transport limitations to photosynthesis.

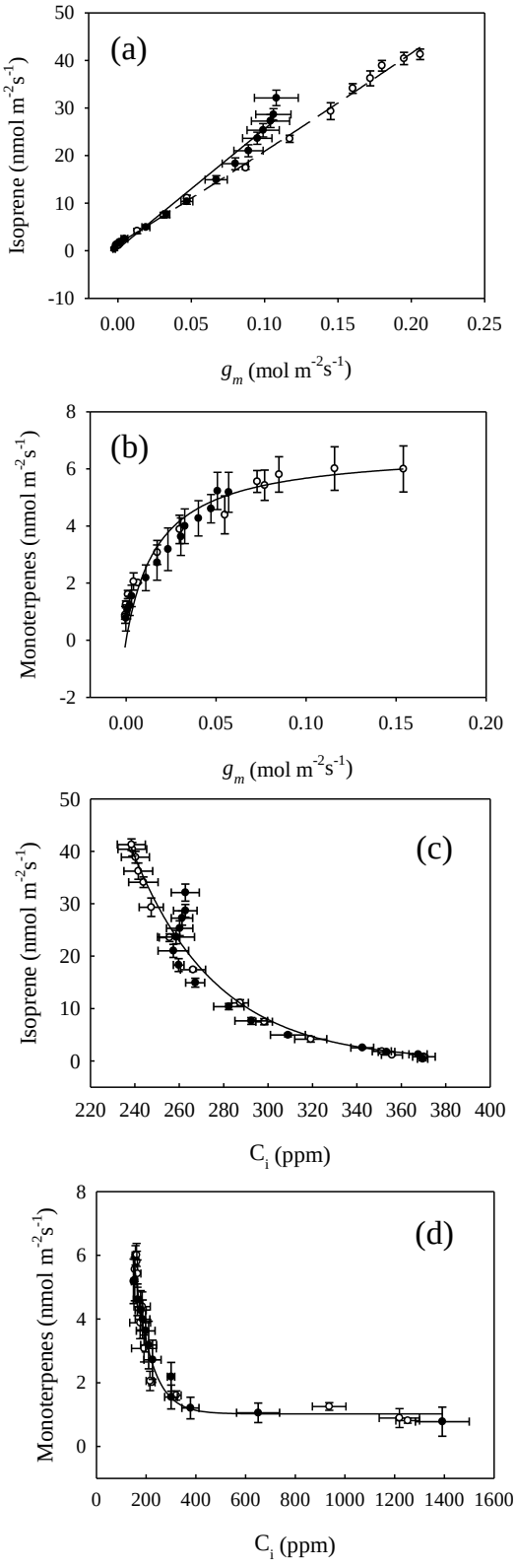

Figure 5.5: effects actinic (○) and blue (●) lights on the relationships between isoprenoids and mesophyll conductance (g_m) in (a) *Populus x euroamericana* (r^2 = 0.997 - P < 0.01 and r^2 = 0.974 - P < 0.01 for measurements made in actinic and blue lights, respectively) and (b) *Quercus ilex* (r^2 = 0.869, P < 0.01) saplings and on the relationships between isoprenoids and leaf intercellular CO_2 concentration (C_i) in (c) *Populus x euroamericana* (r^2 = 0.946, P < 0.01) and (d) *Quercus ilex* (r^2 = 0.938 - P < 0.01). Data are means of 5 plants per treatment ± SEM.

In conclusion, our results confirmed that blue light has a strong and fast effect on photosynthesis, which is mostly mediated by g_m, but that this effect may be reduced by the degree of sclerophylly, as demonstrated by the different responses seen in *Q. ilex*, and *C. reticulata*. We also demonstrated 1) that the fast decline in photosynthesis was mirrored by a concomitant decline in isoprene emission, and by a transient inhibition in total monoterpene emissions irrespective of the species storage capacity for monoterpenes, and 2) that both isoprene and monoterpene emissions were controlled by CO_2 transport limitations. Taken together these results indicate that blue light is a powerful tool to elucidate the plasticity of the primary and secondary metabolism processes.

CHAPTER 6 – Discussion

Sunlight consists of about 4% ultraviolet radiation, 52% infrared radiation, and 44% visible light (Moore *et al.*, 2003). Most of the sunlight that reaches the upper atmosphere is filtered or reflected back to outer space so that only a small portion reaches the earth. The visible wavelengths of light lie from about 390 nm to 760 nm which is just a small section of the entire electromagnetic spectrum of solar radiation. Visible light corresponds roughly to the photosynthetically active radiation (PAR) from 400 nm to 700 nm. These wavelengths contain the right amounts of energy for the biochemical processes and their relative proportion in the available light is of prime importance in determining light quality. Infrared radiation contains minimal energy and only participates indirectly in photosynthesis and other plant reactions. On the other hand ultraviolet light is characterised by high amounts of energy. While UV-C radiation (100-280 nm) is completely absorbed by the ozone layer and atmosphere and UV-B (320-280 nm) is mostly absorbed, UV-A (320-400 nm) radiation completely passes through.

Despite the enormous information available about the effect of light intensities on photosynthesis, conductances and BVOC emissions, very little is known on the influence of short-wave high-energy on these processes. Our studies aim to understand the basic mechanisms of plants physiological responses to UV-B, UV-A and blue light.

Anthropogenic release of halocarbons, has resulted in a depletion of the stratospheric ozone layer, facilitating the passage of increased levels of short wavelength radiation to the biosphere (Gwynn-Jones *et al.*, 1999; Madronich *et al.*, 1998). Montreal Protocol and subsequent agreements has slowed the rate of increase of several ozone-depleting substances in the atmosphere, but the stratospheric levels of bromine and chlorine are expected to continue increasing over the next several years (WMO 1995) with a consequent increase of UV radiation on Earth' surface for the remainder of the decade before beginning a slow recovery (Harley *et al.*, 1996). Increased UV levels is likely to have both direct and indirect implications on photosynthesis (Paul & Gwynn-Jones, 2003) and, in turn, on biogenic volatile organic compounds (BVOCs) emitted by the plants.

