BULGARIAN ACADEMY OF SCIENCES

Institute of Plant Physiology and Genetics

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Physiological role of biogenic isoprene in plants

SUMMARY

A dissertation submited for the degree of Doctor of Science

professional speciality 4.3. Biological sciences,
scientific speciality Plant Physiology

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Sofia, 2019

The dissertation was discussed and admitted to defense at a meeting of an extended scientific seminar of the "Photosynthesis - activity and regulation" laboratory of the Institute of Plant Physiology and Genetics at the Bulgarian Academy of Sciences.

The dissertation consists of 422 pages. The main text is presented in 7 parts, illustrated with 67[1] figures. 379 references are cited. The achievements of 20 scientific publications (19 in Q1 and 1 in Q2, with a total impact factor of 86,851) are summarized.

Acknowledgements

The research involved in the dissertation was made possible by the financial support of: **NATO** (individual fellowships 2000-2001, 2002; projects LST.CLG 978838 / 2002-2003; EAP.RIG 981279 / 2004-2007); **Bulgarian National Science Fund** (TKB-1604 / 2007-2010; B02-8 / 2014-2017); **Ministry of Education and Science** (D01-168 / 2014-2016); **Bilateral projects with Italy** (2004-2006; 2007-2009; 2010-2012) and **Hungary** (2010-2012); **Maria Curie EC** (2011); **Alexander von Humboldt Foundation** (2012-2014).

I would like to express my gratitude to Prof. Francesco Loreto, from National Research Council of Italy, not only for introducing me to the field of biogenic organic compaunds, but also for his valuable guidance and constant support. His positive attitude and constant desire for new discoveries in science has motivated me during all these years. I feel fortunate to have had the opportunity to collaborate with him.

I am also very grateful to Prof. Jörg-Peter Schnitzler, who gave me the opportunity to join his group at the Helmholtz Center - Munich, for his unconditional support and advice for the successful implementation of the project funded by the Alexander von Humboldt Foundation.

A special thanks to my family and friends for their continued support.

The defense will take place on ..... at ..... at the meeting room of the Institute of Plant Physiology and Genetics - BAS. The dissertation is available to those interested in the office of the Institute of Plant Physiology and Genetics, Sofia, Acad. G. Bonchev Str., Bl. 21, fl.2.

[1] The numbering of the figures in the abstract is in line with that of the dissertation and the corresponding articles.

LIST OF ABBREVIATIONS

ATP – adenosine triphosphate

NADPH – nicotinamide adenine dinucleotide phosphate

Φ_{PSII} – operating photochemical efficiency of PSII

$^1O_2$ – singlet oxygen

CD – circular dichroism

C_i – intercellular CO_2 concentration

C_c – chloroplastic CO_2 concentration

FTSW – fraction of transpirable soil water

g_m – mesophyll conductace

g_s – stomatal conductance

MDA – malondialdehyde

MEP – 2-C-methylerythriol 4-phospate

NO – nitrogen oxide

NPQ – nonphotochemical quenching

PSII – photosystem II

RB – Rose Bengal

ROS – reactive oxygen species

SOA – secondary organic aerosols

TL – thermoluminescence

WUE – water used efficiency

ΔA_{515} – electrochromic absorbance transients at 515 nm

OPLS analysis – Orthogonal Partial Least Squares analysis

INTRODUCTION

Globally, plants release vast amounts of volatile organic compounds equivalent to about 1300 Tg of carbon per year, and these compounds are about 10 times more than other classes of volatile matter. The total anthropogenic emissions of organic compounds are estimated to be about 150 Tg of carbon per year. Isoprene is the most abundant and dominant biogenic volatile organic compound emitted from a wide range of plant species. Globaly, it is estimated that isoprene emission (360-750 Tg C yr^{-1}) represent about 44% of the global biogenic volatile organic emissions. Plants do not possess structures where to store isoprene and after its formation it is released into the atmosphere. Owing to its high volatility and reactivity, isoprene plays an important role in atmospheric chemistry. Once isoprene releases into the atmosphere, in the presence of sunlight and nitrogen oxides, hydroxyl radicals initiate reactions leading to the conversion of isoprene to toxic photochemical products (formaldehyde, methacrolein and methyl vinyl ketone). The oxidation of isoprene in the presence of nitrogen oxides forms enormous amounts of peroxides (~100 Tg C yr^{-1}), organic nitrates and peroxyacetyl nitrates, which are toxic in a few ppb. In the presence of nitrogen oxides and hydroxyl radicals, isoprene reacts in the atmosphere, resulting in the formation of carbon monoxide, organic peroxides and tropospheric ozone. Though isoprene is not a greenhouse gas, it can also alter atmospheric chemistry, affecting the residence times of gases that do contribute to the greenhouse effect, such as methane. Isoprene oxidation products are important constituents of secondary organic aerosol particles (SOA). Biogenic SOAs are estimated to be about 10 times higher than those of anthropogenic origin. Increasing biogenic organic emissions by 50% due to climate change will lead to an increase in SOA by 19%, with serious consequences for the radiation balance of earth by scattering and absorbing light and participating in cloud formation. Thus, isoprene emissions have far-reaching impact on air quality, global tropospheric chemistry and climate change, implying that it is essential to investigate the impacts of environmental factors on isoprene emissions to predict global climate change and propose environmental management policies for future.

Given the essential importance of biogenic isoprene for the physical and chemical properties of the atmosphere, including air quality, understanding how environmental factors control isoprene emission from plants is of particular importance for accurate predictions of changes in atmospheric chemistry, for an adequate assessment of the vulnerability and flexibility of terrestrial ecosystems in the face of global climate change, as well as the development of a concept for the selection of suitable plant species for afforestation of areas with different anthropogenic and industrial pollution.

The interest in the study of biogenic isoprene is associated not only because its importance for atmospheric chemistry, but also because the suggested protective role in plants experiencing various stress effects. In non-stressed conditions about 0.5 – 2% of the carbon assimilated during photosynthesis is re-emitted to the atmosphere as isoprene. However, this percentage increases dramatically under stress conditions, and isoprene synthesis is energetically costly for the plants (20 ATP and 14 NADPH molecules per isoprene molecule). What benefit, if any, do plant drive from isoprene emission is a question needs an answer. The reason of this “wasteful” metabolic issue has attracted the attention of many researchers. Due to the strong control of isoprene emission from light and temperature, it is assumed that isoprene has a thermo-protective effect on the basic physiological process in plants - photosynthesis. Another hypothesis has been argued that isoprene as an antioxidant can be a universal mechanism of action that explains both its antioxidant properties and its thermal protection function. However, it remains unclear why some plants would use this antioxidant mechanism while others would not, especially considering the antioxidant systems in chloroplasts and mitochondria.

The present dissertation summarizes the author's contributions to clarifying the physiological role of biogenic isoprene. Through the application of diverse research approaches (studies at physiological, biophysical, biochemical and structural levels), the dissertation provides evidence for the ability of endogenous isoprene to increase plant resistance to abiotic stress, as well as possible mechanisms related to the protective role of isoprene. The interaction between endogenous isoprene and nitric oxide *in planta* has been studied to elucidate the possible joint action of the two molecules under stress (**papers V, VIII and XIII**). Studies examining the role of biogenic isoprene in reducing the negative effects of oxidative stress (**papers I, II, and IX**) are summarized. A substantial part of the dissertation is devoted to the thermoprotective role of isoprene (**papers III, IV, VI, X and XII**). Its contribution to increased plant thermal tolerance is explained by the increased stability of photosynthetic membranes. The hydrophobic isoprene molecule is thought to "embed" into the lipid bilayer of the thylakoid membranes and affect the dynamics of the membrane-bound proteins. The implications of the inhibition of isoprene biosynthesis on the protein and lipid composition, as well as chloroplast ultrastructure have been clarified (**papers XIV, XVII and XVIII**). For the first time, a scheme for the role of isoprene as part of the whole plant protection system has been proposed (**papers XVI**). Its interaction with both non-volatile isoprenoids and metabolites, products of other biosynthetic pathways (eg phenylpropanoids) has been established (**papers XV, XIX and XX**). As an important factor in the atmospheric chemistry, the role of biogenic isoprene in anthropogenic pollution has been investigated (**papers VII and XI**).

AIM:

The main scientific PURPOSE of the dissertation is to reveal the physiological significance of biogenic isoprene as a tool for plant protection against abiotic stress factors.

HYPOTHESES:

I. Environmental factors have a strong effect on biogenic isoprene emission and in the same time it represents a non-trivial carbon loss to the plants, thus it is essential for plant tolerance, which manifests at functional, proteome, metabolic and structural levels.

II. Endogenous isoprene regulates the generation of reactive nitrogen and oxygen species in cells and thus determines plant response to stress.

III. Biogenic isoprene, as part of the plant antioxidant system, works in sync with other protective metabolites, providing better protection against stress.

TASKS:

1. To investigate the possible protective effect of isoprene against various abiotic factors (anthropogenic pollution, high temperature, elevated [CO_2] in the ambient air, ozone, singlet oxygen, drought).

2. To elucidate the interaction between isoprene and nitric oxide *in planta*, focusing on the consequence of this interaction under oxidative stress.

3. To study the changes in the chloroplast protein profile and overall cell proteome, lipid composition of thylakoid membranes and chloroplast ultrastucture of plants altered in their isoprene emission capability.

4. To clarify the relationship between isoprenoids and phenylpropanoids under optimal and stressful conditions.

3. STUDY APPROACHES AND METHODS

3.1. Plant material

The plant material used includes **(1)** plant species emitting isoprene as natural metabolite (*Phragmites australis*, *Platanus orientalis* L., *Platanus* x *acerifolia* L., *Populus nigra*, *Populus* x *canescens*, *Arundo donax*); **(2)** non-isoprene emitting species (*Hakonechloa macra*), and **(3)** plant species with altered ability to emit isoprene by genetic manipulation (*Arabidopsis thaliana*, *Nicotiana tabacum* cv. Samsun; *Populus* x *canescens*); **(4)** leaves with manipulated isoprene emission: through chemical inhibition by fpsmidomycin, which specifically blocks MEP pathway in the chloroplasts (Zeidler et al. 1998); leaves, developed under elevated [CO_2] atmosphere; leaves in different developmental stages, as well as plants in different ages (**Fig. 3**).

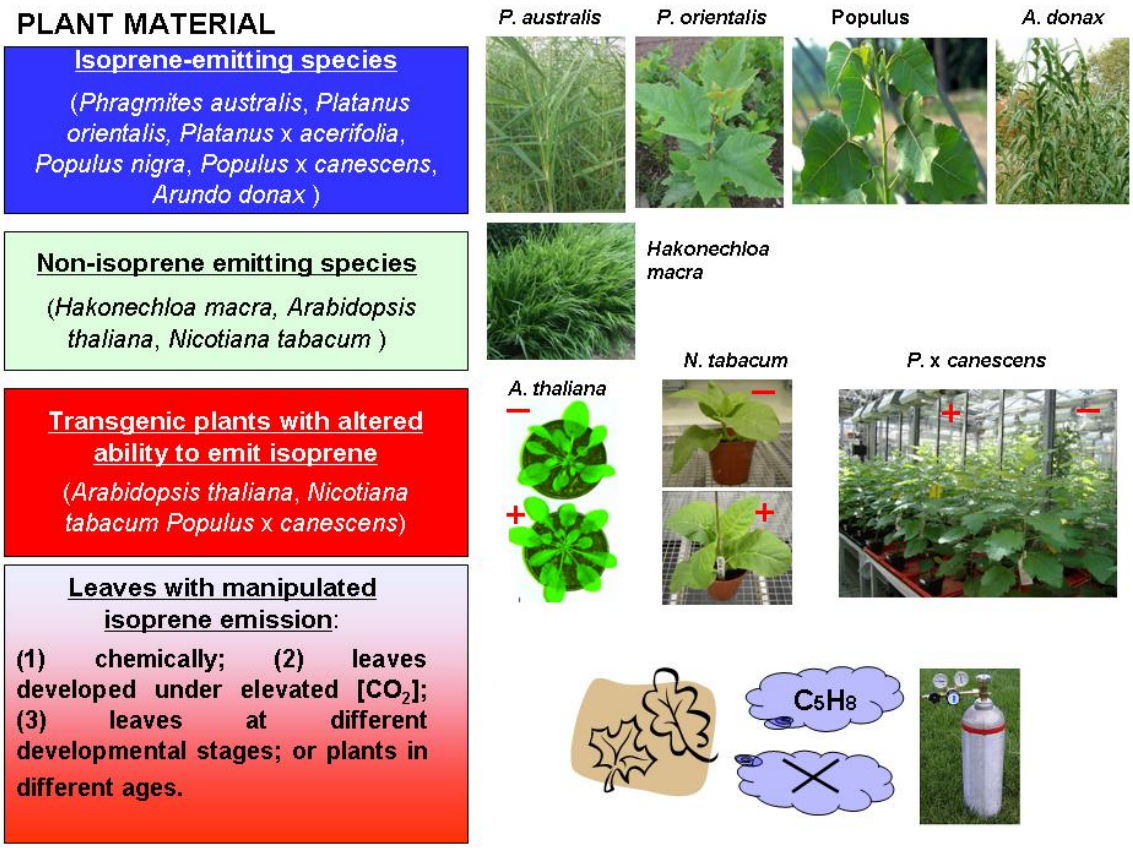

Fig. 3. Plant material used

3.2. Methods

3.2.A. Physiological analyses

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3.2.D3. phenylpropanoids (hydroxicinamic acids; деривати на quercetin, kaempferol, luteolin, apigenin).
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3.2.D14. Superoxide dismutase (EC 1.15.1.1)
3.2.D15. Antioxidant (FRAP) and antiradical activity ($DPPH^{\bullet}$).
3.2.E. Structural analysis – light microscopy and transmission electron microscopy
3.2.F. Statistical analysis

4. RESULTS AND DISCUSSION

4.1. Antioxidant role of biogenic isoprene

Many plants invest carbon to form isoprene. The role of isoprene in plants is unclear, but many experiments showed that isoprene may have a role in protecting plants from thermal damage. A more general antioxidant action has been recently hypothesized on the basis of the protection offered by exogenous isoprene in nonemitting plants exposed to acute ozone doses.

The antioxidant capacity of biogenic isoprene was studied in a series of experiments. We used different plant species (*Phragmites australis* and transgenic tobacco). Oxidative stress was induced by ozone fumigation and treatment with Rose Bengal (a dye which generate singled oxygen upon illumination).

4.1.A. Endogenous isoprene protects photosynthetic apparatus against oxidative stress due to ozone fumigation **(paper I. Loreto & Velikova – Plant Physiology 127:1781-1787, 2001)**

Phragmites australis leaves with inhibited isoprene emision by feeding fosmidomycin became more sensitive to ozone than those leaves forming isoprene. Photosynthesis, stomatal conductance, and chlorophyll fluorescence parameters were significantly affected by ozone only in leaves on which isoprene was not formed (**Fig. I.2**). The protective effect of isoprene was more evident when the leaves were exposed for a long time (8 h) to

relatively low (100 nL L^{-1}) ozone levels than when the exposure was short and acute (3 h at 300 nL L^{-1}).

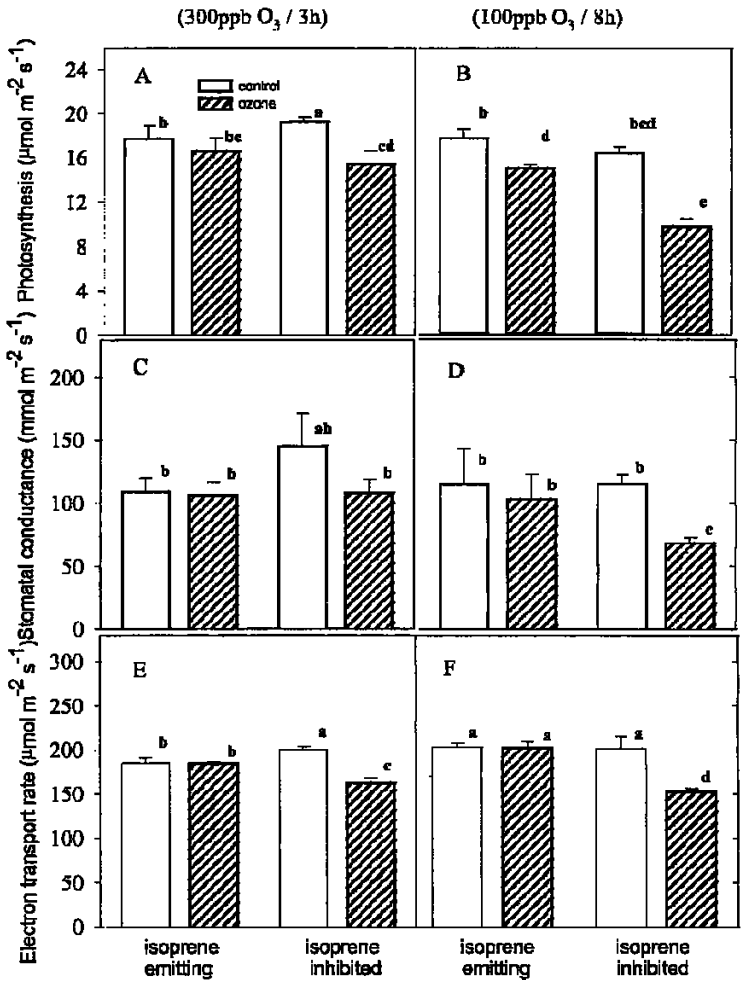


Fig. I.2. Effect of short-term (3 h, 300 nL L^{-1}; left) and long-term (8 h, 100 nL L^{-1}; right) ozone treatments on photosynthesis (**A and B**), stomatal conductance (**C and D**), and electron transport rate (**E and F**) of *P. australis* leaves (Loreto & Velikova – Plant Physiology 127:1781-1787, 2001).