Our first aim was to assess the direct effects of high UV-B radiation on both non-emitting and isoprene-emitting *Nicotiana tabacum* plants, increasing the knowledge about the mechanisms of primary and secondary metabolism processes to UV-B irradiance. In this study, both types of tobacco plants were exposed to doses of UV-B radiation at several different intensities from values of 3 W m^{-2} to strong dose of 15 W m^{-2}. These doses were very strong when compared with natural

occurring UV-B radiation as the highest dose rate registered was 3.96 W m^{-2} (Lhasa, Tibet, China) (Norsang *et al.*, 2009). Using these high unrealistic UV-B doses was necessary to fully analyse and understand the hypothetical protection role of isoprene on the photosynthetic apparatus. We found that the mechanisms regulating BVOC emissions were not very sensitive to high UV-B, because emission in transgenic plants decreased only at very high UV-B intensities. However, isoprene was able to mitigate UV-B effect in isoprene-emitting plants, resulting in faster recovery compared with wild-type plants. Transgenic tobacco plants also experienced a minor cell wall damage at high 15 W m^{-2} intensity if compared to wild-type plants. Furthermore, the isoprene-induced protection was combined with an independent higher activation of the ascorbate-glutathione cycle as mechanism for ROS scavenging.

However these protective mechanisms were evident only at very high and unrealistic UV-B doses. Consequently, isoprene emission does not exert any protective mechanisms at natural occurring UV-B levels.

The second aim of this study was to assess the direct effects of short-term, acute exposure to UV-A on photosynthesis and BVOC emissions from hybrid poplar saplings. We treated *Populus x euroamericana*, a strong isoprene emitter, with acute UV-A radiations, performing the experiment in the middle of summer and then repeating it towards the end of the same summer season.

For this experiment we used realistic UV-A intensities ranged from 30 W m^{-2} and 120 W m^{-2}, because 30 W m^{-2} is a radiation intensity which usually occurs on sunny summer days and 90 W m^{-2} represents real-world episodic UV-A radiation stress and up to unrealistic 120 W m^{-2} intensity. During the experiment performed in the middle of the summer, we showed that both photosynthesis and BVOC emissions were very sensitive to UV-A doses higher than ambient level, while the ambient treatment (30 W m^{-2}) did not cause any significant change in the time course of both photosynthesis and isoprene emission. This results provide further evidence that photosynthesis and isoprene emission can be uncoupled under environmental stresses (Brilli *et al.*, 2007; Loreto & Schnitzler 2010; Centritto *et al.*, 2011a). Methanol showed the highest emission peak after the first applied UV-A intensity (60 W m^{-2}) suggesting that the strongest damages of cellular structures occurred during the first UV-A treatment. Measurements were performed again in plants exposed to acute UV-A doses at the end of the summer. The results were rather different as both photosynthesis, isoprene and methanol emission were unaffected by high UV-A doses. Analyses performed *in situ* with a portable fluorimetric sensor showed that epidermal flavonoid levels increased significantly over the summer in the adaxial surface of the leaf protecting the plants against UV-A radiation. This experiment provides a further evidence that ambient UV are able to

elicit the production of UV-protective pigments that are able to shield leaves as observed by Li *et al.* (1993), Burchard *et al.* (2000) and Agati *et al.* (2011) and then nullifying the impact on photosynthesis and isoprene emission. To our knowledge this is the first study that showed the seasonality response of BVOC emission to UV.

The relationship between isoprenoid emissions and diffusional limitation to photosynthesis were studied in response to blue light. A wide range of plant processes such as phototropism, photomorphogenesis, stomatal opening are influenced by blue light (Whitelam & Halliday, 2007). We used three different plant species, a strong isoprene emitter (*Populus x euroamericana*), a strong monoterpene emitter with a small storage pool (*Quercus ilex*) and a monoterpene emitter with a big storage pool (*Citrus reticulata)*.

Our results confirmed the strong and fast effect of blue light on photosynthesis, which was mostly mediated by g_m. It was also confirmed that g_m declined very rapidly upon exposure to blue light. Despite the factor causing g_m fast decline is still unknown, Kaldenhoff (2012) has hypnotized that aquaporins can be involved as a regulatory mechanism. Furthermore, isoprene emission was highly affected by blue light whereas monoterpenes emissions were in general not affected by blue light irrespective of the species storage capacity for monoterpenes. However, it was also clearly shown, for the first time to our knowledge, that both isoprene and monoterpene emissions were controlled by CO_2 transport limitations. These results can indicate that blue light is a powerful tool to elucidate the plasticity of the primary and secondary metabolism processes.

Taken collectively, our findings add to the understanding of the basic physiology of plant response to UV radiation and to blue light, providing an important notion in the discussion of the effects of light quality on the mechanisms of primary and secondary metabolism processes which are major components in many global physicochemical processes as well as ecological process.

CHAPTER 7 – References

Abukassem I, Bero M (2012). Radiometric characterization of radiochromic gel material applied for UVA radiation dosimetry. *Radiation Measurements*, 47: 34-39.

Affek H, Yakir D (2003). Natural abundance carbon isotope composition of isoprene reflects incomplete coupling between isoprene synthesis and photosynthetic carbon flow. *Plant Physiology*, 131: 1727–1736.

Aganchich B, Wahbi S, Loreto F, Centritto M (2009). Partial root zone drying: regulation of photosynthetic limitations and antioxidant enzymatic activities in young olive (*Olea europaea*) saplings. *Tree Physiology*, 29: 685-696.

Agati G, Biricolti S, Guidi L, Ferrini F, Fini A, Tattini M (2011). The biosynthesis of flavonoids is enhanced similarly by UV radiation and root zone salinity in *L. vulgare* leaves. *Journal of Plant Physiology*, 168: 204–212.

Agati G, Tattini M (2010). Multiple functional roles of flavonoids in photoprotection. *New Phytologist*, 186: 786-793.

Albert K, Mikkelsen T, Ro-Poulsen H (2008). Ambient UV-B radiation decreases photosynthesis in high arctic *Vaccinium uliginosum*. *Physiologia Plantarum*, 133: 199–210.

Ballaré C, Caldwell M, Flint S, Robinson A, Bornman J (2011). Effects of solar ultraviolet radiation on terrestrial ecosystems. Patterns, mechanisms, and interactions with climate change. *Photochemical and Photobiological Sciences*, 10: 226-241.

Ballaré C, Scopel A, Stapleton A, Yanovsky M (1996). Solar ultraviolet-B radiation affects seedling emergence, DNA integrity, plant morphology, growth rate, and attractiveness to herbivore insects in *Datura ferox*. *Plant Physiology*, 112: 161–170.

Banaś A, Aggarwal C, Łabuz J, Sztatelman O, Gabryś H (2012). Blue light signalling in chloroplast movements. *Journal of Experimental Botany*, 63.

Behnke K, Ehlting B, Teuber M, Bauerfeind M, Louis S, Hänsch R, Polle A, Bohlmann J, Schnitzler J (2007). Transgenic, nonisoprene emitting poplars don't like it hot. *The Plant Journal*, 51: 485–499.

Behnke K, Loivamaki M, Zimmer I, Rennenberg H, Schnitzler J, Louis S (2010). Isoprene emission protects photosynthesis in sunfleck exposed grey poplar. *Photosynthesis Research*, 104: 5–17.

Ben Ghozlen N, Moise N, Latouche G, Martinon V, Mercier L, Besançon E, Cerovic, Z (2010). Assessment of grapevine maturity using new portable sensor: non-destructive quantification of anthocyanins. *Journal International des Sciences de la Vigne et du Vin*, 44: 1–8.

Bernacchi C, Calfapietra C, Centritto M Valladares F (2012). Global Change and Photosynthesis. In: Terrestrial Photosynthesis in a Changing Environment: A Molecular, Physiological, and Ecological Approach (Flexas J., Loreto F. and Medrano H. eds.). Cambridge University Press, in press.

Bernacchi C, Singsaas E, Pimentel C, Portis A Jr, Long S (2001). Improved temperature response functions for models of Rubisco-limited photosynthesis. *Plant, Cell and Environment*, 24: 253-259.

Bilger W, Veit M, Schreiber L, Schreiber U (1997). Measurement of leaf epidermal transmittance of UV radiation by chlorophyll fluorescence. *Physiologia Plantarum*, 101: 754-763.

Bjorn L, Callaghan T, Gehrke C, Gwynn-Jones D, Lee J, Johanson U, Sonesson M, Buck N (1999). Effects of ozone depletion and increased ultraviolet-B radiation on northern vegetation. *Polar Research*, 18: 331–337.

Blande J, Turunen K, Holopainen J (2009). Pine weevil feeding on Norway spruce bark has a stronger impact on needle VOC emissions than enhanced ultraviolet-B radiation. *Environmental Pollution*, 157: 174–180.

Bornman J (1989). Target sites of UV-B radiation in photosynthesis of higher plants. *Journal of Photochemistry and Photobiology*, 4: 145–58.

Brasseur G, Chatfield R (1991). The fate of biogenic trace gases in the atmosphere. In Trace Gas Emission by Plants. Sharkey T, Holland B, Money H, eds. New York, Academic Press: 1-28.

Brilli F, Barta C, Fortunati A, Lerdau M, Loreto F, Centritto M (2007). Response of isoprene emission and carbon metabolism to drought in white poplar (*Populus alba*) saplings. *New Phytologist*, 175: 244-254.

Brilli F, Ciccioli P, Frattoni M, Prestininzi M, Spanedda AF, Loreto F (2009). Constitutive and herbivore-induced monoterpenes emitted by *Populus x euroamericana* leaves are key volatiles that orient *Chrysomela populi* beetles. *Plant, Cell and Environment*, 32: 542–552.

Brilli F, Ruuskanen T, Schnitzhofer R, Müller M, Breitenlechner M, Bittner V, Wohlfahrt G, Loreto F, Hansel A (2011). Detection of plant volatiles after leaf wounding and darkening by proton transfer reaction ‘‘time-of-flight’’ mass spectrometry (PTR-TOF). *PLos ONE*, Vol. 6, Issue 5, e20419.

Burchard P, Bilger W, Weissenböck G (2000). Contribution of hydroxycinnamates and flavonoids to epidermal shielding of UV-A and UV-B radiation in developing rye primary leaves as assessed by ultravioletinduced chlorophyll fluorescence measurements. *Plant Cell and Environment*, 23: 1373-1380.

Buschmann, C (2007). Variability and application of the chlorophyll fluorescence emission ratio red/far-red of leaves. *Photosynthesis Research*, 92: 261-271.

Caldwell M, Dawson T, Richards J (1998). Hydraulic lift: consequences of water efflux from the roots of plants. *Oecologia*, 113: 151–161.

Caldwell M, Flint S (1994). Stratospheric ozone reduction, solar UV-B radiation and terrestrial ecosystems. *Climatic Change*, 28: 375–394.

Caldwell M, Flint S (1997). Uses of biological spectral weighting functions and the need of scaling for the ozone reduction problem. *Plant Ecology*, 128: 66-76.

Caldwell M, Teramura A, Tevini M (1989). The changing solar ultraviolet climate and the ecological consequences for higher plants. *Trends in Ecology and Evolution*, 4: 363–366.

Casati P, Walbot V (2003). Gene expression profiling in response to ultraviolet radiation in *Zea mays* genotypes with varying amounts of flavonoids. *Plant Physiology*, 132: 1739-1754.

Casati P, Walbot V (2004). Crosslinking of ribosomal proteins to RNA in vivo after UV-B irradiation of maize leaves. *Plant Physiology*, 136: 3319-3332.

Centritto M, Brilli F, Fodale R, Loreto F (2011a). Different sensitivity of isoprene emission, respiration, and photosynthesis to high growth temperature coupled with drought stress in black poplar (*Populus nigra*). *Tree Physiology* 31, 275-286.

Centritto M, Lauteri M, Monteverdi MC, Serraj R (2009). Leaf gas exchange, carbon isotope discrimination, and grain yield in contrasting rice genotypes subjected to water deficits during the reproductive stage. *Journal of Experimental Botany*, 60: 2325-2339.

Centritto M, Loreto F, Chartzoulakis K (2003). The use of low [CO2] to estimate diffusional and non-diffusional limitations of photosynthetic capacity of salt-stressed olive saplings. *Plant Cell and Environment*, 26: 585-594.

Centritto M, Nascetti P, Petrilli L, Raschi A, Loreto F (2004). Profiles of isoprene emission and photosynthetic parameters in hybrid poplars exposed to free-air CO_2 enrichment. *Plant, Cell and Environment*, 27: 403-412.

Centritto M, Tognetti R, Leitgeb E, Střelcová K, Cohen S (2011b). Above ground processes - Anticipating climate change influences. In: Bredemeier, M., Cohen, S., Godbold, D.L., Lode, E., Pichler, V., Schleppi, P., eds. Forest Management and the Water Cycle: An Ecosystem-Based Approach. Ecological Studies 212, Springer, 31-64.

Chameides W, Lindsay R, Richardson J, Kianf C (1988). The role of biogenic hydrocarbons in urban photoschemical smog: Atlanta as a case-study. *Science*, 241: 1473-1475.

Christie J, Briggs W (2001). Blue light sensing in higher plants. *The Journal of Biological Chemistry*, 276 (15): 11457-11460.

Christophe A, Moulia B, Varlet-Grancher C (2006). Quantitative contributions of blue light and PAR to the photocontrol of plant morphogenesis in *Trifolium repens* (L.). *The Journal of Experimental Botany*, 57: 2379–2390.