Isoprene quenched the amount of H_2O_2 formed in leaves (**Fig. I.5**) and reduced lipid peroxidation (**Fig. I.6**) of cellular membranes caused by ozone. These results indicate that isoprene may exert its protective action at the membrane level, although a similar effect could be obtained if isoprene reacted with ozone before forming active oxygen species. Irrespective of the mechanism, our results suggest that endogenous isoprene has an important antioxidant role in plants.

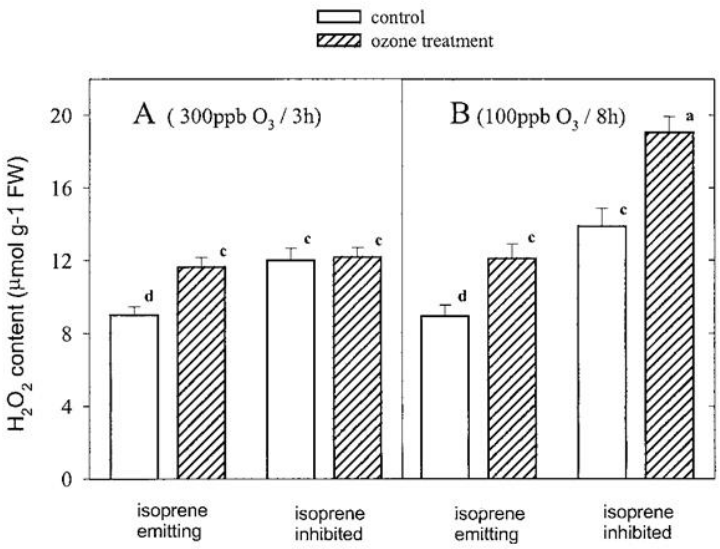


Fig. I.5. Effect of short-term (3 h, 300 nL L^{-1}; **A**) and long-term (8 h, 100 nL L^{-1}; **B**) ozone treatments on the content of H_2O_2 in *P. australis* leaves (Loreto & Velikova – Plant Physiology 127:1781-1787, 2001).

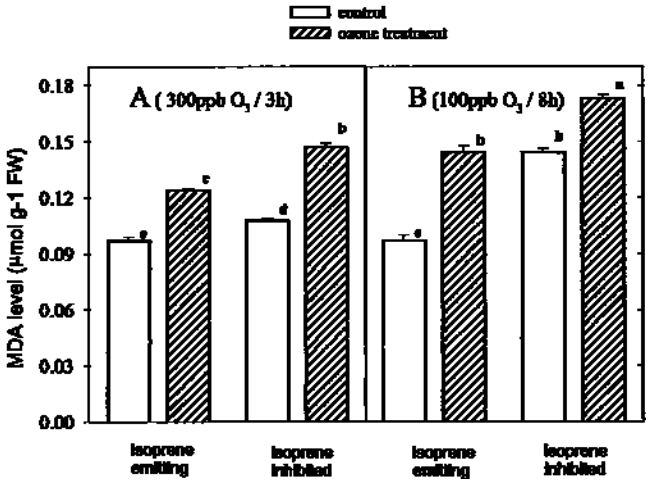


Fig. I.6. Effect of short-term (3 h, 300 nL L^{-1}; **A**) and long-term (8 h, 100 nL L^{-1}; **B**) ozone treatments on the content of MDA in *P. australis* leaves (Loreto & Velikova – Plant Physiology 127:1781-1787, 2001).

For the first time, we demonstrated that endogenous isoprene has an important antioxidant role in plants. When the oxidation potential becomes high, such as under acute or prolonged ozone exposures, isoprene quenches ozone-dependent reactive oxygen species reducing the damage at the membrane level and probably the consequent damage at biochemical and physiological levels (paper I. Loreto & Velikova 2001).

4.1.B. Endogenous isoprene protects Phragmites australis leaves against singlet oxygen (**paper II. Velikova et al. – Physiologia Plantarum122:219-225, 2004**)

Singlet oxygen ($^1O_2$) is highly reactive and toxic oxygen species and their generation is localized mainly in the chloroplasts. The singlet oxygen level cannot be controlled enzymatically and plants have evolved other mechanisms, namely a network of molecules

with conjugated double bonds (delocalized p electrons) which can take up and transfer energy with ease, such as carotenoids and quinone derivatives. We have **hypothesized** that isoprene is involved in the protection against $^1O_2$ by assuming that, similar to carotenoids, its conjugated double bonds mediate energy transfer. The hydrophobicity of the isoprene molecule may also allow interactions with non-polar membrane components, possibly resulting in membrane stabilization (Sharkey & Yeh 2001). We aimed at determining whether endogenous isoprene protects leaves from oxidative stress caused by singlet oxygen.

The possible protective role of endogenous isoprene against oxidative stress caused by singlet oxygen ($^1O_2$) was studied in the isoprene-emitting plant *Phragmites australis*. Leaves emitting isoprene and leaves in which isoprene synthesis was inhibited by fosmidomycin were exposed to increasing concentrations of $^1O_2$ generated by Rose Bengal (RB) sensitizer at different light intensities. In isoprene-emitting leaves, photosynthesis (**Fig. II.2A,B**) and H_2O_2 (**Fig. II.4A**) and malonyldialdehyde (MDA) (**Fig. II.4B**) contents were not affected by low to moderate $^1O_2$ concentrations generated at light intensities of 800 and 1240 μmol m^{-2} s^{-1}.

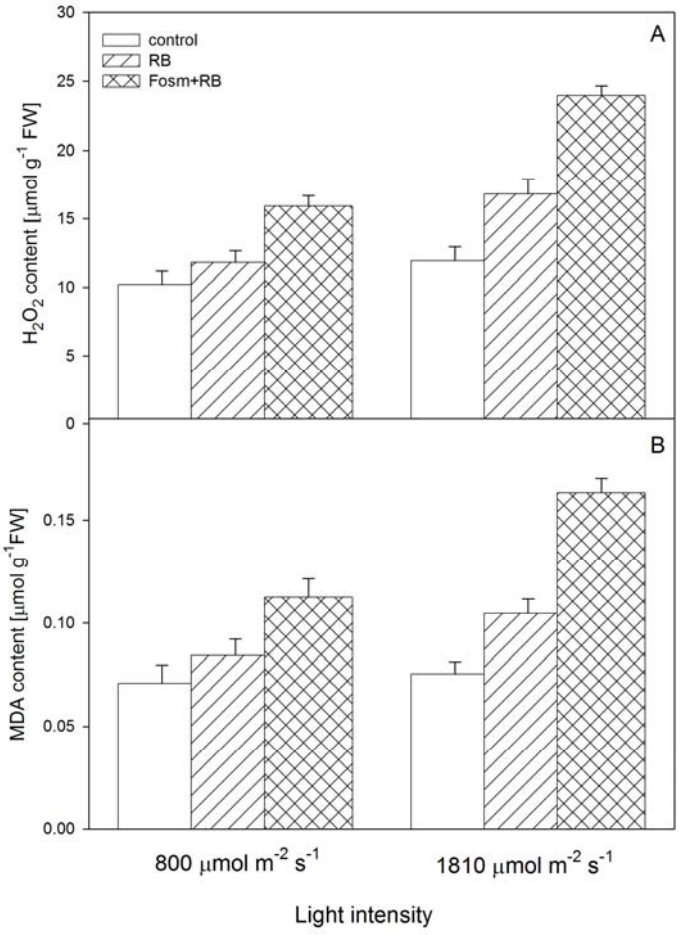


Fig. II.4. Effect of Rose Bengal (RB) feeding on H_2O_2 **(A)** and malonyldialdehyde (MDA) **(B)** contents of *Phragmites australis* leaves. RB effect on isoprene-emitting (striped bars) and isopreneinhibited (cross-striped bars) leaves is compared with the values in non-treated leaves (controls, open bars) at light intensities of 800 and 1810 μmol m^{-2} s^{-1}. Measurements on RB-fed leaves were performed at the end of the 4-h treatment. Control – isoprene-emitting leaves; RB – RB-treated isoprene-emitting leaves; Fosm + RB – isoprene-inhibited leaves treated with RB (Velikova et al. – Physiologia Plantarum122:219-225, 2004).

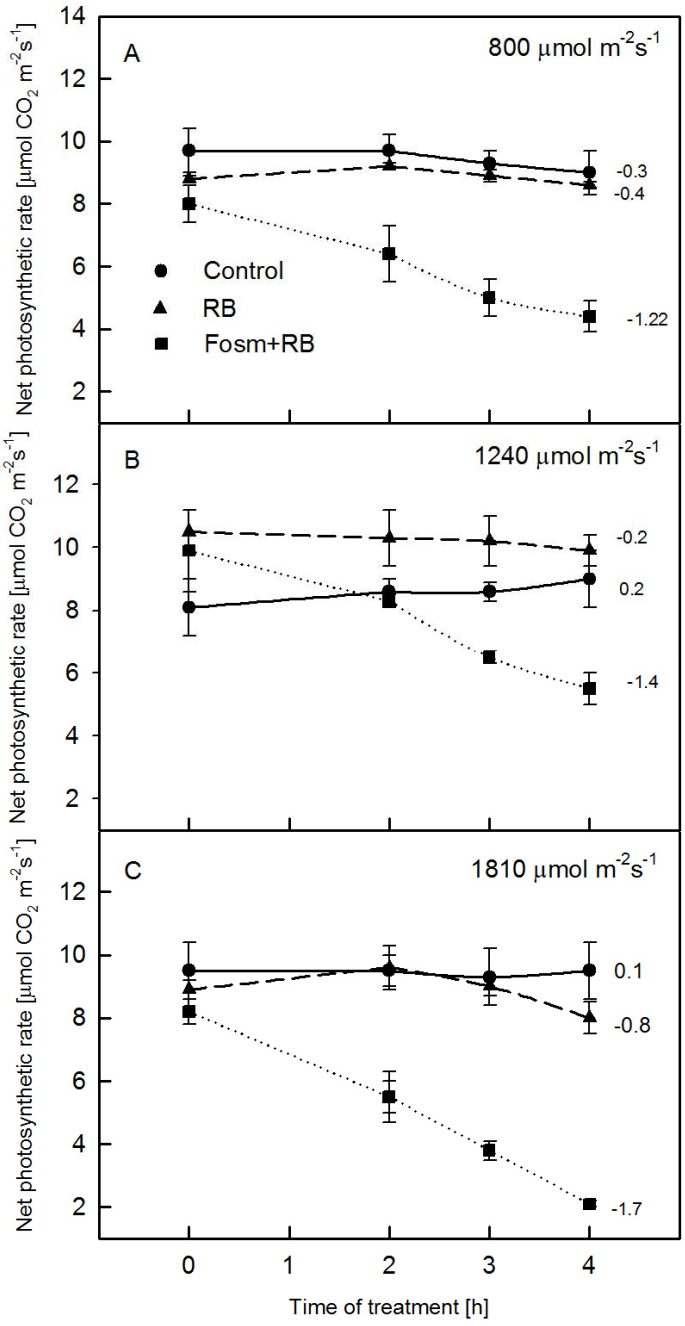


Fig. II.2. Effect of Rose Bengal (RB) feeding on the net photosynthetic rate of *Phragmites australis* leaves at a leaf temperature of 30°C and different light intensities: **(A)** 800 µmol m^{-2} s^{-1}; **(B)** 1240 µmol m^{-2} s^{-1}; **(C)** 1810 µmol m^{-2} s^{-1}. Control – isoprene-emitting leaves; RB – RB-treated isoprene-emitting leaves; Fosm + RB – isoprene-inhibited leaves treated with RB (Velikova et al. – Physiologia Plantarum122:219-225, 2004).

In isoprene-emitting leaves symptoms of damage on photosynthesis (**Fig. II.2A,B**) and reactive oxygen accumulation (**Fig. II.4**) started to be observed when high levels of $^1O_2$ were generated by very high light intensity (1810 µmol m^{-2} s^{-1}). A dramatic decrease in photosynthetic performance and an increase in H_2O_2 and MDA levels were measured in isoprene-inhibited RB-fed leaves, but photosynthesis was not significantly inhibited in leaves in which the isoprene leaf pool was reconstituted by fumigating exogenous isoprene. The inhibition of photosynthesis in isoprene-inhibited leaves was linearly associated with the light intensity and with the consequently formed $^1O_2$. Hence,

physiological levels of endogenous isoprene may supply protection against $^1O_2$. The protection mechanisms may involve a direct reaction of isoprene with $^1O_2$. Moreover, as it is a small lipophilic molecule, it may assist hydrophobic interactions in membranes, resulting in their stabilization. The isoprene conjugated double bond structure may also quench $^1O_2$ by facilitating energy transfer and heat dissipation. This action is typical of other isoprenoids, but we speculate that isoprene may provide a more dynamic protection mechanism as it is synthesized promptly when high light intensity produces $^1O_2$ (II. Velikova et al. 2004).

4.1.C. Biogenic isoprene protects transgenic tobacco plants from oxidative stress (**parer IX. Vickers et al. – Plant, Cell and Environment 32:520-531, 2009**)

Transgenic tobacco plants (*IspS*) capable of releasing isoprene as a natural metabolite have been used to further elucidate the antioxidant properties of endogenous isoprene. The responses of these plants to ozone were compared with those of isoprene non-emitting tobacco plants (WT). Non-emiting plants are characterized by typical visible damage resulting from ozone treatment, while isoprene-emitting plants are better protected and exhibit minor visible damage (**Fig. IX.3**).

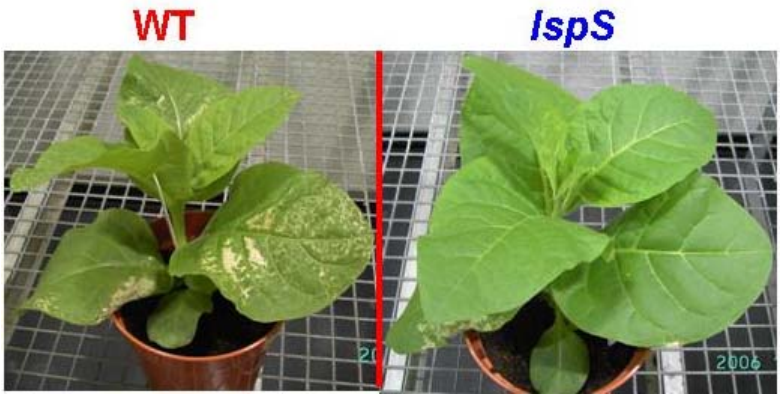


Fig. IX.3. Phenotype of ozone-fumigated isoprene non-emitting (WT) and isoprene-emitting (*IspS*) tobacco plants. Plants were fumigated with ozone (200 ppb) for 6 h d^{-1} over 2 days (Vickers et al. – Plant, Cell and Environment 32:520-531, 2009).

Ozone treatment resulted in a decrease in photosynthesis in both emitting and non-emitting plants (paper IX. Vickers et al. 2009). The magnitude of this decrease was significantly greater in non-emitting plants relative to emitting plants after the second fumigation. The difference in response between non-emitting and emitting plants was not caused by differences in CO_2 availability or ozone uptake, because stomatal conductance was unchanged between the emitting and nonemitting plants.

Iisoprene-emitting transgenic tobacco plants had substantially lower H_2O_2 (**Fig. IX.5a,b**) and lipid peroxidation (**Fig. IX.5c,d**) levels following ozone fumigation. Isoprene is unlikely to be effective as an antioxidant in the aqueous phase, and is therefore unlikely to have directly scavenged H_2O_2. Isoprene is highly hydrophobic, and is likely to partition into the lipid phase in membranes (Siwko et al. 2007). It has been proposed that isoprene may physically stabilize membranes and/or behave as an antioxidant (Sharkey & Singsaas 1995; Loreto & Velikova 2001; Loreto et al. 2001; Sharkey et al. 2001; Affek & Yakir 2002), presumably preventing lipid peroxidation and minimizing oxidative damage to membranes.