Close D, Mcarthur C, Hagerman A, Davies N, Beadle C (2007). Phenolic acclimation to ultraviolet-A irradiation in *Eucalyptus nitens* seedlings raised across a nutrient environment gradient. *Photosynthetica*, 45: 36-42.

Cojocariu C, Escher P, Haeberle K-H, Matyssek R, Rennenberg H, Kreuzwieser J (2005). The effect of ozone on the emission of carbonyls from leaves of adult *Fagus sylvatica*. *Plant, Cell and Environment*, 28: 603–611.

Copolovici L, Niinemets U (2010). Flooding induced emissions of volatile signalling compounds in three tree species with differing waterlogging tolerance. *Plant, Cell and Environment*, 33: 1582–1594.

Day T, Ruhland C, Grobe C, Xiong F (1999). Growth and reproduction of Antarctic vascular plants in response to warming and UV radiation reductions in the field. *Oecologia*, 119: 24-35.

De Gouw J, Warneke C (2007). Measurements of volatile organic compounds in the Earth's atmosphere using proton-transferreaction mass spectrometry. *Mass Spectrometry Reviews*, 26: 223-257.

Delwiche C, Sharkey T (1993). Rapid appearance of ^{13}C in biogenic isoprene when $^{13}CO_2$ is fed to intact leaves. *Plant, Cell and Environment*, 16: 587–591.

Di Carlo P, Brunw W, Martinez M, Harder H, Lesher R, Ren X, Thornbelly T, Carroll M, Young V, Shepson P, Riemer D, Ape E, Campbell C (2004). Missing OH reactivity in a forest: evidence for unknown reactive VOCs. *Science*, 304: 722-725.

Donaghy K (2007). Climate Change and Planning: Responding to the Challenge. *Town Planning Review*, 78(4).

Duysens L (1952). Transfer of excitation energy in photosynthesis. *Ph*D thesis, University of Utrecht, the Netherlands.

Etiope G, Ciccioli P (2009). Earth's degassing – A missing ethane and propane source. *Science*, 323, 5913, 478.

Evans J (1987). The dependence of quantum yield on wavelength and growth irradiance. Australian *Journal of Plant Physiology*, 14: 69-79.

Fall R (1999). Biogenic emissions of VOCs from higher plants. In: Hewitt, C.N. (Ed.), Reactive Hydrocarbons in the Atmosphere. Academic Press, San Diego, CA, 41–96.

Fall R (2003). Abundant oxygenates in the atmosphere: a biochemical perspective. *Chemical Reviews*, 103: 4941–4951.

Fall R, Wildermuth M (1998). Isoprene synthase: From biochemical mechanism to emission algorithm. *Journal of Geophysical Research*, 103: 25599-25609.

Faubert P, Tiiva P, Rinnan Å, Räsänen J, Holopainen J, Holopainen J, Kyrö E, Rinnan R (2010). Non-methane biogenic volatile organic compound emissions from a subarctic peatland under enhanced UV-B radiation. *Ecosystems*, 13: 860-873.

Filella I, Peñuelas J, Seco R (2009). Short-chained oxygenated VOC emissions in *Pinus halepensis* in response to changes in water availability. *Acta Physiologiae Plantarum*, 31: 311-318.

Flexas J, Ribas-Carbó M, Diaz-Espejo A, Galmés J, Medrano H (2008). Mesophyll conductance to CO_2: current knowledge and future prospects. *Plant, Cell and Environment*, 31: 602-621.

Forster P, Ramaswamy V, Artaxo P, Berntsen T, Betts R, Fahey D, Haywood J, Lean J, Lowe DC, Myhre G, Nganga J, Prinn R, Raga GMS, Van Dorland R (2007). Changes in Atmospheric Constituents and in Radiative Forcing. In: S. Solomon et al. (Editors), Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, U.K.

Fortunati A, Barta C, Brilli F, Centritto M, Zimmer I, Schnitzler J, Loreto F (2008). Isoprene emission is not temperature-dependent during and after severe drought stress: a physiological and biochemical analysis. *The Plant Journal*, 55: 687-697.

Fowler D, Flechard C, Skiba U, Coyle M, Cape NJ (1998). The atmospheric budget of oxidised nitrogen and its role in ozone formation and deposition. *New Phytologyst*, 139: 11-23.

Foyer C, Trebst A, Noctor G (2006). Signaling and integration of defense functions of tocopherol, ascorbate and glutathione. In Photoprotection, Photoinhibition, Gene Regulation, and Environment (eds B. Demmig-Adams, W.W. Adams III & A.K. Mattoo), pp. 241–268. Springer, Dordrecht, the Netherlands.

Frohnmeyer H, Staiger D (2003). Ultraviolet-B radiation-mediated responses in plants balancing damage and protection. *Plant Physiology*, 133: 1420-1428.

Funk J, Mak J, Lerdau M (2004). Stress-induced changes in carbon sources for isoprene production in Populus deltoides. *Plant, Cell and Environment*, 27: 747–755.

Galbally I, Kirstine W (2002). The production of methanol by flowering plants and the global cycle of methanol. *Journal of Atmospheric Chemistry*, 43: 195–229.

Galmés J, Flexas J, Keys AJ, Cifre J, Mitchell RAC, Madgwick PJ, Haslam RP, Medrano H, Parry MA (2005). Rubisco specificity factor tends to be larger in plant species from drier habitats and in species with persistent leaves. *Plant, Cell and Environment*, 28: 571-579.

Galmés J, Medrano H, Flexas J (2007). Photosynthetic limitations in response to water stress and recovery in Mediterranean plants with different growth forms. *New Phytologist*, 175: 81-93.

Gamon JA, Field CB, Bilger W, Björkman O, Fredeen AL, Peñuelas J (1990). Remote sensing of the xanthophyll cycle and chlorophyll fluorescence in sunflower leaves and canopies. *Oecologia*, 85: 1-7.

Gamon JA, Peñuelas J, Field CB (1992). A narrow-waveband spectral index that tracks diurnal changes in photosynthetic efficiency. *Remote Sensing Environment*, 41: 35-44.

Gamon JA, Serrano L, Surfus JS (1997). The photochemical reflectance index: An optical indicator of photosynthetic radiation use efficiency across species, functional types, and nutrient levels. *Oecologia*, 112: 492-501.

Gelsor N, Pingcuo N, Wangmu T, Kjeldstad B, Chen Y-C, Frette Ø, Stamnes JJ, Bhattarai BK, Dhalback A (2011). Solar UV Irradiance Measurements at Four Sites in Tibet. *Journal of the Institute of Engineering*, 8: 75-86.

Genty B, Briantais JM, Baker NR (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta*, 990: 87-92.

Gershenzon J, Dudareva N (2007). The function of terpene natural products in the natural world. *Nature Chemical Biology*, 3: 408-414.

Grote R, Niinemets U (2008). Modeling volatile isoprenoid emissions--a story with split ends. *Plant biology*, 10(1): 8-28.

Guenther A, Archer S, Greenberg J, Harley P, Helmig D, Klinger L, Vierling L, Wildermuth M, Zimmerman P, Zitzer S (1999). Biogenic hydrocarbon emissions and landcover/ climate change in a subtropical savannah. *Physics and Chemistry of the Earth Part B – Hydrology Oceans and Atmosphere*, 24(6): 659–667.

Guenther A, Zimmerman P, Harley P, Monson R, Fall R (1993). Isoprene and monoterpene emission rate variability: model evaluations and sensitivity analyses. *Journal of Geophysical Research*, 98: 12609-12617.

Guenther AB, Hewitt CN, Erickson D (1995). A global model of natural volatile organic compound emissions. *Journal of Geophysical Research Atmospheres*, 100: 8873-8892.

Guidolotti G, Calfapietra C, Loreto F (2011). The relationship between isoprene emission, CO_2 assimilation and water use efficiency across a range of poplar genotypes. *Physiologia Plantarum*, 142: 297-304.

Gurney RJ, Foster JL, Parkinson CL (1993). Atlas of satellite observations related to global change. *Cambridge University Press*, pp. 470, Cambridge.

Gwynn-Jones D, Johanson U, Phoenix GK, Gehrke C, Callaghan TV, Bjorn LO, Sonesson M, Lee JA (1999). UV-B impacts and interactions with other co-occurring variables of environmental change: an arctic perspective. In: Rozema J. (Ed.), Stratospheric ozone depletion. The effects of enhanced UV-B radiation on terrestrial ecosystems. Backhuys, Leiden, The Netherlands, 187–201.

Häder DP (1996). Effects of solar radiation on local and German wheat seedlings in a Chilean high mountain station. *Journal of Photochemistry and Photobiology B: Biology*, 35: 181-187.

Harley P, Deem G, Flint S, Caldwell M (1996). Effects of growth under elevated UV-B on photosynthesis and isoprene emission in *Quercus gambelii* and *Mucuna pruriens*. *Global Change Biology*, 2: 149-154.

Harley P, Greengerg J, Niinemets Ü, Guenther A (2007). Environmental controls over methanol emission from leaves. *Biogeosciences*, 4(6): 1083-1099.

Harley P, Loreto F, Di Marco G, Sharkey T (1992). Theoretical considerations when estimating the mesophyll conductance to CO_2 flux by analysis of the response of photosynthesis to CO_2. *Plant Physiology*, 98: 1429-1436.

Hegglin MI, Shepherd TG (2009). Large climate-induced changes in ultraviolet index and stratosphere-to-troposphere ozone flux. *Nature Geoscience*, 2: 687-691.

Herman JR (2010). Global increase in UV irradiance during the past 30 years (1979-2008) estimated from satellite data. *Journal of Geophysical Research* 115.

Inada K (1976). Action spectra for photosynthesis in higher plants. *Plant and Cell Physiology*, 17: 355–365.

IPCC (2007)a. Climate change 2007: the physical sciences bases. IPCC Fourth Assessment Report Chap. (3).

IPCC (2007)b. Climate change 2007: the physical sciences bases. IPCC Fourth Assessment Report Chap. (2).

Izaguirre MM, Scopel AL, Baldwin IT, Ballaré CL (2003). Convergent responses to stress. Solar ultraviolet-B radiation and *Manduca sexta* herbivory elicit overlapping transcriptional responses in field-grown plants of *Nicotiana longiflora*. *Plant Physiology*, 132: 1755-1767.

Jansen MAK, Gaba V, Greenberg BM (1998). Higher plants and UV-B radiation: balancing damage, repair and acclimation. *Trends in Plant Science*, 3: 131–135.

Jordan BR (1996). The effects of ultraviolet-B radiation on plants: a molecular perspective. *Advances in Botanical Research*, 22: 97–162.

Kaldenhoff R (2012). Mechanisms underlying CO_2 diffusion in leaves. *Current Opinion in Plant Biology*.

Karl T, Fall R, Jordan A, Lindinger W (2001). On-line analysis of reactive VOCs from urban lawn mowing. *Environmental Science and Technology*, 35: 2926–2931.

Karl T, Fall R, Rosenstiel TN, Prazeller P, Larsen B, Seufert (2002). On-line analysis of the $^{13}CO_2$ labeling of leaf isoprene suggests multiple subcellular origins of isoprene precursors. *Planta*, 215: 894–905.

Kasahara M, Kagawa T, Oikawa K, Suetsugu N, Miyao M, Wada M (2002). Chloroplast avoidance movement reduces photodamage in plants. *Nature*, 420: 829–832.

Kavouras LJ, Mihalopoulus N, Stephanou EG (1998). Formation of atmospheric particles from organic acids produced by forest. *Nature*, 395: 683-686.

Kesselmeier J, Staudt M (1999). Biogenic volatile organic compounds (VOC): an overview on emission. *Journal of Atmospheric Chemistry*, 33: 23–88.

Klem K, Ač A, Holub P, Kováč D, Špunda V, Robson TM, Urban O (2012). Interactive effects of PAR and UV radiation on the physiology, morphology and leaf optical properties of two barley varieties. *Environmental and Experimental Botany*, 75: 52-64.

Koning RE (1994). Light. *Plant Physiology Information Website*, http://plantphys.info/plant_physiology/light.shtml.

Kotilainen T, Tegelberg R, Julkunen-Tiitto R, Lindfors A, Aphalo PJ (2008). Metabolite specific effects of solar UV-A and UV-B on alder and birch leaf phenolics. *Global Change Biology*, 14: 1294-1304.

Kotilainen T, Tegelberg R, Julkunen-Tiitto R, Lindfors A, O'Hara RB, Aphalo PJ (2010). Seasonal fluctuations in leaf phenolic composition under UV manipulations reflect contrasting strategies of alder and birch trees. *Physiologia Plantarum*, 140: 297-309.

Kreuzwieser J, Harren FJM, Laarhoven LJ, Boamfa I, te Lintel-Hekkert S, Scheerer U, Hüglin C, Rennenberg H (2001). Acetaldehyde emission by the leaves of trees-correlation with physiological and environmental parameters. *Physiologia Plantarum*, 113: 41-49.