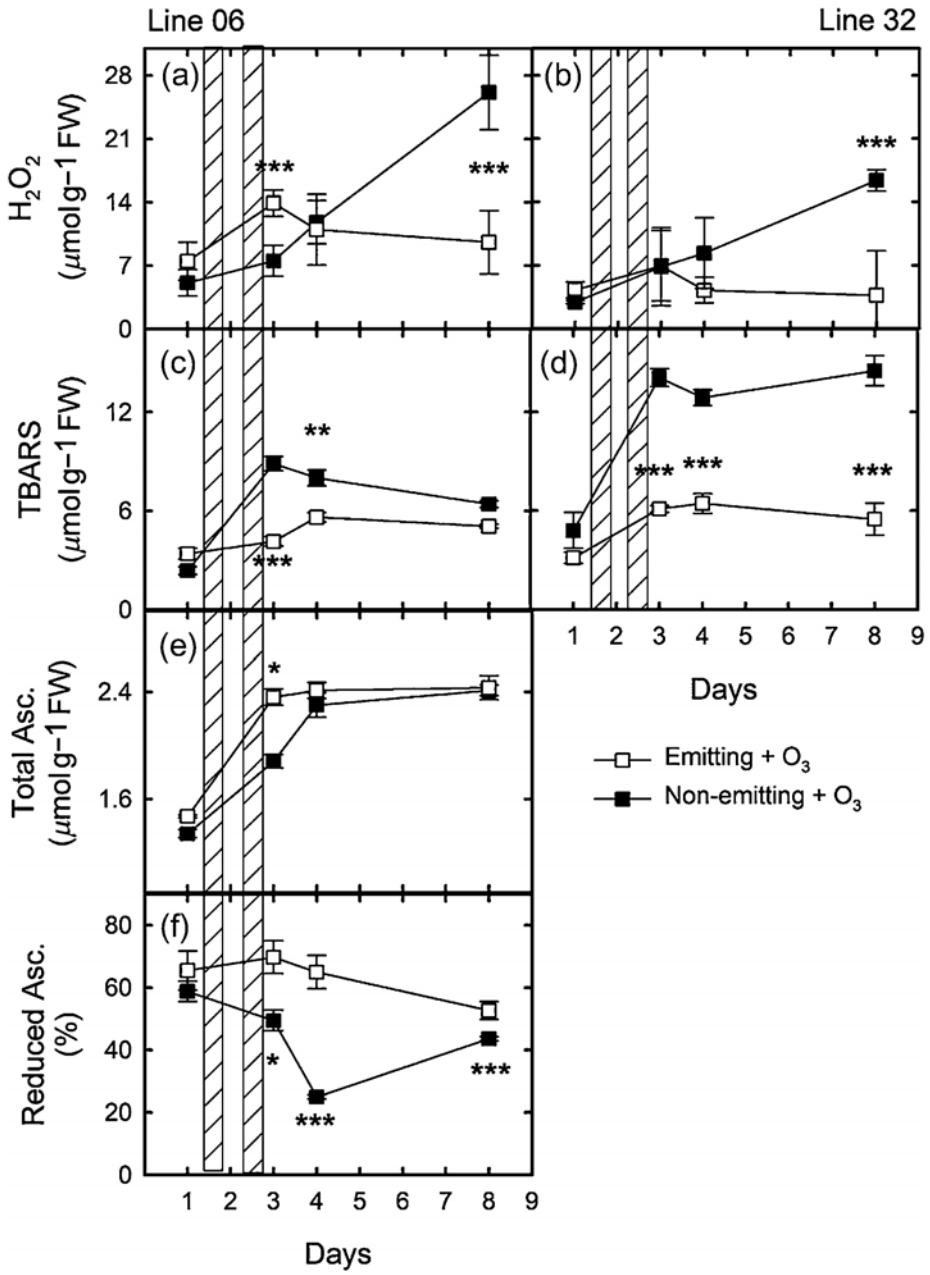


Fig. IX.5. Biochemical data collected from tobacco plants (lines 6 and 32) fumigated with 120 ppb ozone, 6 h d^{-1} for 2 days. Measurements were taken before fumigation, immediately after fumigation and at 1 and 5 d post-fumigation. H_2O_2 levels are shown in **(a,b)**. Lipid peroxidation products were measured using the thiobarbituric acid-reactive substances (TBARS) test **(c,d)**. In line 6, ascorbate levels were also analysed; total ascorbate (Asc.) is shown in **(e)** and reduced Asc. is shown in **(f)**. Ozone fumigation events are represented by cross-hatched bars (Vickers et al. – Plant, Cell and Environment 32:520-531, 2009).

Aqueous and lipid-phase antioxidant capacities are linked (e.g. through ascorbate - mediated regeneration of tocopherol moieties) (Foyer et al. 2006), so an increase in lipid-phase antioxidant capacity may be expected to impact on aqueous-phase antioxidant capacity. Although the total Asc. pools displayed relatively little variation in transgenic tobacco (**Fig. IX.5e**), the amount of reduced ascorbate in isoprene-emitting tobacco plants was greater than in non-emitting plants after ozone fumigation (**Fig. IX.5f**). This shows that isoprene-emitting plants have a reduced requirement for antioxidant capacity in the

aqueous phase, most likely as an indirect consequence of lipid-phase protection by isoprene (paper IX. Vickers et al. 2009).

4.2. Termoprotective role of biogenic isoprene

4.2.A. The inhibition of isoprene emission causes more significant impairments in photosynthetic function after high temperature treatment (**papers III. Velikova et al. – Agriculture, Ecosystems & Environment 106: 209-2017, 2005; IV. Velikova & Loreto – Plant, Cell and Environment 28: 318-327, 2005; VI. Velikova et al. – Functional Plant Biology 33: 931-940, 2006**)

The thermoprotective role of biogenic isoprene has been studied in the leaves of *Phragmites australis* and *Platanus orientalis*. The experiments were performed with (1) leaves in which isoprene emission is chemically manipulated with fosmidomycin (Velikova et al. 2005; Velikova & Loreto 2005, papers III and IV), and (2) leaves originating from different age plants *P. orientalis*, which naturally emitt different amounts of isoprene (Velikova et al. 2006, paper VI).

In the first experiment isoprene-emitting and isoprene-inhibited *P. australis* laves were exposed to different temperatures (30, 38, 44 and 48°C) in the light (840 μmol m^{-2} s^{-1}) and were maintained for 15 min at each temperature before carrying the measurements. Gas exchange measurements were done at each temperature point, and determination of H_2O_2 and malonyldialdehyde (MDA), catalase and peroxidase activity - at the final point, 48°C. Leaves continuously exposed to 30°C served as controls (paper III, Velikova et al. 2005). We wanted to know if isoprene inhibition increased the membrane damage in heat stressed *P. australis* leaves, and if this was associated with a reduced thermotolerance. At each temperaturetested photosynthesis was slightly but not significantly higher in isoprene-emitting than in isoprene-inhibited leaves (**Fig. III.2A**). The H_2O_2 and the MDA contents in leaves after the high temperature treatment increased in both isoprene-emitting and isoprene-inhibited leaves (**Fig. III.3**). These results suggested no substantial change in thermotolerance under the specific conditions of our treatment.

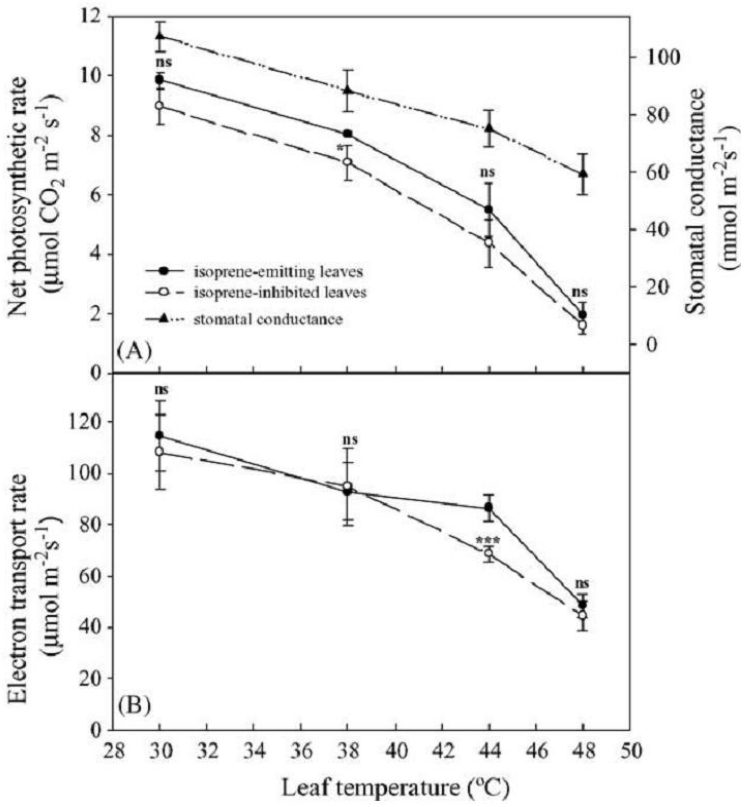


Fig. III.2. Net photosynthesis, stomatal conductance **(A)** and electron transport rate **(B)** in isoprene-emitting and isoprene-inhibited *P. australis* leaves at 30, 38, 44 and 48°C for 15 min each. Measurements were performed on fully expanded leaves at 840 μmol m^{-2} s^{-1} PPFD (Velikova et al. – Agriculture, Ecosystems & Environment 106:209-217, 2005).

It should be noted that isoprene inhibition was incomplete and the residual emission showed the same temperature-dependence of the emission from isoprene-emitting leaves, peaking at 44°C. At this temperature a significantly higher electron transport rate was observed in isoprene-emitting leaves than in isoprene-inhibited leaves (**Fig. III.2B**) suggesting that isoprene may have facilitated electron flow through the photosynthetic/photorespiratory cycles. More H_2O_2 (**Fig. III.3A**) and malonyldialdehyde (**Fig. III.3B**) contents and higher catalase (**Fig. III.4A**) and peroxidase (**Fig. III.4B**) activities were observed in isoprene-inhibited than in isoprene-emitting leaves. These changes were less evident after exposure to the temperature ramp up to 48°C than in leaves maintained at 30°C. This suggests that isoprene, independent of the temperature stress, effectively reduces the accumulation of reactive oxygen species and protects membrane from denaturation.

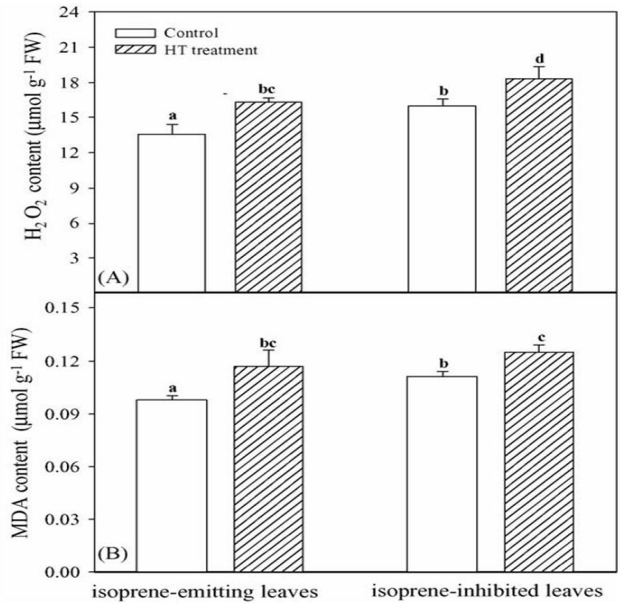


Fig. III.3. H_2O_2 content **(A)** and malonyldialdehyde (MDA) level **(B)** in isoprene-emitting and isoprene-inhibited *P. australis* leaves before (controls, 30°C) and after a high temperature treatment. The leaf discs were exposed to increasing temperatures (30, 38, 44 and 48°C) in the light (840 µmol m^{-2} s^{-1}) and were maintained for 15 min at each temperature. The biochemical measurements were carried out at the end of the exposure at 48°C (Velikova et al. – Agriculture, Ecosystems & Environment 106:209-217, 2005).

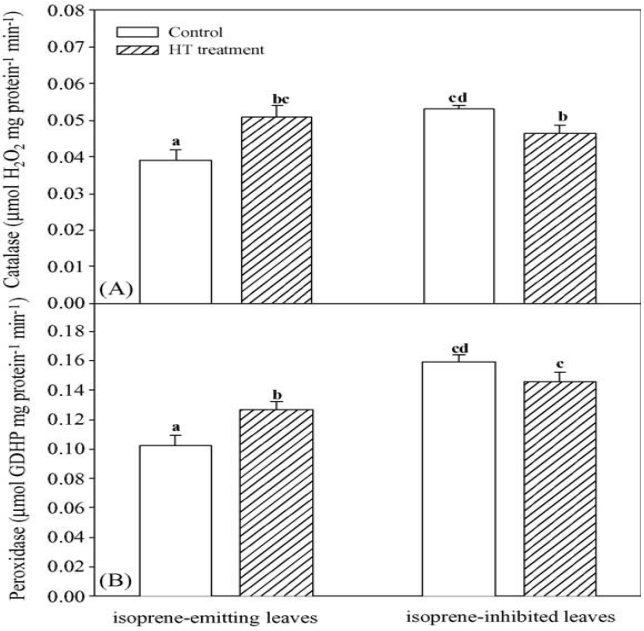


Fig. III.4. Catalase **(A)** and peroxidase **(B)** activities in isoprene-emitting and isoprene-inhibited *P. australis* leaves before (controls, 30°C) and after a high temperature treatment. Other explanations as in Fig. III.3 (Velikova et al. – Agriculture, Ecosystems & Environment 106:209-217, 2005).

Those results induced us to further explore the role of isoprene in thermotolerance (paper III. Velikova & Loreo 2005). We investigated the direct effect of exposure to high temperature stress (38°C) at different light intensities (500, 1000 и 1500 µmol m^{-2} s^{-1}), and for a period reasonably similar to that experienced in nature (1.5 h), and the recovery from this stress in isoprene-emitting and isoprene-inhibited *P. australis* leaves. Our objectives were to determine whether isoprene protection is mediated by light, and whether isoprene presence also induces a faster or stronger recovery from stress.

Moderately high temperature treatment (38°C for 1.5 h) reduced photosynthesis (**Fig. IV.2**), stomatal conductance, and photochemical efficiency of photosystem II in isoprene-emitting, but to a significantly lower extent than in isoprene-inhibited *Phragmites australis* leaves. Isoprene inhibition and high temperature independently, as well as together, induced lipid peroxidation, increased level of H_2O_2, and increased catalase and peroxidase activities. However, leaves in which isoprene emission was previously inhibited developed stronger oxidative stress under high temperature with respect to isoprene-emitting leaves.

The heaviest photosynthetic stress was observed in isoprene-inhibited leaves exposed to the brightest illumination (1500 μmol m^{-2} s^{-1}) and, in general, there was also a clear additive effect of light excess on the formation of reactive oxygen species, antioxidant enzymes, and membrane damage. The increased thermotolerance capability of isoprene-emitting leaves may be due to isoprene ability to stabilize membranes or to scavenge reactive oxygen species. Irrespective of the mechanism by which isoprene reduces thermal stress, isoprene-emitting leaves are able to quickly recover after the stress. This may be an important feature for plants coping with frequent and transient temperature changes in nature.

In this study we (paper IV. Velikova & Loreto 2005) confirmed that thermotolerance is reduced when isoprene synthesis is inhibited (Sharkey & Singsaas 1995; Singsaas et al. 1997) and revealed that recovery from high temperature stress is also restrained in the absence of isoprene. Whereas it has been shown that thermotolerance may be increased by isoprene at temperatures higher than 40°C (Singsaas et al. 1997; Sharkey et al. 2001), in our experiments we show that a better thermal protection may be induced by isoprene also at temperatures below 40°C. Perhaps this underlies two different mechanisms of thermal protection associated with isoprene production. In any case, the incomplete recovery of photosynthesis in isoprene-inhibited leaves indicates that the photosynthetic structures are permanently damaged in these leaves.