Kreuzwieser J, Kuehnemann F, Martis A, Rennenberg H, Urban W (2000). Diurnal pattern of acetaldehyde emission by flooded poplar trees. *Physiologia Plantarum*, 108: 79–86.

Kreuzwieser J, Scheerer U, Rennenberg H (1999). Metabolic origin of acetaldehyde emitted by poplar (*Populus tremula* × *P. alba*) trees. *Journal of Experimental Botany*, 50: 757–765.

Lai R (1999). World soils and the greenhouse effect. *IGBP Newsletter* 37: 4-5.

Laisk A, Loreto F (1996). Determining photosynthetic parameters from leaf CO_2 exchange and chlorophyll fluorescence: rubisco specificity factor, dark respiration in the light, excitation distribution between photosystems, alternative electron transport rate and mesophyll diffusion resistance. *Plant Physiology*, 110: 903-912.

Leicht SA, Silander Jr JA (2006). Differential responses of invasive *Celastrus orbiculatus* (Celastraceae) and native *C. scandens* to changes in light quality. *American Journal of Botany*, 93(7): 972-977.

Li F-R, Peng S-L, Chen B-M, Hou Y-P (2010). A meta-analysis of the responses of woody and herbaceous plants to elevated ultraviolet-B radiation. *Acta Oecologica*, 36: 1-9.

Li J, Ou-Lee T, Raba R, Amundson RG, Last RL (1993). Arabidopsis flavonoid mutants are hypersensitive to UV-B Irradiation. *The Plant Cell*, 5: 171-179.

Lindinger W, Hansel A, Jordan A (1998). Proton-transfer-reaction mass spectrometry (PTR-MS): On-line monitoring of volatile organic compounds at pptv level. *Chemical Society Reviews*, 27: 347-354.

Loreto F, Centritto M (2008). Leaf carbon assimilation in a water-limited world. *Plant Biosystems*, 142: 154-161.

Loreto F, Centritto M, Barta C, Calfapietra C, Fares S, Monson R (2007). The relationship between isoprene emission rate and dark respiration rate in white poplar (*Populus alba* L.) leaves. *Plant, Cell and Environment*, 30: 662-669.

Loreto F, Schnitzler JP (2010). Abiotic stresses and induced BVOCs. *Trends in Plant Science*, 15: 154-166.

Loreto F, Sharkey T (1990). A gas- exchange study of photosynthesis and iso- prene emission in Quercus rubra L. *Planta*, 182: 523–31.

Loreto F, Tsonev T, Centritto M (2009). The impact of blue light on leaf mesophyll conductance. *Journal of Experimental Botany*, 60: 2283-2290.

Loreto F, Velikova V (2001). Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. *Plant Physiology*, 127: 1781–1787.

Madronich S, McKenzie R, Bjorn R, Caldwell M (1998). Changes in biologically active ultraviolet radiation reaching the Earth's surface. *The Journal of Photochemistry and Photobiology B: Biology*, 46: 5-19.

McCree KJ (1972). The action spectrum, absorptance and quantum yield of photosynthesis incrop plants. *Agricultural Meteorology*, 9: 191-216.

McKenzie RL, Aucamp PJ, Bais AF, Björn LO, Ilyas M, Madronich S (2011). Ozone depletion and climate change: impacts on UV radiation. *Photochemical & Photobiological Sciences*, 10: 182-198.

Meyer M, Seibt U, Griffiths H (2008). To concentrate or ventilate? Carbon acquisition, isotope discrimination and physiological ecology of early land plant life forms. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 363: 2767-2778.

Mitchell JFB (1989). The "Greenhouse" effect and climate change. *Reviews of Geophysics*, VOL. 27, 1: 115-139.

Mittler R (2002). Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science*, 7: 405–410.

Monson R, Fall R (1989). Isoprene emission from aspen leaves. The influence of environment and relation to photosynthesis and photorespiration. *Plant Physiology*, 90:267–74.

Monson R, Lerdau M, Sharkey T, Schimel T, Fall R. (1995). Biological aspects of constructing volatile organic-compound emission inventories. *Atmospheric Environment*, 29: 2989-3002.

Moore R, Clark W, Vodopich D (2003). Botany. 2nd ed. New York, NY: McGraw-Hill companies, Inc. p. 136-137.

Morales L, Tegelberg R, Brosché M, Keinänen M, Lindfors A, Aphalo P (2010). Effects of solar UV-A and UV-B radiation on gene expression and phenolic accumulation in *Betula pendula* leaves. *Tree Physiology*, 30: 923-34.

Morales L, Tegelberg R, Brosché M, Lindfors A, Siipola S, Aphalo P (2011). Temporal variation in epidermal flavonoids due to altered solar UV radiation is moderated by the leaf position in *Betula pendula*. *Physiologia Plantarum*, 143: 261-70.

Mpoloka S (2008). Effects of prolonged UV-B exposure in plants. *Journal of Biotechnology*, 7(25): 4874-4883.

Nemecek-Marshall M, MacDonald R, Franzen J, Wojciechowski C, Fall R (1995). Methanol Emission from Leaves. Enzymatic Detection of Gas-Phase Methanol and Relation of Methanol Fluxes to Stomatal Conductance and Leaf Development. *Plant Physiology*, 108: 1359-1368.

Niinemets Ü (2010). Mild versus severe stress and BVOCs: thresholds, priming and consequences. *Trends in Plant Science*, 15: 145-153.

Niinemets Ü, Monson R, Arneth A, Ciccioli P, Kesselmeier J, Kuhn U, Noe S, Peñuelas J, Staudt M (2010). The leaf-level emission factor of volatile isoprenoids: caveats, model algorithms, response shapes and scaling. *Biogeosciences*, 7: 1809-1832.

Niinemets Ü, Wright I, Evans J (2009). Leaf mesophyll diffusion conductance in 35 Australian sclerophylls covering a broad range of foliage structural and physiological variation. *Journal of Experimental Botany*, 60: 2433-2449.

Niinemets Ü, Tenhunen J, Harley P, Steinbrecher R (1999). A model of isoprene emission based on energetic requirements for isoprene synthesis and leaf photosynthetic properties for Liquidambar and Quercus. *Plant Cell and Environment*, 22: 1319-1335.

Noctor G, Foyer C (1998). Ascorbate and glutathione: Keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular*, 49: 249–279.