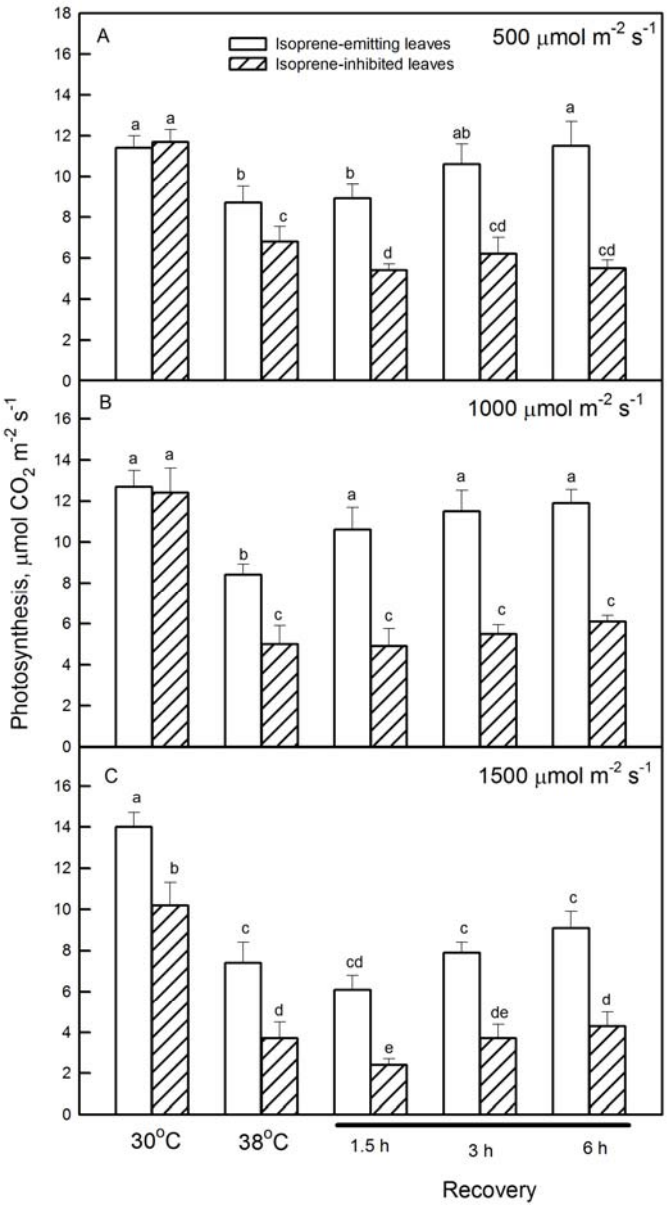


Fig. IV.2. Photosynthesis of *Phragmites australis* leaves in response to a high temperature treatment (38°C for 1 h 30 min) at increasing light intensities **(panels A, B, C)** and after recovery from heat treatment at 30°C. Photosynthesis was measured three times during recovery (after 1.5, 3 and 6 h) (Velikova & Loreto – Plant, Cell and Environment 28:318-327, 2005).

The phenomenon of enhanced plant thermotolerance by isoprene was also studied in *Platanus orientalis* plants (paper VI. Velikova et al. 2006). The purpose of this study was to test the possible thermotolerance induced by isoprene in leaves of *P. orientalis* characterised by different isoprene emission levels, namely (1) leaves developing from 1- and 2-year-old plants, (2) leaves in which isoprene biosynthesis was inhibited by fosmidomycin, and (3) leaves that were supplied exogenous isoprene after inhibiting endogenous isoprene synthesis with fosmidomycin. We found that the isoprene emission rate was higher in leaves developed from 2-year old plants than in leaves developed from 1-year-old plants (**Fig. VI.1**). Moreover, isoprene emission was particularly enhanced following the heat treatment in 2-year old plants, which shows that the isoprene emission capacity of older plants is very large and may be sustained even when photosynthesis is reduced by environmental stresses.

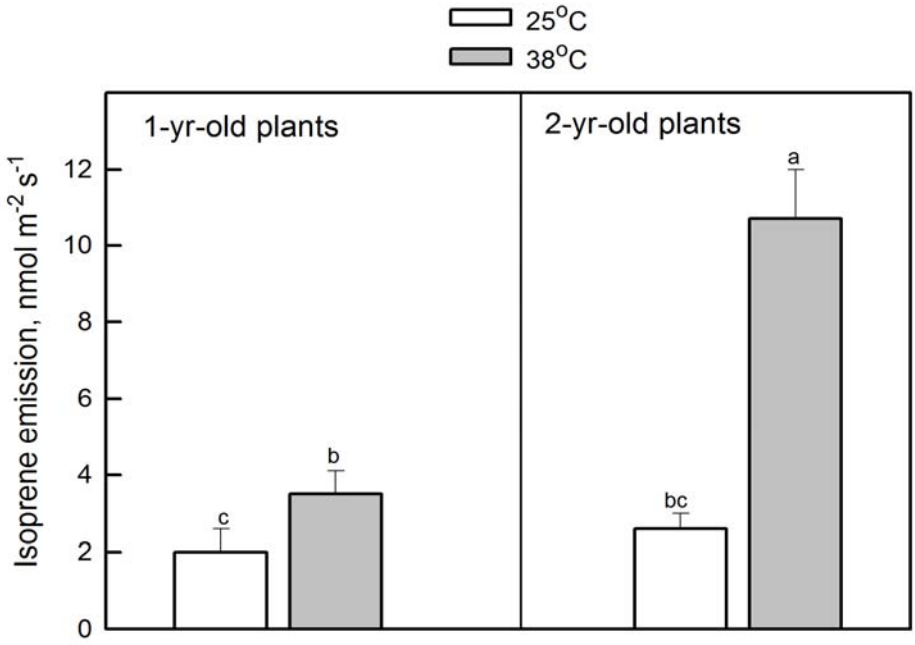


Fig. VI.1. Effect of high temperature treatment (38°C for 4 h) on isoprene emission in leaves of 1- and 2-year-old *Platanus orientalis* plants (Velikova et al. – Functional Plant Biology 33:931-940, 2006).

After a high temperature treatment (38°C for 4 h), photosynthetic activity (**Fig. VI.2**), hydrogen peroxide content, lipid peroxidation and antiradical activity were preserved in isoprene emitting leaves of 1- and 2-year-old plants (paper VI. Velikova et al. 2006). However, heat inhibited photosynthesis and PSII efficiency, caused accumulation of H2O2, and increased allindices of membrane damage and antioxidant capacity in leaves of plants of both ages in which isoprene was inhibited by fosmidomycin. In isoprene-inhibited leaves fumigated with exogenous isoprene during the heat treatment, the negative effects on photosynthetic capacity were reduced. These results further support the notion that isoprene plays an important role in protecting photosynthesis against damage at high temperature. It is suggested that isoprene is an important compound of the non-enzymatic defence of plants against thermal stress, possibly contributing to scavenging of reactive oxygen species and membrane stabilising capacity, especially in developed plants (paper VI. Velikova et al. 2006).

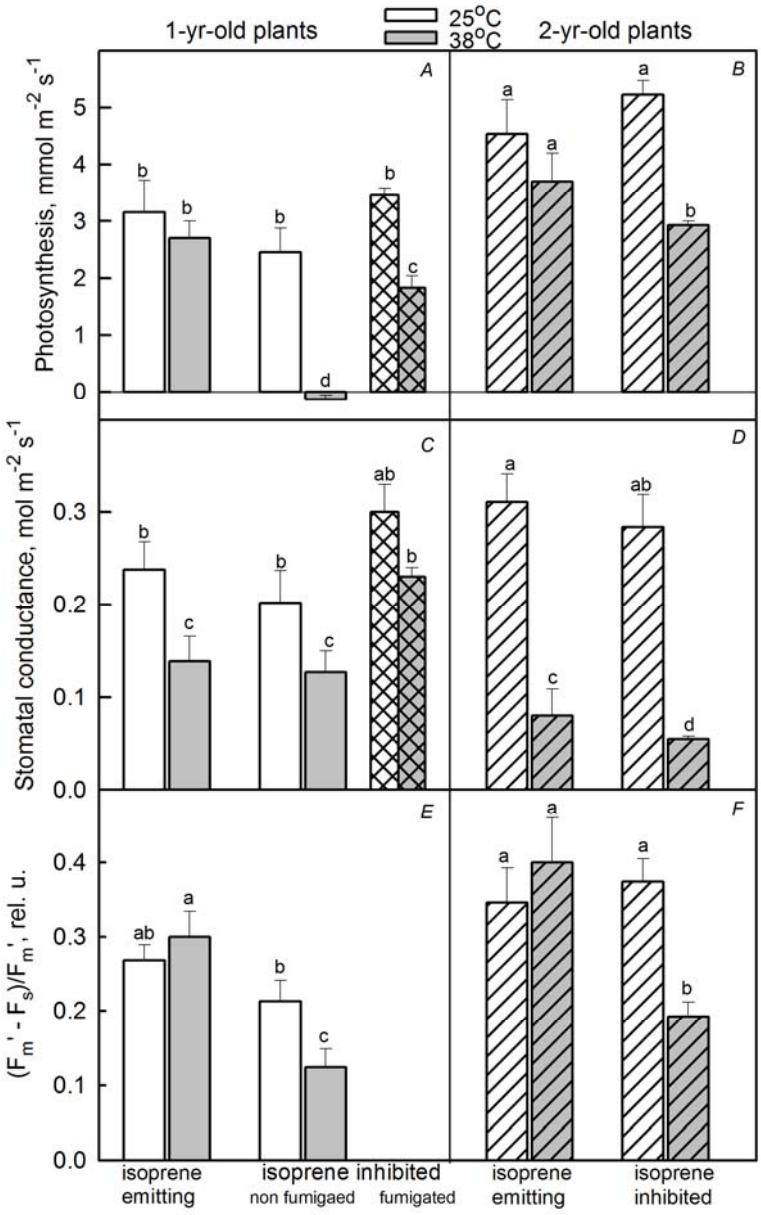


Fig. VI.2. Photosynthesis **(A, B)**, stomatal conductance **(C, D)** and photochemical quantum efficiency of PSII **(E, F)** in isoprene-emitting and isoprene-inhibited leaves of 1- and 2-year-old *Platanus orientalis* plants in response to high temperature (38°C for 4 h) (Velikova et al. – Functional Plant Biology 33:931-940, 2006).

4.2.B. The combined effect of elevated atmospheric [CO_2] and high temperature has an adverse effect on isoprene emission, plant functional and structural characteristics (**X. Velikova et al. – Environmental Pollution 157: 2629-2637, 2009**)

To investigate the interactive effects of increasing [CO_2] and heat wave occurrence on isoprene (IE) and methanol (ME) emissions, *Platanus orientalis* was grown for one month in ambient (380 μmol mol^{-1}) or elevated (800 μmol mol^{-1}) [CO_2] and exposed to high temperature (HT) (38°C/4 h). Two types of leaves were used. Leaves that had already reached their fully expanded state before the onset of the CO_2 fumigations are termed as ‘‘pre-existing’’ leaves, while leaves that developed during the CO_2 fumigations are termed as ‘‘newly-emerged’’ leaves (**Fig. X.1**).

Fig. X.1. Photograph of 2-year-old *Platanus orientalis* seedling after 1-month growth at 800 μmol mol^{-1} CO_2. Two types of leaves were used in the experiments. Leaves developed and reached maturity during the fumigation with 800 μmol mol^{-1} [CO_2] are termed as ‘‘newly-emerged’’; and leaves already reached their fully expanded state when the μmol mol^{-1} [CO_2] fumigation was implemented are termed as ‘‘preexisting’’ (Velikova et al. – Environmantal Pollution 157:2629-2637, 2009).

It was **hypothesized** that (1) the exposure to elevated [CO_2] might positively affect photosynthesis and that this could reduce the negative effect of heat on carbon metabolism; and (2) CO_2 enrichment might inhibit isoprene biosynthesis, which in turn could reduce thermotolerance and worsen the heat stress for photosynthesis.

Heat treatment alone did not cause any unfavorable effects on photosynthesis and chloroplast ultrastructure of pre-existing leaves, which are characterized by significantly higher isoprene emission. Elevated [CO_2] significantly reduced IE in both leaf types, whereas it increased ME in newly-emerged leaves only (**Fig. X.4**). The growth-promoting effect of elevated [CO_2] was not as clear as expected, but in agreement with our first hypothesis, elevated [CO_2] was able to counteract the negative impact of high temperature stress in pre-existing leaves. However, high temperature induced a considerable decrease of photosynthesis and electron transport rate (**Fig. X.2**), increase of methanol emission (**Fig. X.4c,d**) and changes of the chloroplast ultrastructure and membrane integrity in newly-emerged leaves, especially in elevated [CO_2] environment. This supports our second hypothesis that leaves with inhibited isoprene emission are more sensitive to heat stress. **Thus**, in a warmer and elevated [CO_2] environment, *Platanus orientalis* is likely be more sensitive to climate-induced damage than in the present climate. This investigation also confirms that isoprene emission rate is uncoupled from photosynthesis and it is likely to be reduced under future elevated [CO_2] levels. On the other hand, elevated [CO_2] by stimulating leaf growth may increase methanol emission. Moreover, high temperature not only increases ME, but is likely to contrast the inhibitory effects of elevated [CO_2] on isoprene emission, confirming that temperature is the main factor affecting the rate of biogenic emissions.We believe that these findings may be useful for a better parameterization of large-scale emission models on the impact of BVOC emissions on air quality on regional and global scales (paper X. Velikova et al. 2009).

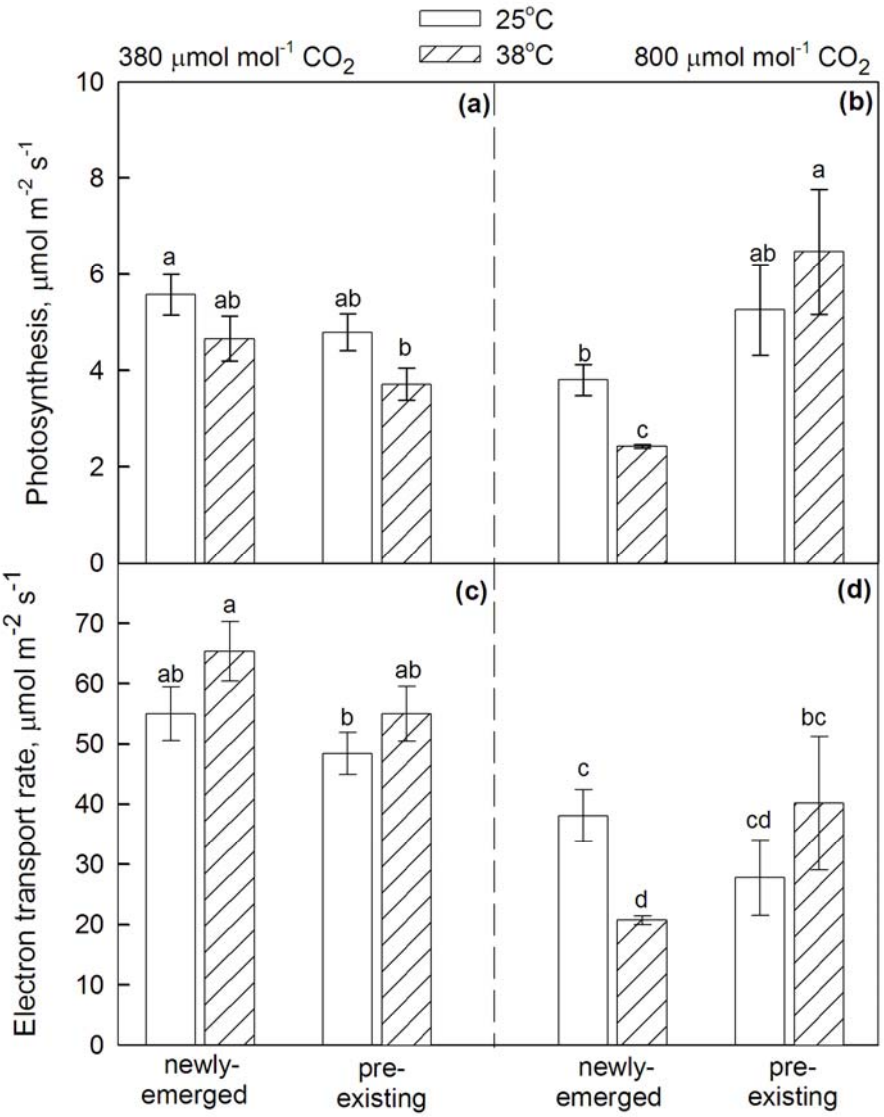


Fig. X.2. Photosynthesis **(a, b)** and electron transport rate **(c, d)** in newly-emerged and pre-existing *Platanus orientalis* leaves, before (white bars) and after a 4-h heat stress (38°C) (hatched bars) (Velikova et al. – Environmantal Pollution 157:2629-2637, 2009).

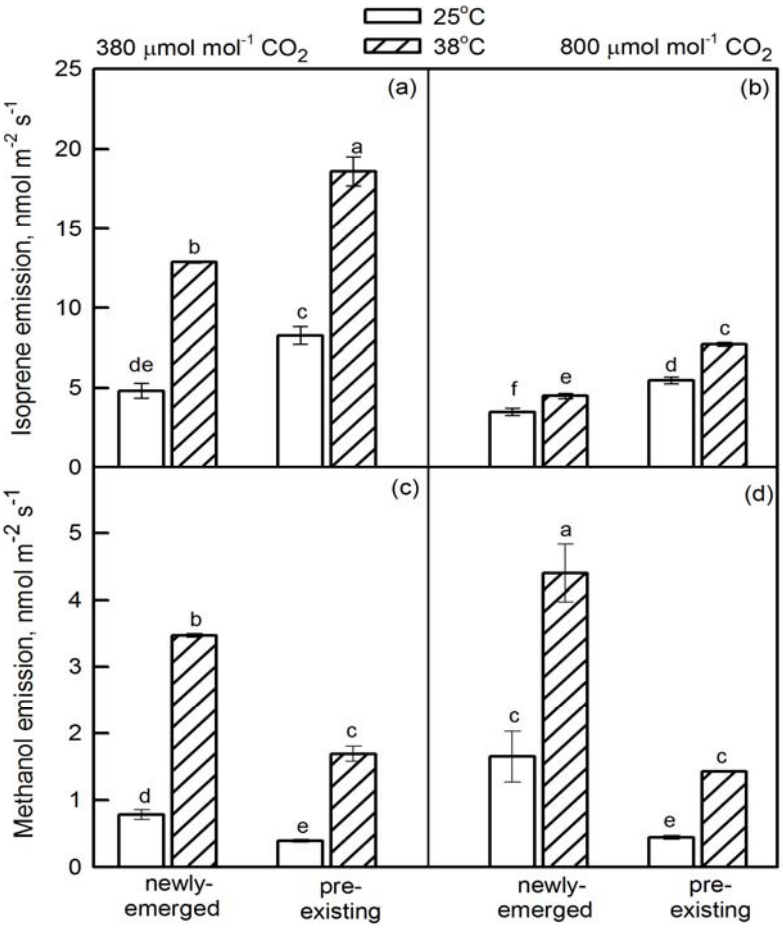


Fig. X.4. Isoprene **(a, b)**, and methanol **(c, d)** emissions in newly-emerged and pre-existing *Platanus orientalis* leaves, before (white bars) and after a 4-h heat stress (38°C) (hatched bars) (Velikova et al. – Environmantal Pollution 157:2629-2637, 2009).

4.2.C. Biogenic isoprene improves thylakoid membrane stability – biophysical approaches (paper XII. Velikova et al. – Plant Physiology 157: 905-916, 2011)

The effect of biogenic isoprene on the functional activity of thylakoid membranes has been investigated using three biophysical techniques. Two plant species were used as a model. Arabidopsis (*Arabidopsis thaliana*), which does not normally make isoprene, was engineered with an isoprene synthase gene from kudzu (*Pueraria lobata*) so that wild-type (wt) plants (non-emitting isoprene) could be compared to the transformed plants that do make isoprene (*IspS*). Leaves of *Platanus* (plane tree) normally do make isoprene but this was inhibited by fosmidomycin so that emitting (water-fed) and nonemitting (fosmidomycin-fed) leaves could be compared. The thermal stability of the thylakoid membranes was characterized with biophysical approaches not previously used in isoprene studies, namely circular dichroism (CD) spectrosocopy, electrochromic absorbance transients (ΔA_{515}), and thermoluminescence (TL).

CD spectra in wild-type and *IspS* Arabidopsis plants showed considerable differences at 20°C. In particular, it was found that the amplitude of the main CD band (at [+]694 nm) was lower in wild-type than in *IspS* plants (**Fig. XII.2A,B**), suggesting that constitutive presence of isoprene might determine structural changes in the thylakoid membranes. While, the CD spectra of *Platanus* leaves at 25°C were not affected by the fosmidomycin treatment suppressing isoprene biosynthesis (**Fig. XII.3A,B**).

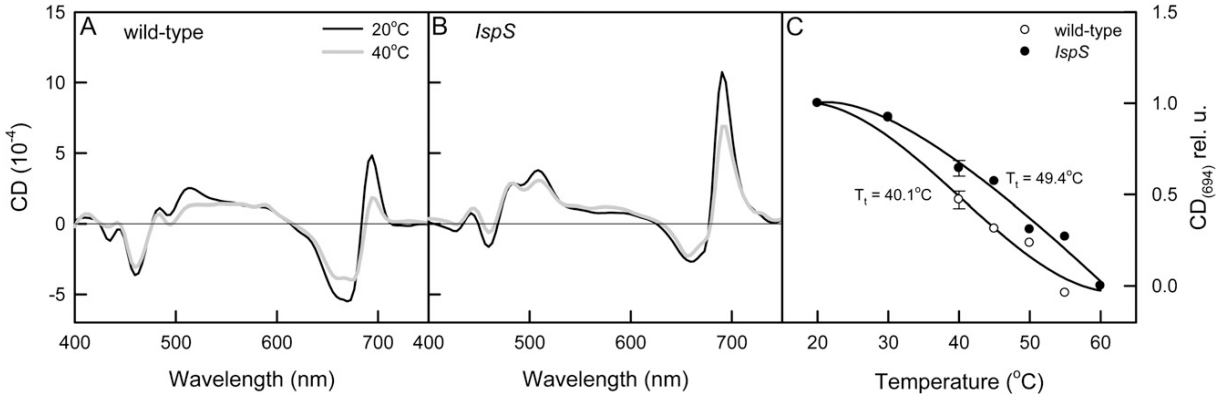
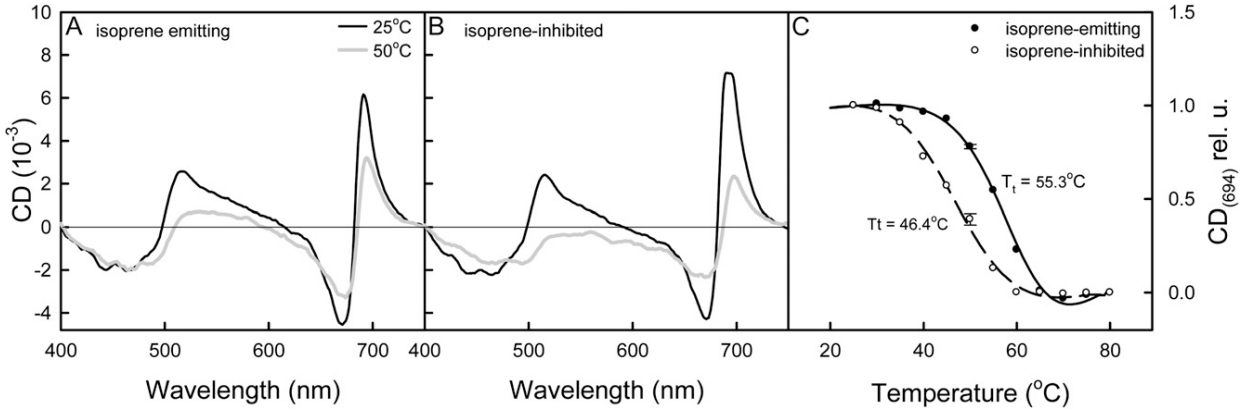


Fig. XII. 2. CD spectra measured in *Arabidopsis* wild type, which do not emit isoprene **(A)**, and *IspS* isoprene-emitting **(B)** plants. CD was recorded in leaves at 20°C (black line) and 40°C (gray line). Temperature dependences of the intensity of the (+)694 nm band for the wild type (white circles) and *IspS* (black circles) are shown in C. In **C**, the temperature at which the intensity of the CD band is 50% of its value at 20°C (transition temperature, Tt) is shown (Velikova et al. – Plant Physiology 157:905-916, 2011).



Фиг. XII. 3. CD spectra measured in isoprene-emitting **(A)**, and isoprene-inhibited **(B)** *Platanus* leaves. CD was recorded in at 25°C (black line) and 50°C (gray line). Temperature dependences of the intensity of the (+)694 nm band for the isoprene-inhibited (white circles) and isoprene-emitting (black circles) are shown in C. In **C**, the temperature at which the intensity of the CD band is 50% of its value at 20°C (transition temperature, Tt) is shown (Velikova et al. – Plant Physiology 157:905-916, 2011).

To investigate the possible role of isoprene in the conformational stability of chloroplast membranes subjected to high temperatures, measurements of CD spectra were performed in wild type and *IspS Arabidopsis* leaves after 10 min incubations at 20°C, 30°C, 40°C, 45°C, 50°C, 55°C, and 60°C. At 40°C the amplitude of the main band at (+)694 nm was considerably lower in wild-type than in *IspS* leaves (**Fig. XII.2A,B**), while the weaker, excitonic bands at (+)440 and (2)650 nm and the excitonic band pair (+)482/(2)470 were not affected by isoprene. The transition temperature of the band at (+)694 nm, was shifted to higher temperature in *IspS* leaves; the transition temperatures were 40.1°C and 49.4°C in wild type and *IspS* leaves, respectively (**Fig. XII.2C**).

The CD band at (+)694 nm was completely missing in isoprene-inhibited *Platanus* leaves already at 55°C and in isoprene-emitting leaves over 60°C (data not shown). The transition temperature was shifted to lower value (46.4°C) in isoprene-inhibited leaves, compared to isoprene-emitting leaves (55.3°C; **Fig. XII.3C**). These results indicat that the heat stability of chiral macrodomains of chloroplast membranes, and specifically the stability of ordered arrays of light-harvesting complex II photosystem II in the stacked region of the thylakoid grana, was improved in the presence of isoprene.

Electrochromic absorbance changes at 515 nm (ΔA_{515}) are used to monitor ion permeability of membranes because of the effect on the electrical potential across the thylakoid membranes. In particular, the decay kinetics of the absorbance changes are proportional to the ion flux across the thylakoid membranes and are sensitive indicators of thylakoid membrane intactness, i.e. of the ability of membranes to maintain the light-induced transmembrane electric field. The decay of ΔA_{515} was faster in the absence of isoprene when leaves of *Arabidopsis* and *Platanus* were exposed to high temperature, indicating that isoprene protects the thylakoid membranes against leakiness at elevated temperature (**Table XII.1**).

Table XII.1. Half-times ($t_{1/2}$, ms) of the decay kinetics of the flash-induced electrochromic absorbance changes at 515 nm in detached Arabidopsis and *P. orientalis* leaves. In Arabidopsis, the measurements were performed at 20°C after preincubating the wild type (non-emitting) and *IspS* (isoprene-emitting) leaves for 5 min at 20°C or 40°C in the dark. In *Platanus*, the preincubation of the control (isoprene-emitting) and fosmidomycin-treated (isoprene-inhibited) leaves was done at 25°C or 45°C for 4 h in weak white light (40 µmol photons m^{-2} s^{-1}) (Velikova et al – Plant Physiology 157:905-916, 2011).

Arabidopsis thaliana				*Platanus orientalis*			
IspS		wild type		isoprene-emitting		isoprene-inhibited	
20°C	40°C	20°C	40°C	25°C	45°C	25°C	45°C
57.8±6.5	52.0±6.0	61.2±2.8	**37.1±3.8***	83.3±5.4	**65.2±2.4***	74.5±4.4	**45.0±1.1****

TL measurements were performed to assess the possibility of isoprene-induced alterations to PSII primary photochemistry for direct estimation and comparison of redox properties of PSII. The wild type *Arabidopsis* leaves showed a main TL B band ($S_2Q_B^-$) peaking at 23.0°C ± 1.3°C (**Fig. XII. 4A**). In *IspS* leaves, the peak position of the B band was up shifted by about 10°C, to 32.6°C ± 1.0°C (**Fig. XII. 4C**), showing a significant increase in the activation energy for $S_2Q_B^-$ charge recombination.

In *Platanus* leaves the inhibition of isoprene emission by fosmidomycin did not cause significant changes in the main B-band temperature (**Fig. XII. 4 B and D**). The B band induced by a single flash in isoprene-inhibited and isoprene-emitting leaves peaked at 33.1°C ± 2.0°C and 35.8° C ± 2.5°C, respectively.

The results obtained provide, for the first time, experimental evidence that isoprene increases the thermotolerance of plants (Sharkey & Singsaas 1995; Loreto & Schnitzler 2010), improving the integrity and functionality of photosynthetic membranes under high temperature conditions (paper XII. Velikova et al. 2011).

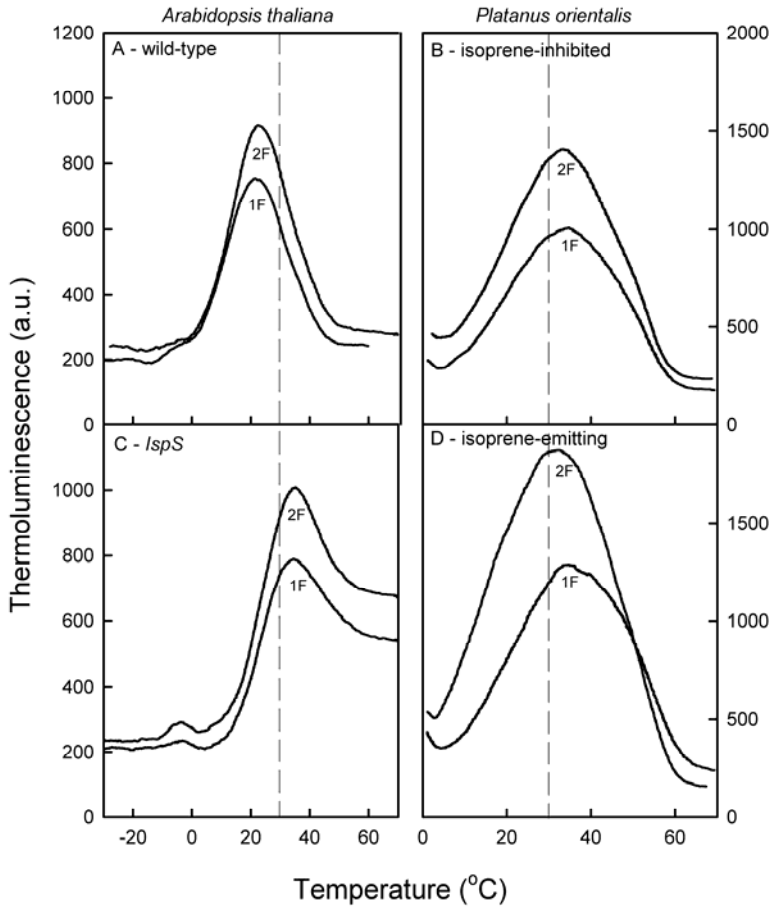


Fig. XII. 4. TL curves of wild type **(A)** and IspS **(C)** *Arabidopsis* leaves and of isoprene-inhibited **(B)** and isoprene-emitting **(D)** *Platanus leaves* after illumination with one or two single-turnover flashes of white saturating light at 1°C. TL was measured during heating of the samples to 70°C in darkness with a ramp of temperature at constant rate of 0.6°C s^{-1} (Velikova et al – Plant Physiology 157:905-916, 2011).

4.3. Effect of drought on isoprenoids in transgenic tobacco plants (**paper XV.** Tattini et al. – Plant, Cell and Environment 37:1950-1964, 2014)

Transgenic tobacco (*Nicotiana tabacum* cv. Samsun) plants were used in this study. We aimed at investigating the protective effect of isoprene against severe drought stress in transgenic plants that naturally produce isoprene and to understand the cosequences of isoprene biosynthesis on plant metabolism, more specifically the relationship between isoprene and non-volatile isoprenoids (carotenoids and abscisic acid), as well as other secondary metabolites with protective function (carbohydrates and phenylpropanoids) under optimal and stress conditions. Plants are subjected to progressive drought (68, 34 and 24% FTSW) with subsequent rehydration (FTSW = 96%).

Isoprene emission was stimulated by mild drought and declined significantly under sever drought (FTSW = 24%) (**Fig. XV.1**), when photosynthesis was significantly inhibited (**Fig. XV.2a**).