Norsang G, Kocbach L, Tsoja W, Stamnes J, Dahlback A, Nema P (2009). Ground-based measurements and modeling of solar UV-B radiation in Lhasa, Tibet. *Atmospheric Environment*, 43: 1498-1502.

Oguchi R, Terashima I, Chow W (2009). The involvement of dual mechanisms of photoinactivation of Photosystem II in *Capsicum annuum* L. plants. *Plant and Cell Physiology*, 50(10): 1815–1825.

Ohko Y, Tryk D, Hashimoto K, Fujishima A (1998). Autoxidation of acetaldehyde initiated by TiO2 photocatalysis under weak UV illumination. *The Journal of Physical Chemistry B* , 102: 2699-2704.

Ohnishi N, Allakhverdiev S, Takahashi S, Higashi S, Watanabe M, Nishiyama Y, Murata N (2005). Two-step mechanism of photodamage to photosystem II: step 1 occurs at the oxygen-evolving complex and step 2 occurs at the photochemical reaction center. *Biochemistry*, 44: 8494–8499.

Peñuelas J, Llusià J, Asensio D, Munné-Bosch S (2005). Linking isoprene with plant thermotolerance, antioxidants, and monoterpene emissions. *Plant, Cell and Environment*, 28: 278-296.

Rasmussen RA (1972). What do hydrocarbons from trees contribute to air pollution? *Journal of Air Pollution*, 22: 537-542.

Reifenrath K, Müller C (2007). Species-specific and leaf-age dependent effects of ultraviolet radiation on two Brassicaceae. *Phytochemistry*, 68: 875-885.

Ren W, Tan H, Wu J, Deng Y, Wu Y, Tang Y, Cui X (2010). UV light spectral response of photosynthetic photochemical efficiency in alpine mosses. *Journal of Plant Ecology*, 3: 17-24.

Rodeghiero M, Niinemets Ü, Cescatti A (2007). Major diffusion leaks of clamp-on leaf cuvettes still unaccounted: how erroneous are the estimates of Farquhar et al. model parameters? *Plant, Cell and Environment*, 30: 1006-1022.

Rosenstiel T, Ebbets A, Khatri W, Fall R, Monson R (2004). Induction of poplar leaf nitrate reductase: a test of extrachloroplastic control of isoprene emission rate. *Plant Biology*, 6: 12-21.

Rosenstiel T, Potosnak M, Griffin K, Fall R, Monson R (2003). Increased CO_2 uncouples growth from isoprene emission in an agriforest ecosystem. *Nature*, 421: 256-259.

Rozema J, Gieskes W, van der Geijn S, Nola C, de Boois H (1997). Special issue: UV-B and biosphere. **Plant Ecology**, 128: 1-319.

Ruuskanen T, Kolari P, Bäck J, Kulmala M, Rinne J, Hakola H, Taipale R, Raivonen M, Altimir N, Hari P (2005). On-line field measurements of monoterpene emissions from Scots pine by proton-transfer-reaction mass spectrometry. *Boreal Environment Research*, 10: 553-567.

Sanadze G (1969). Light-dependent excretion of molecular isoprene. *Progress in Photosynthesis Research*, 2: 701–706.

Sanadze G, Dzhaiani G, Tevzadze I (1972). Incorporation into the isoprene molecule of carbon from $^{13}CO_2$ assimilated during photosynthesis. *Soviet Plant Physiology*, 19: 17–20.

Sävenstrand H, Brosché M, Strid A (2002). Regulation of gene expression by low levels of ultraviolet-B radiation in *Pisum sativum*: isolation of novel genes by suppression subtractive hybridisation. *Plant Cell Physiology*, 43: 402–410.

Schnabl N, Weissenböck G, Scharf H (1986). In vivo microspectrophotometric characterization of flavonol glycosides in *Vicea faba* guard and epidermal cells. *Journal of Experimental Botany*, 37: 61-72.

Schnitzler JP, Graus M, Kreuzwieser J, Heizmann U, Rennenberg H, Wisthaler A (2004). Contribution of different carbon sources to isoprene biosynthesis in poplar leaves. *Plant Physiology*, 135: 152–160.

Schurgers G, Arneth A, Holzinger R, Goldstein A (2009). Process-based modelling of biogenic monoterpene emissions combining production and release from storage. *Atmospheric Chemistry and Physics*, 9: 3409-3423.

Sharkey T (2005). Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions, rubisco deactivation, reactive oxygen species, and thermotolerance provided by isoprene. *Plant, Cell and Environment*, 28: 269–277.

Sharkey T, Chen X, Yeh S (2001). Isoprene increases thermotolerance of fosmidomycin-fed leaves. *Plant Physiology*, 125: 2001–2006.

Sharkey T, Loreto F (1993). Water stress, temperature, and light effects on the capacity for isoprene emission and photosynthesis of kudzu leaves. *Oecologia*; 95: 328-333.

Sharkey T, Raschke K (1981). Effect of light quality on stomatal opening in leaves of *Xanthium strumarium* L. *Plant Physiology*, 68: 1170-1174.

Sharkey T, Singsaas E (1995). Why plants emit isoprene. *Nature*, *374*: 769.

Sharkey T, Yeh S (2001). Isoprene emission from plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, 52: 407–436.

Shi Z, Cheng R, Liu S, Sorrentino G, Centritto M (2008). Carbon assimilation, δ13C and water relations of *Elaeagnus angustifolia* grown at two groundwater depths in the Minqin desert, China. *Plant Biosystems*, 142: 525-532.

Shi Z, Liu S, Liu X, Centritto M (2006). Altitudinal variation in photosynthetic capacity, diffusional conductance, and δ13C of butterfly bush (Buddleja davidii Franch.) plants growing at high elevations. *Physiologia Plantarum*, 128: 722-731.

Silver G, Fall R (1991). Enzymatic synthesis of isoprene from dimethylallyl diphosphate in aspen leaf extracts. *Plant Physiology*, 97: 1588–1591.

Silver G, Fall R (1995). Characterization of aspen isoprene synthase, an enzyme responsible for leaf isoprene emission to the atmosphere. *Journal of Biological Chemistry*, 270: 13010–13016.

Singsaas E, Lerdau M, Winter K, Sharkey T (1997). Isoprene increases thermotolerance of isoprene-emitting leaves. *Plant Physiology*, 115: 1413–1420.

Singsaas E, Sharkey T (2000). Regulation of isoprene synthesis during high temperature stress. *Plant, Cell and Environment*, 23: 751–757.

Siwko M, Marrink S, de Vries A, Kozubek A, Uitercamp A, Mark A (2007). Does isoprene protect plant membranes from thermal shock? A molecular dynamics study. *Biochimica et Biophysica Acta*. 1768: 198–296.