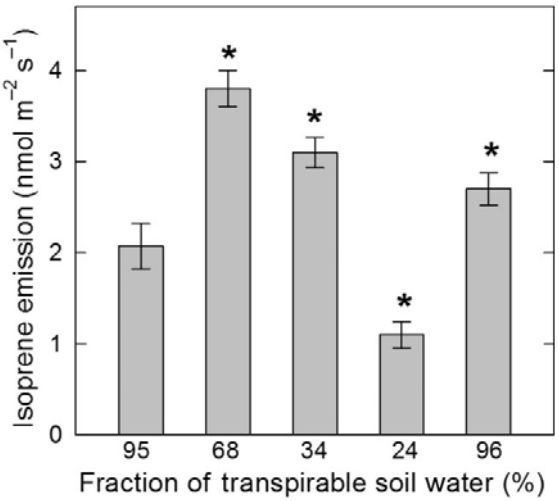


Fig. XV.1. Isoprene emission from isoprene-emitting tobacco leaves (line 12) during drought stress (FTSW=68, 34 and 24%) and subsequent re-watering (FTSW=96%) (Tattini et al. – Plant, Cell and Environmnet 37:1950-1964, 2014).

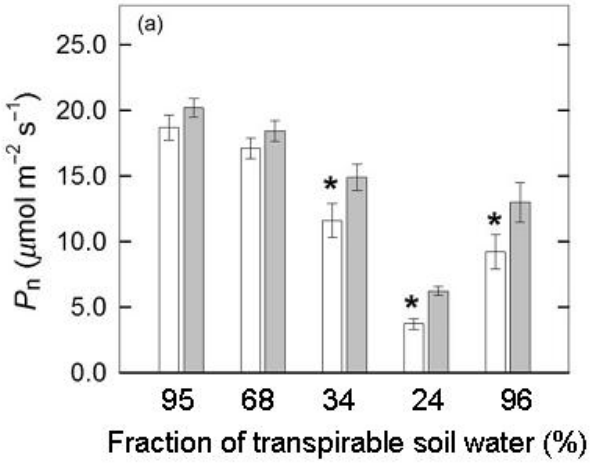


Fig. XV.2. Photosynthesis (P_n, **a**) in non-emitting (white bars) and isoprene-emitting (grey bars) tobacco plants (line 12) during drought stress (FTSW=68, 34 and 24%) and subsequent re-watering (FTSW=96%) (Tattini et al. – Plant, Cell and Environmnet 37:1950-1964, 2014).

Drought stress had a minor effect on foliar carotenoids in non-emitting plants, whereas in isoprene-emitting plants, it stimulated the biosynthesis of antheraxanthin and zeaxanthin and consequently enhanced de-epoxidation (DES) of violaxanthin cycle pigments, during severe drought and on re-watering (**Fig. XV.4c,d,f**). We conclude that drought-induced isoprene biosynthesis may up-regulate MEP-derived isoprenoid metabolism, which also produces carotenoids.

In plants under mild drought, no difference is observed between ABA levels in isoprene-emitting and non-emitting plants (Ryan et al. 2014). However, in our experiment isoprene-emitting plants accumulated significantly higher levels ABA than non-emitting plants under severe drought and re-watering (**Fig. XV.5**). This might be due to both the stimulation of the MEP pathway (in leaves, isoprene and ABA are both synthesized through the same biosynthetic pathway – the MEP pathway in the chloroplasts, see Lichtenthaler et al. 1997) and the transport of ABA synthesized by roots (Simonneau et al. 1998) or shoots (Christmann et al. 2005).

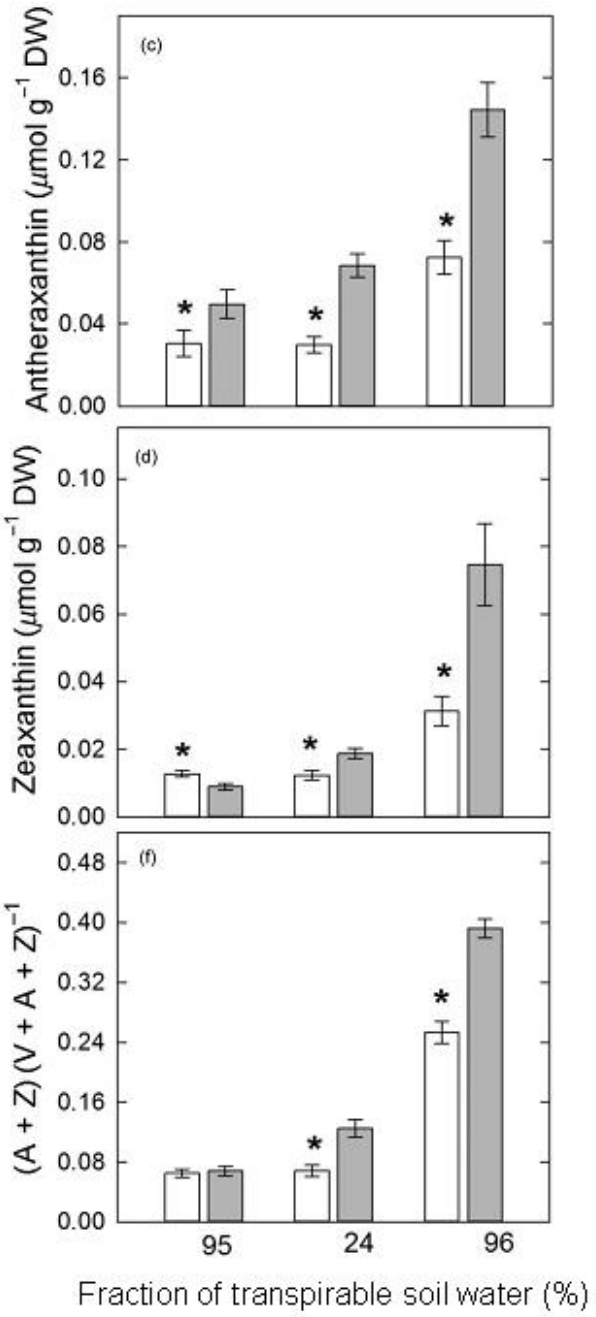


Fig. XV.4. Concentration of antheraxanthin **(c)**, zeaxanthin **(d)** and de-epoxidation state of violaxanthin cycle pigments **(f)** in non-emitting (white bars) and isoprene-emitting (grey bars) tobacco leaves (line 12) during droght stress (FTSW=24%) and subsequent re-watering (FTSW=96%) (Tattini et al. – Plant, Cell and Environmnet 37:1950-1964, 2014).

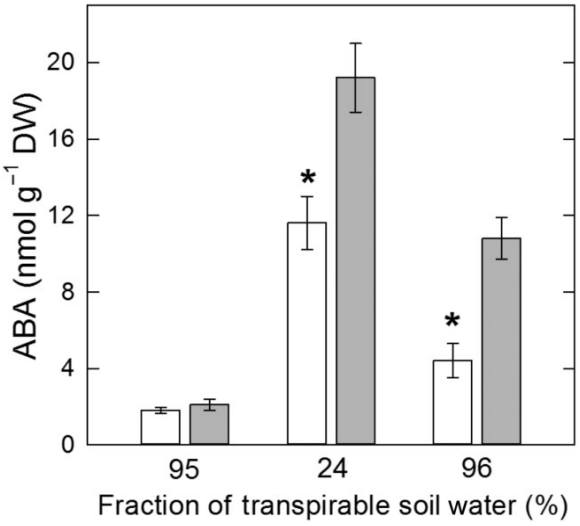


Fig. XV.5. Concentration of abscisic acid (ABA) in leaves of non-emitting (white bars) and isoprene-emitting (grey bars) tobacco (line 12) during drought stress (FTSW=24%) and subsequent re-watering (FTSW=96%) (Tattini et al. – Plant, Cell and Environmnet 37:1950-1964, 2014).

The higher ABA levels we observed in isoprene-emitting plants relative to non-emitting plants under severe drought correlates with the increased starch degradation and increased hexoses levels observed in isoprene-emitting plants (**Fig. XV.6**). The high hexose levels are sustained after re-watering, although starch levels recover. This is consistent with the partial recovery of photosynthesis, which is clearly then sufficient to re-stock the starch pool. ABA and soluble sugars can act individually and/or in concert in promoting the biosynthesis of phenylpropanoids, particularly flavonoids (Koch 1996; Abe et al. 1997; Tossi et al. 2009; Luo et al. 2012). In our isoprene-emitting tobacco leaves, the concentration of effective antioxidant phenylpropanoids, namely chlorogenic acid isomers and quercetin 3-O-rutinoside was higher than in non-emitting plants during and after drought stress (**Fig. XV.7**).

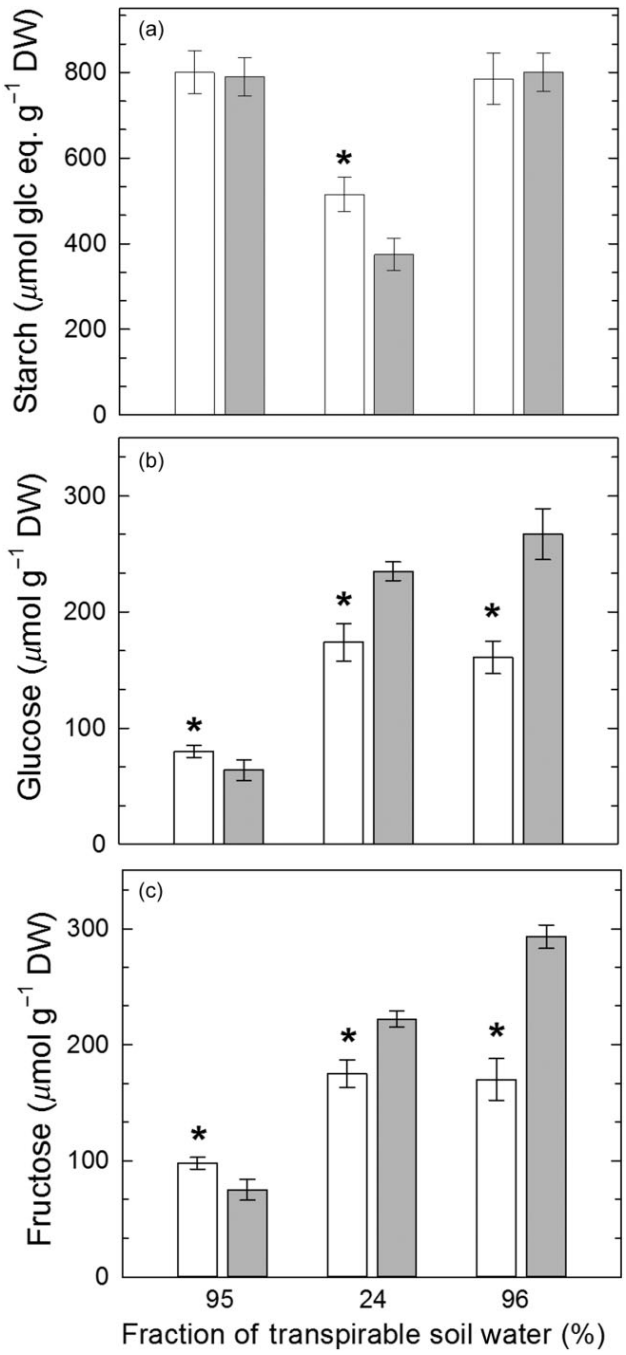


Fig. XV.6. Concentration of starch **(a)**, glucose **(b)** and fructose **(c)** in leaves of non-emitting (white bars) and isoprene-emitting (grey bars) tobacco (line 12) during drought stress (FTSW=24%) and subsequent re-watering (FTSW=96%) (Tattini et al. – Plant, Cell and Environmnet 37:1950-1964, 2014).

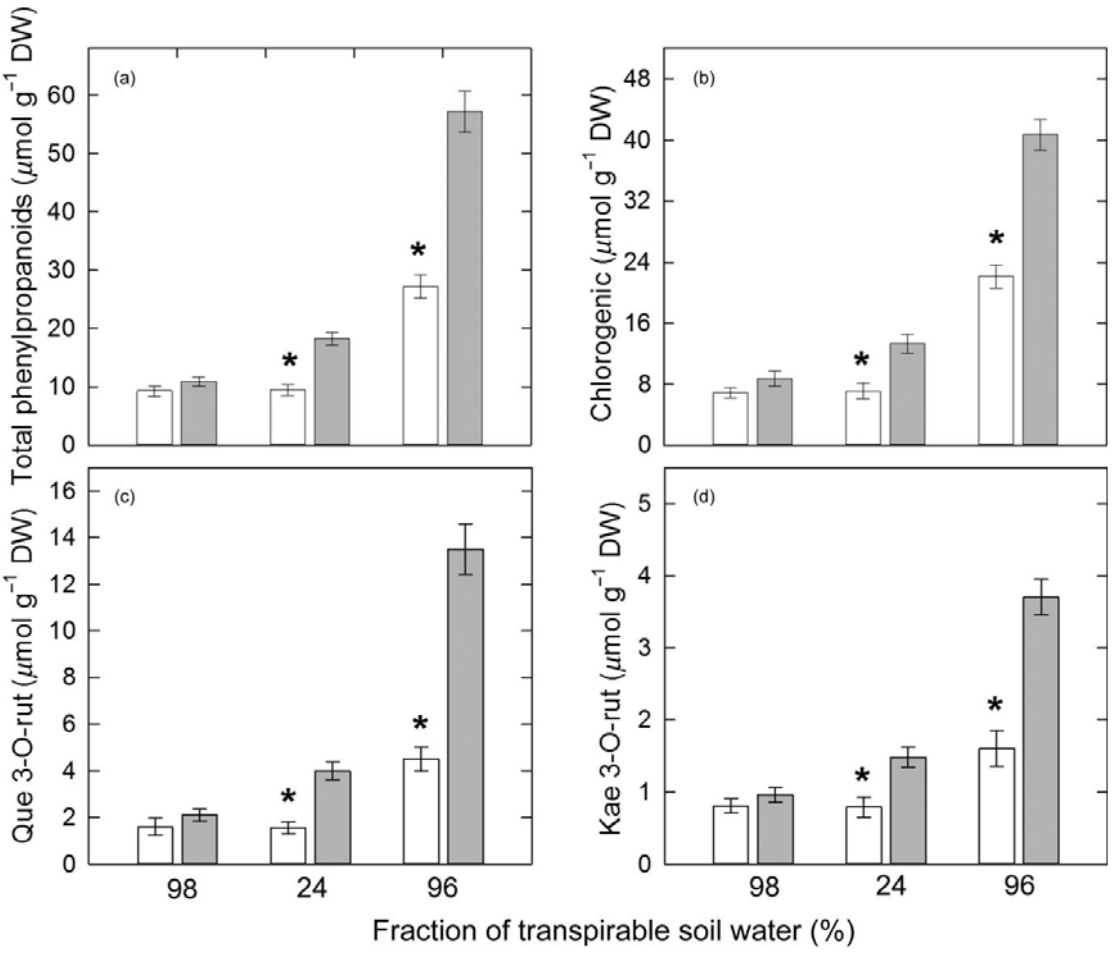


Fig. XV.7. Concentration of total **(a)** and individual phenylpropanoids [chlorogenic = chlorogenic acid isomers **(b)**; Que 3-O-rut = quercetin 3-O-rutinoside **(c)**; Kae 3-O-rut = kampferol 3-O-rutinoside **(d)**] in leaves of non-emitting (white bars) and isoprere-emitting (grey bars) tobacco (line 12) during drought stress (FTSW=24%) and subsequent re-watering (FTSW=96%) (Tattini et al. – Plant, Cell and Environmnet 37:1950-1964, 2014).

It is suggested that isoprene production in transgenic tobacco triggered different responses, depending upon drought severity. Under drought, the observed trade-off between isoprene and non-volatile isoprenoids suggests that in isoprene-emitting plants isoprene acts as a short-term protectant, whereas non-volatile isoprenoids protect against severe, long-term damage. After drought, it is suggested that the capacity to emit isoprene might up-regulate production of non-volatile isoprenoids and phenylpropanoids, which may further protect isoprene-emitting leaves.

4.4. Endogenous isoprene and nitric oxide interaction *in planta* (papers V. Velikova et al. – New Phytologist 166:419-426, 2005; VIII. Velikova et al. – Plant, Cell and Environment 31:1882-1894, 2008; XIII. Velikova et al. – Plant Signaling & Behavior 7:139-141, 2012)

Isoprene is a highly reactive volatile compound and reactions with other gaseous compounds emitted by leaves are theoretically possible.