Staudt M, Joffre R, Rambal S (2003). How growth conditions affect the capacity of Quercus ilex leaves to emit monoterpenes. *New Phytologist*, 158: 61-73.

Staudt M, Seufert G (1995). Light-dependent emission of monoterpenes by holm oak (*Quercus ilex* L.). *Naturwissenschaften*, 82: 89-92.

Staudt M, Seufert G, Kotzias D, Spartà C, Ciccioli P (1993). Holm oak (*Quercus ilex*) a strong emitter of monoterpenes, in *Proceedings of the 1st Italian Symphosium on the Strategies and Techniques for the Monitoring of the Atmosphere*, edited by P.Ciccioli, *Società Chimica Italy*, 579-586.

Sullivan J, Teramura A, Ziska L (1992). Variation in UV-B sensitivity in plants from a 3,000-m elevational gradient in Hawaii. *American Journal of Botany*, 79: 737–743.

Sun P, Grignetti A, Liu S, Casacchia R, Salvatori R, Pietrini F, Loreto F, Centritto M (2008). Associated changes in physiological parameters and spectral reflectance indices in olive (*Olea europaea* L.) leaves in response to different levels of water stress. *International Journal of Remote Sensing*, 29: 1725-1743.

Taiz L, Zeiger E (2002). Plant Physiology, Ed 3. Sinauer Associates, Sunderland, MA, pp 591–623.

Takahama U, Oniki T (1992). Regulation of peroxidase-dependent oxidation of phenolics in the apoplast of spinach leaves by ascorbate. *Plant Cell Physiology*, 33: 379-387.

Taylor A, Assmann S (2001). Apparent absence of a redox requirment for blue light activation of pump current in broad bean guard cells. *Plant Physiology*, 125: 329-338.

Teramura A, Sullivan J (1994). Effects of UV-B radiation on photosynthesis and growth of terrestrial plants. *Photosynthesis Research*, 39: 463–473.

Terashima I, Hanba Y, Tholen D, Niinemets Ü (2011). Leaf functional anatomy in relation to photosynthesis. *Plant Physiology*, 155: 108-116.

Tholen D, Boom C, Noguchi K, Ueda S, Katase T, Terashima I (2008). The chloroplast avoidance response decreases internal conductance to CO_2 diffusion in *Arabidopsis thaliana* leaves. *Plant Cell and Environment*, 31: 1688-1700.

Tiiva P, Rinnan R, Faubert P, Räsänen J, Holopainen T, Kyrö E, Holopainen J (2007). Isoprene emission from a subarctic peatland under enhanced UV-B radiation. *New Phytologist*, 176: 346-355.

Toome M, Randjärv P, Copolovici L, Niinemets Ü, Heinsoo K, Luik A, Noe S (2010). Leaf rust induced volatile organic compounds signalling in willow during the infection. *Planta*, 232: 235-243.

Tsuboi H, Wada M (2011). Chloroplasts can move in any direction to avoid strong light. *Journal of Plant Research*, 124: 201-210.

Turtola S, Sallas L, Holopainen J, Julkunen-Tiitto R, Kainulainen P (2006). Long-term exposure to enhanced UV-B radiation has no significant effects on growth or secondary compounds of outdoor-grown Scots pine and Norway spruce seedlings. *Environmental Pollution*, 144: 166-171.

UNEP, United Nations Environment Programme (2002). Scientific Assessment of Ozone Depletion: 2002. Global Ozone Research and Monitoring Project 47, World Meteorological Organization, Nairobi, Kenya.

UNEP, United Nations Environment Programme (2010). Environmental effects of ozone depletion and its interactions with climate change: 2010 assessment. Nairobi, Kenya.

Velikova V (2008). Isoprene as a tool for plant protection against abiotic stresses. *Journal of Plant Interactions*, 3: 1-15.

Velikova V, Loreto F (2005). On the relationship between isoprene emission and thermotolerance in *Phragmites australis* leaves exposed to high temperatures and during the recovery from heat stress. *Plant, Cell and Environment*, 28: 318-327.

Velikova V, Tsonev T, Loreto F, Centritto M (2011). Changes in photosynthesis, mesophyll conductance to CO_2, and isoprenoid emissions in *Popolus nigra* plants exposed to excess nickel. *Environmental Pollution*, 159: 1058-1066.

Vickers CE, Possell M, Cojocariu C, Velikova VB, Laothawornkitkul J, Ryan A, Mullineaux PM, Hewitt CN (2009). Isoprene synthesis protects transgenic tobacco plants from oxidative stress. *Plant Cell and Environment*, 32: 520-531.

Wada M, Kagawa T, Sato Y (2003). Chloroplast movement. *Annual Review of Plant Biology*, 54: 455–468.

Wellman E (1983). UV radiation in photomorphogenesis. - In: Shropshire W & Mohr JRH (eds.), Encyclopedia of plant physiology: New series 16B: 745-756. Springer-Verlag, Berlin, Heidelberg, New York, Tokyo.

Welp L, Keeling R, Meijer H, Bollenbacher A, Piper S, Yoshimura K, Francey R (2011). Interannual variability in the oxygen isotopes of atmospheric CO2 driven by El Niño. *Nature*, 477(7366): 579-82.

Whitelam G, Halliday K (2007). Light and plant development. Oxford: Blackwell Publishing.

Wildermuth M, Fall R (1996). Light-dependent isoprene emission. Characterization of a thylakoid-bound isoprene synthase in *Salix discolor* chloroplasts. *Plant Physiology*, 112: 171-182.

Winter T, Rostás M (2008). Ambient ultraviolet radiation induces protective responses in soybean but does not attenuate indirect defense. *Environmental Pollution*, 155: 290-297.

WMO (1995). Scientific Assessmetit of Ozone Depletion: 1994. Global Ozone Research and Monitoring Project of the World Meteorological Organization.

Yang Y, Yao Y (2008). Photosynthetic responses to solar UV-A and UV-B radiation in low-and high-altitude populations of *Hippophae rhamnoides*. *Photosynthetica*, 46: 307-311.

Zepp R, Erickson D, Paul N, Sulzberger B (2007). Interactive effects of solar UV radiation and climate change on biogeochemical cycling. *Photochemical & Photobiological Sciences*, 6: 286-300.

Zimmer W, Bruggemann N, Emeis S, Giersch C, Lehning A, Steinbrecher R, Schnitzler J-P (2000). Process-based modeling of isoprene emission by oak leaves. *Plant Cell and Environment*, 23: 585-595.