Isoprene reduces the amount of H_2O_2 formed in ozonated leaves (paper I. Loreto & Velikova 2001), and quenches singlet oxygen (Affek & Yakir 2002; paper II. Velikova et

al. 2004), thus providing a general protection against reactive oxygen species (Vickers et al. 2009b). Our **hypothesis** is that isoprene could also react with nitric oxide (NO) or with peroxynitrite ($ONOO^-$) formed by NO–ROS interaction, reducing the presence of these compounds which may be harmful for many biological molecules (Lipton et al. 1993), and also indirectly modulating the amount of NO reacting with ROS to initiate cellular hypersensitive responses. **For the first time** we demonstrated that that NO accumulated only in isoprene-inhibited *P. australis* leaves that were exposed to ozone, while NO was not detected in isoprene-emitting or isoprene-inhibited non-ozonated leaves, or in isoprene-emitting leaves exposed to ozone (**Fig. V.3**). We showed that isoprene-inhibited poplar leaves exposed to oxidative stress (Rose Bengal) emit higher amounts of NO (**Fig. VIII.4a**), and that the best protection of the photosynthetic apparatus against oxidative damage occurs indeed in NO-enriched, isoprene-emitting leaves (**Fig. VIII.4c**).

If isoprene can modulate NO presence in leaves, then it not only protects leaves against stress, but may also indirectly influence plant response to stress. NO is induced in almost every stress response analysed so far (Grün et al. 2006). Exogenous NO supplied as a gas or produced by the NO donor SNP had a beneficial effect on leaves that were exposed to oxidative stresses by reducing photosynthesis inhibition (**Fig. VIII.4c**) and accumulation of ROS and other products of membrane degradation. At present, NO is a well-established signalling molecule, and it is suggested that the level of NO available for signalling is a balance between its rate of synthesis and removal (Wilson et al. 2008). In *4.5.B.* the interaction between isoprene and nitric oxide and protein nitrosylation is discussed (paper XVIII).

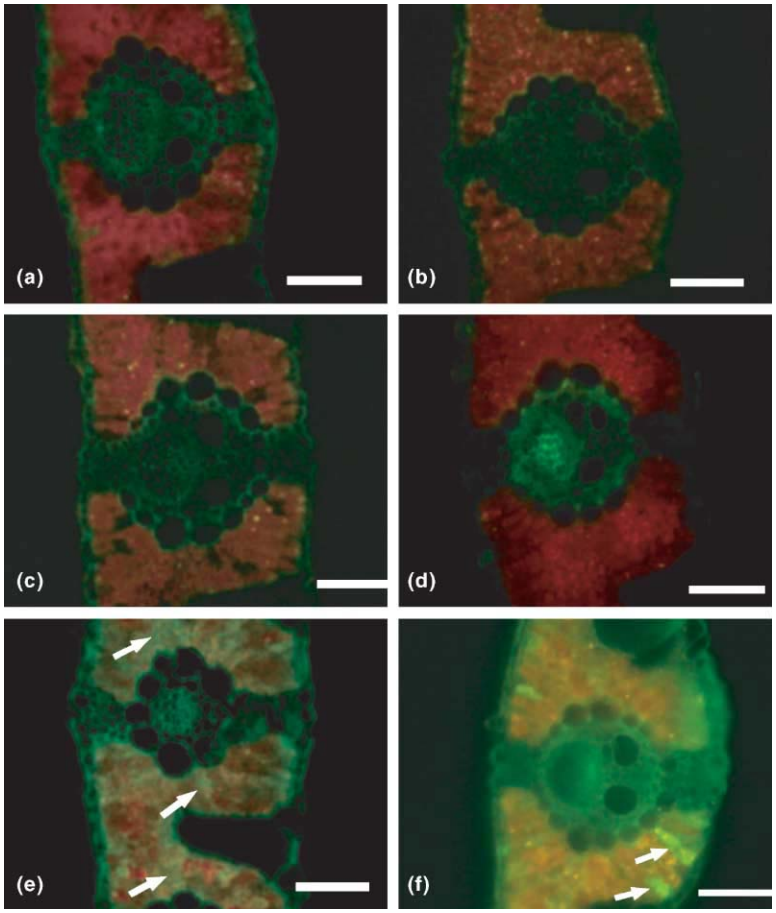


Fig. V.3. Nitric oxide (NO) localization in mesophyll cells of *Phragmites australis*. NO (bright yellow-green fluorescence, see arrows) was not significantly revealed by the fluorescence probe in isoprene-emitting leaves **(a)**, and in isoprene-inhibited leaves **(b)** that were not exposed to ozone. NO was also not detected in ozonated isoprene-emitting leaves **(c)**, and in ozonated isoprene-inhibited leaves in which NO was scavenched by c-PTIO **(d)**. In the absence of NO-scavenging compounds, NO diffusely colonized the mesophyll cells of ozonated isoprene-inhibited leaves as shown in two different leafsections (**e, f**) (Velikova et al. – New Phytologist 166:419-426, 2005).

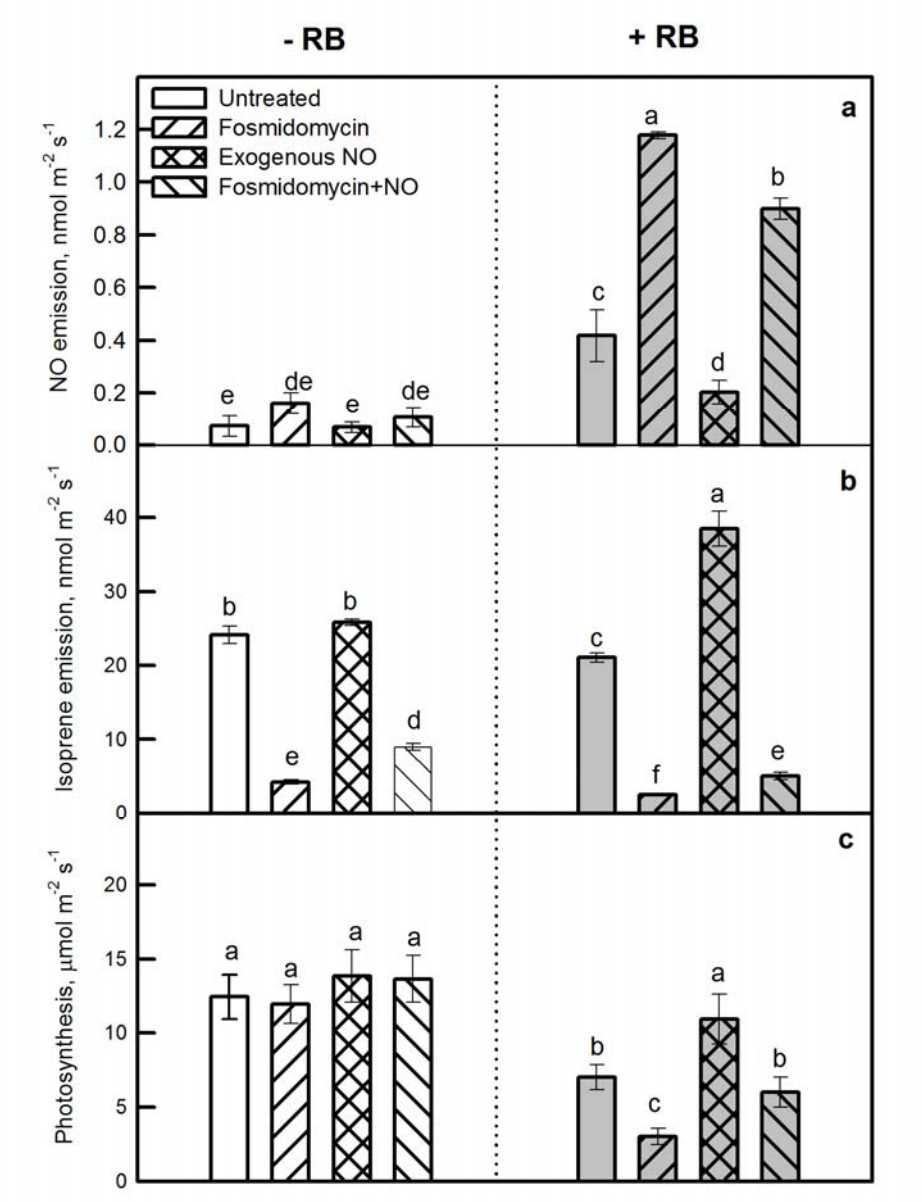


Fig. VIII.4. Nitric oxide (NO) **(a)** and isoprene emissions rates **(b)**, and photosynthesis **(c)** in non-stressed (-RB) and RB-treated (+RB) *Populus nigra* leaves (Velikova et al. – Plant, Cell and Environment 31:1882-1894, 2008).

The amounts of NO and H_2O_2, two key molecules in plant signaling inducing hypersensitive responses to stress (Delledonne et al. 2001; Delledonne 2005) were measured in wild-type and transgenic (*IspS*) plants before stress at growth temperature

(22°C) and after exposure to heat (38°C; **Fig. XIII.1**) for 48 h. A significant increase of H_2O_2, and NO was found in both wild-type and isoprene-emitting heat-stressed Arabidopsis plants (**Fig. XIII.1**). However, the accumulation of H_2O_2 was substantially higher in wild-type than in *IspS* lines, whereas NO increased similarly in the two plants compared with pre-stress conditions. By changing the balance between NO and H_2O_2 under heat stress conditions, isoprene emission may also modify the signaling pattern of these molecules (Delledone et al. 2001).

When isoprene is not produced (wild-type *Arabidopsis*), the simultaneous accumulation of H_2O_2 and NO may trigger cell death signaling, whereas in isoprene-emitting plants (*IspS*) the accumulation of H_2O_2 and NO may not reach toxic levels or their ratio may not be adequate. Consequently, the signaling action of the two molecules might be dimmed and hypersensitive responses might be prevented.

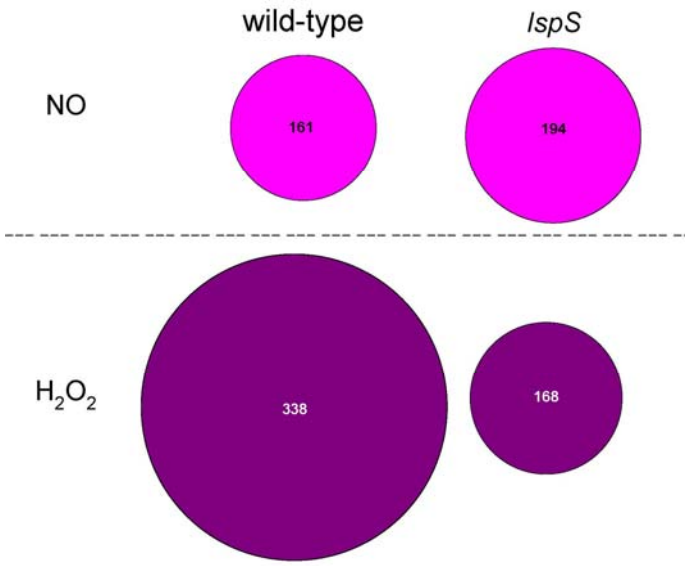


Fig. XIII.1. Increased NO (pink) and H_2O_2 (purple) contents in wild-type (non-emitting) and transgenic *IspS* (*isoprene-emitting*) *Arabidopsis* plants after exposure to heat (38°C for 4 h). Velues are present increase with respect to pools of NO and H_2O_2 detected in wild-type and *IspS* plants grown at 22°C (Velikova et al. – Plant Signaling & Behavior 7:139-141, 2012).

It is noteworthy that the level of H_2O_2 is significantly lower in isoprene-emitting *Arabidopsis* plants, while in terms of nitric oxide concentration the differences between the wild-type and the mutant are significantly smaller. The relatively lower increase (168%) of H_2O_2 in *IspS* could be an indirect consequence of the retained functionality of the photosynthetic regulatory mechanisms in these plants, which is due to the ability of isoprene to stabilize membranes (paper XII. Velikova et al. 2011).

If ROS damage membranes and damaged membranes lead to ROS production then a feedforward loop can occur. Stresses that make ROS (e.g. ozone) and stresses that damage membranes (e.g. heat) will activate the feedforward cycle and lead to H_2O_2 accumulation and eventually cell death. Isoprene could stop this feedforward cycle in either of two ways: (1) quenching the ROS, and (2) stabilizing membranes (paper XIII. Velikova et al. 2012). If isoprene quenches ROS the products of the isoprene/ROS interaction need to be considered. One prominent product is methylvinyl ketone, which could be cytotoxic (Vollenweider et al. 2000). The results of Velikova et al. (2011) allow speculation that

membrane stabilization is a major mechanism by which isoprene helps plants tolerate abiotic stress of many different forms (paper XII. Velikova et al. 2011).

4.5. Proteins, lipids and chloroplast ultrastructure of plants with inhibited isoprene emission (papers **XIV. Velikova et al. – Journal of Proteome Research 13:2005-2018, 2014, XVII. Velikova et al. – Plant Physiology 168: 859-870, 2015, XVIII. Vanzo et al. – Plant Physiology 170: 1945-1961, 2016**)

4.5.A. Genetic manipulation of isoprene emission remodels the chloroplast proteome (paper **XIV. Velikova et al. – Journal of Proteome Research 13:2005-2018, 2014**)

We tested the **hypothesis** that suppression of isoprene production in the poplar plants by genetic engineering would cause changes in the chloroplast protein profile, which in turn would compensate for changes in chloroplast functionality and overall plants performance under abiotic stress. This is the **first study** to specifically address changes in the chloroplast protein profile due to the altered ability of plants to emit isoprene.

We applied ICPL analysis, which is based on isotopic labeling of all free amino groups in proteins. This method enables quantitative proteome profiling of highly complex protein mixture and has never been previously used for plant chloroplasts. ICPL labeling allowed the quantification of 119 chloroplastic proteins, which were annotated by searching against in the *Populus trichocarpa* genome sequences (Phytozome v9.1, http://www.phytozome.net). We clustered the 119 proteins in 8 functional categories. The main group (29.4% of the total number of proteins) comprised proteins associated with photosynthetic light reactions, proton transport, oxidation− reduction, the Calvin cycle, and the oxidative phosphopentose pathway. "Ribosomal proteins" represented the next prominent group (19.3%), followed by the category of "Structural role" (16.0%) summarizing proteins involved in protein synthesis, binding, and folding. Proteins clustered in the "Metabolism" group (12.6%) are assigned to various metabolic processes. "Histones" represented 7.6% of the overall number of proteins. Only a few proteins were related to "Stress" (1.7%) and "Others" (2.5%). A total of 10.9% labeled proteins were not functionally annotated or are still not yet identified (**Fig. XIV.2**).

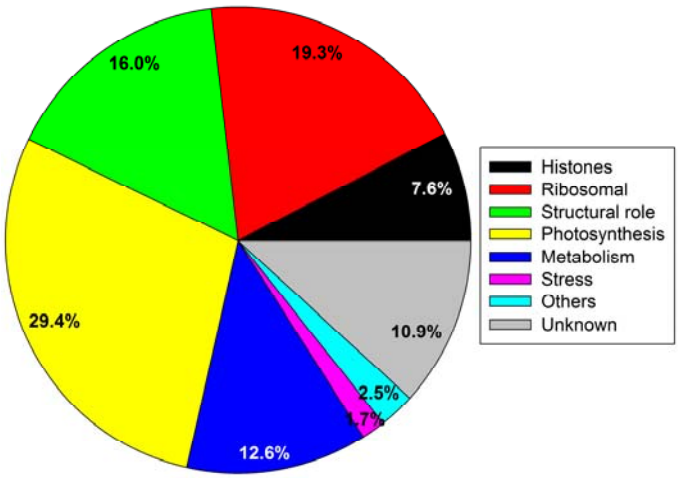


Fig. XIV.2. Functional summary of the 119 chloroplast proteins identified using the ICPL technique in non-emitting and isoprene-emitting grey poplar lines. Identified proteins are clustered

in eight categories, according to their functions (Velikova et al. – Journal of Proteome Research 13:2005-2018, 2014).

In **conclusion**, the inhibition of isoprene production in poplar implied that the down-regulation of proteins related to photosynthesis light reactions, redox regulation, and oxidative stress defense and several proteins with structural activity that are responsible for lipid metabolism alteration occurred (**Fig. XIV.5**). These changes were the consequences of alternative defense mechanisms such as photorespiration and nonphotochemical quenching that needed to compensate for the absence of isoprene. Indeed, the lower amounts of peroxiredoxin and ascorbate peroxidase indicated their overoxidation in the presence of increased levels of ROS. Overall, the present proteomic analysis revealed that the absence of isoprene in poplar leaves remodels the chloroplast protein profile to cope against oxidative stress. These data strongly support the idea that isoprene improves thylakoid membrane structure and regulates the production of ROS.

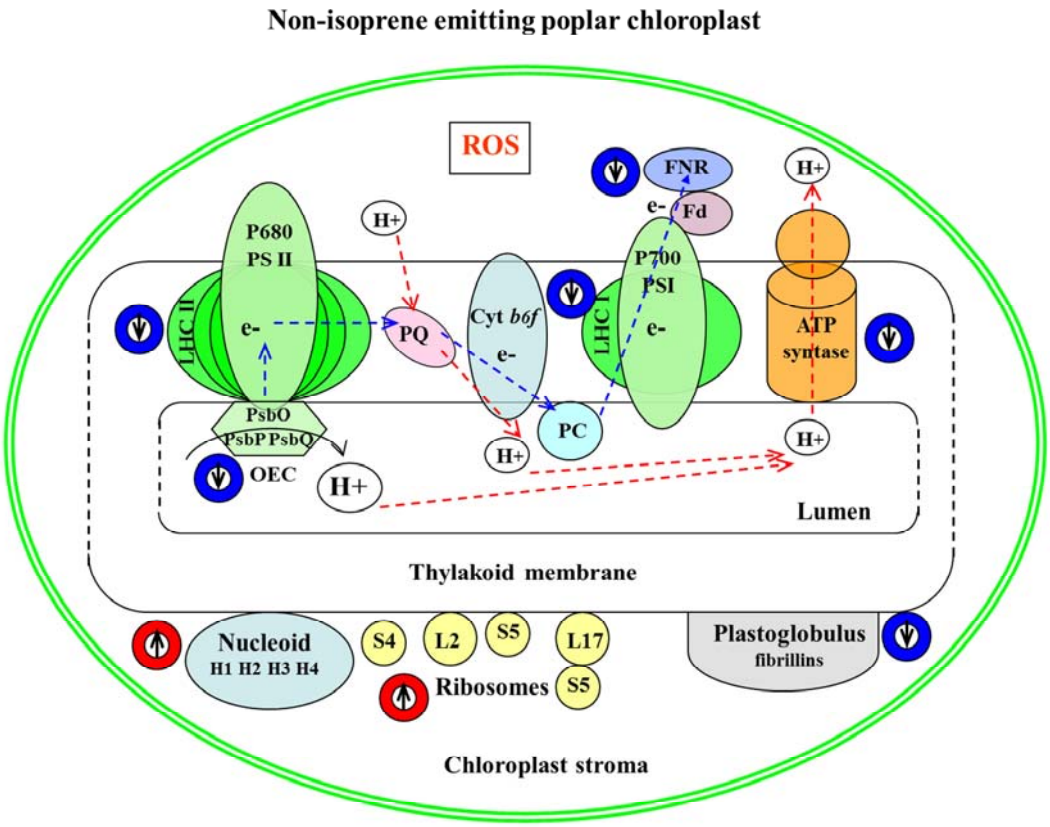


Fig. XIV. 5. Suborganelle structure of non-isoprene emitting poplar chloroplasts. Blue symbols with the arrow pointing down indicate the spatial loci where proteins were down-regulated and red symbols with the arrow pointing up indicate the spatial loci where proteins were up-regulated. Isoprene suppression caused significant down-regulation of specific proteins related to photosynthetic electron transport, redox regulation and oxidative stress defence, and several proteins with structural role, which responsible for the alteration of lipid metabolism. The chloroplast proteome of transgenic poplar were further characterized by a higher abundance of hystones and ribosomal proteins, which may be linked to a higher protein turnover in non-isoprene emitting poplar chloroplasts (Velikova et al. – Journal of Proteome Research 13:2005-2018, 2014).

4.5.B. Inhibition of isoprene emission in poplar modulates protein S-nitrosylation (paper **XVIII. Vanzo et al. – Plant Physiology 170: 1945-1961, 2016**)

Nitric oxide (NO) has been found to accumulate only in isoprene-inhibited ozonated leaves (paper V. Velikova et al. 2005), these leaves emit higher amounts of nitric oxide (paper VIII. Velikova et al. 2008). Nitric oxide exerts its signaling action by directly altering proteins through posttranslational modifications (PTMs; i.e. *S*-nitrosylation, metal nitrosylation, and Tyrosine nitration). *S*-Nitrosylation, the covalent binding of NO to the thiol side of protein-Cys residues to form nitrosothiols, is regarded as the most important PTM of NO signaling in plants (Moreau et al. 2010).

- ***Isoprene suppression results in slight modification of the S-nitroso-proteome of grey poplar plants under unstressed conditions***

Isoprene-emitting and non-emitting genotypes exhibited only minor differences in the *S*-nitrosylation pattern of unstressed plants. Five of the discriminant proteins were found to be more *S*-nitrosylated in non-isoprene emitting plants. These are Rubisco activase, a-N-arabinofuranosidase (ARA), phosphoribulokinase (PRK), HSP70, and O-acetylserine(thiol)lyase (OAS-TL). By contrast, only one protein, a PSII assembly protein, was less *S*-nitrosylated in the in non-isoprene emitting genotype compared with the isoprene-emitting genotype.

- ***Acute ozone fumigation stimulates NO emission and modifies the S-nitroso-proteome of isoprene-emitting and non-emitting grey poplar***

Under control conditions, emissions of NO did not differ significantly between non-emitting and isoprene-emitting poplar genotypes, although a tendency to higher emission from the non-emitting genotype was observed (**Fig. XVIII.3**). Emissions of NO were induced rapidly after the ozone exposure in both genotypes, but NO emissions were much more induced in non-emitting shoots. The finding that non-emitting poplar emits significantly higher rates of NO upon ozone fumigation compared with the natural isoprene-emitting genotype is an indication that isoprene interferes in the signaling pathway activated by NO - ROS interactions.

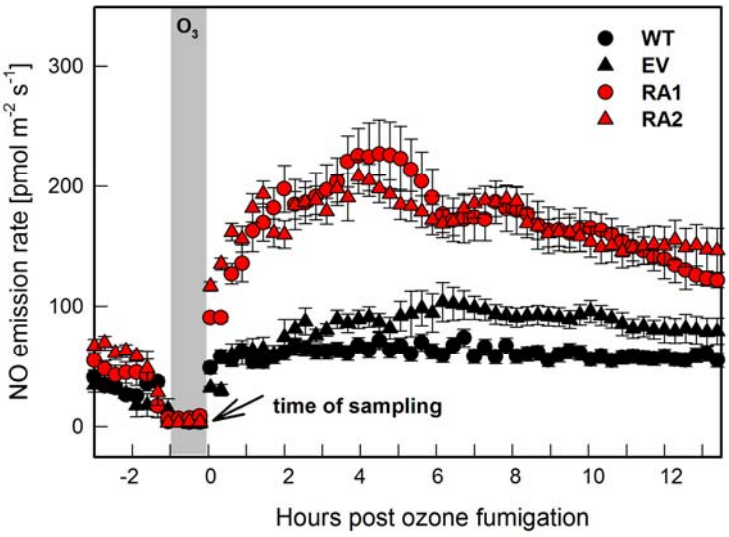


Fig. XVIII.3. Time-course curves of NO emission rates in shoots of **isoprene-emitting (WT and EV)** and **non-emitting (RA1 and RA2)** grey poplar before and after ozone fumigation. The vertical grey bar indicates the period of ozone fumigation (Vanzo et al. – Plant Physiology 170:1945-1961, 2016).

Irrespective to the plant genotypes, ozone induced strong changes in the *S*-nitroso-proteome. Principal component analysis (PCA) revealed that the pronounced differences in the abundance of *S*-nitrosylated proteins between non-emitting and isoprene-emitting genotypes appear after ozone treatment, as indicated by a clear separation between ozonated NE and IE samples in the first and second principal components (Supplemental **Fig. XVIII.S1A**). The functional categorization of the 203 *S*-nitrosylated proteins revealed a strong dominance of proteins related to photosynthetic processes (21%), followed by protein synthesis, degradation and folding processes (19%), and redox regulation and signaling (8%; Supplemental **Fig. XVIII.S1B**; Supplemental **Table XVIII.S2**). For clarity, **Fig. XVIII.5** presents the results of an OPLS analysis (Orthogonal Partial Least Squares), which identifies discriminant proteins able to discriminate between isoprene-emitting and non-emitting genotypes. in the profiles of inseparable and separating poplar lines. The separation between treatments and genotypes can be explained by the 63 discriminant *S*-nitrosylated proteins out of a total of 203 (Supplemental **Table XVIII.S4**).

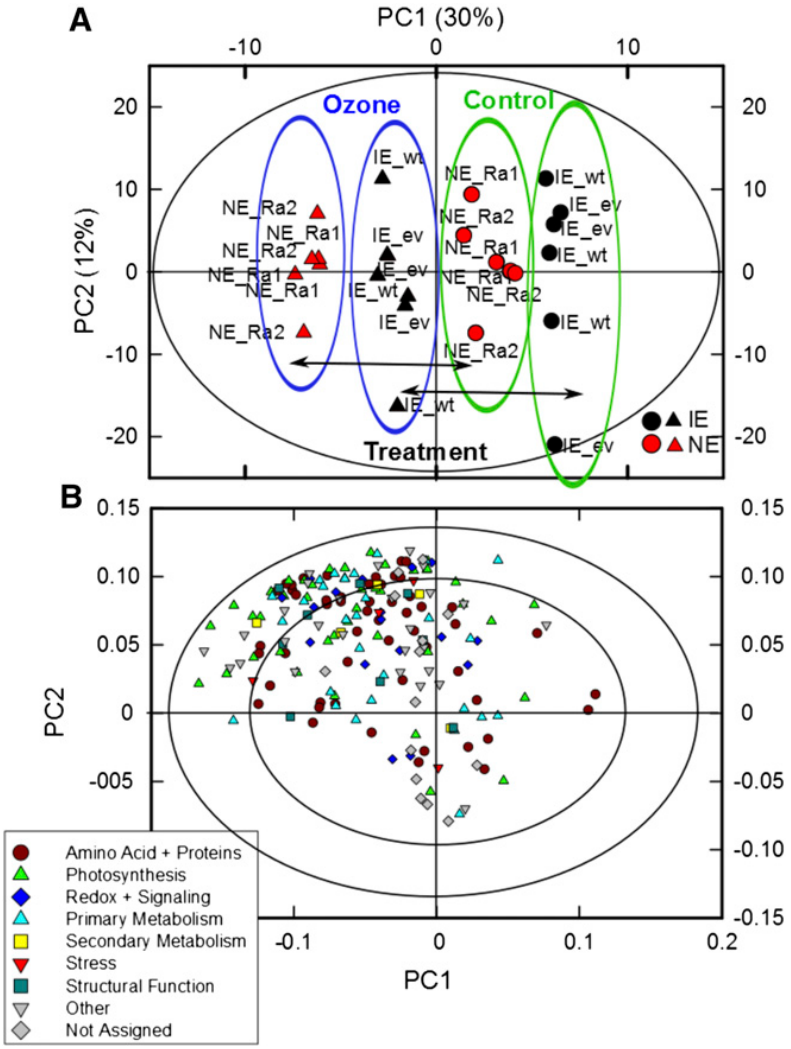


Fig. XVIII.5. Score **(A)** and loading **(B)** plots of the OPLS of *S*-nitrosylated protein abundance from control and ozone samples of isoprene-emitting (IE; wt and ev) and non-emitting (NE; Ra1 and Ra2) genotypes. **(A)** Plants were divided into the ozone group (triangles; n=12) and the control group (circles; n=12); **(B)** Each functional group of proteins is indicated with different colors. The outer and inner ellipses indicate 100% and 75% explained variance, respectively. Each point represents an independent plant in the score plot and an individual protein in the loading plot (Vanzo et al. – Plant Physiology 170:1945-1961, 2016).

Overall, the data strongly support the hypothesis (Vickers et al. 2009b) that unsaturated volatile isoprenoids such as isoprene can alter signaling pathways by modulating to what extent and how rapidly ROS and NO signaling molecules are generated within a cell, thus likely modulating the velocity and extent of the physiological response upon biotic and abiotic stress (Ahlfors et al. 2009; Wang et al. 2013).

4.5.C. Knocking down of isoprene emission modifies the lipid matrix of thylakoid membranes and influences the chloroplast ultrastucture in poplar (paper **XVII. Velikova et al. – Plant Physiology 168: 859-870, 2015**)

One of the proposed biological functions of isoprene is the stabilization of thylakoid membrane structures through modification of the lipid environment and organization of the pigment-protein complexes in thylakoid membranes (paper XII. Velikova et al. 2011). We

demonstrated that the total amount of monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG) and phospholipids is reduced in chloroplasts when isoprene biosynthesis is blocked (**Fig. XVII.1**).

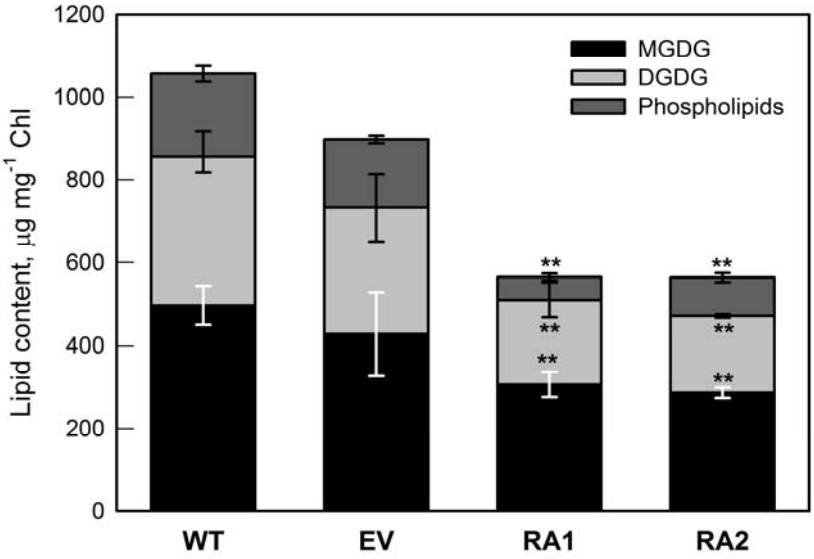


Fig. XVII.1. Lipid contents in isolated chloroplasts of isoprene-emitting (WT, EV) and non-emitting (RA1, RA2) poplar. Asterisks indicate significant differences from the wild-type (WT): **, $P < 0.01$ (Velikova et al. – Plant Physiology 168:859-870, 2015).

The reduced amount of galactolipids and PLs and significant alterations in the chloroplast proteome (paper XIV. Velikova et al. 2014) were associated with considerable changes in chloroplast ultrastructure of non-emitting poplar genotypes (**Fig. XVII.3B,D**), thus indicating the important role of isoprene in structural organization of plastidic membranes. Indeed, the chloroplasts of non-emitting plants were characterized by a less developed membrane system, with shorter and fewer grana stacks (**Fig. XVII.4E**) and longer stroma thylakoids. Non-emitting chloroplasts contained more plastoglobules and smaller starch grains than isoprene-emitting chloroplasts (**Fig. XVII.3 B and D**). Non-emitting chloroplasts were also in close structural contact with mitochondria through relatively large associative regions. A relatively large number of non-emitting chloroplasts were undeveloped (data not shown).

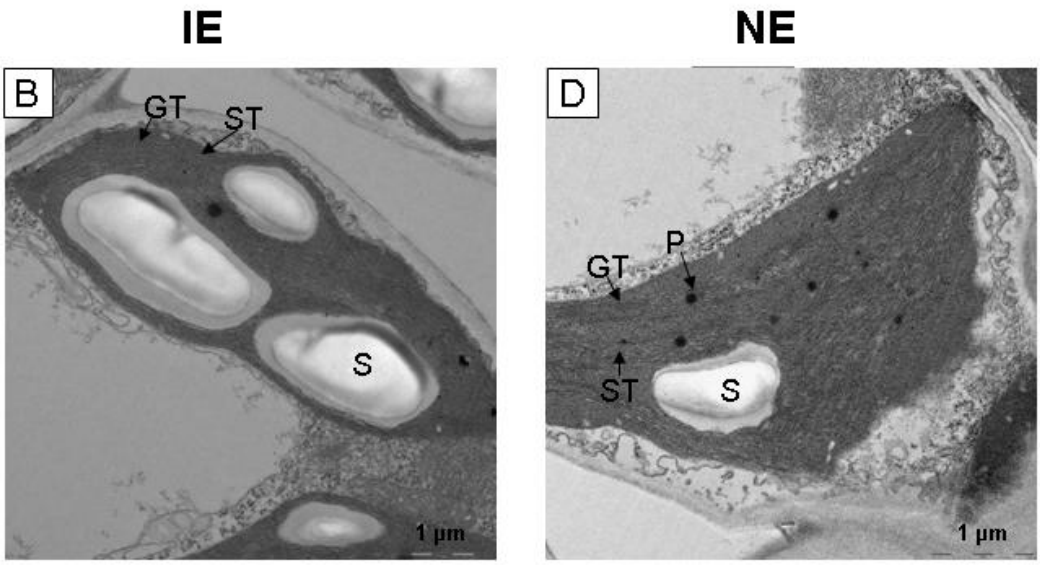


Fig. XVII.3. Transmission electron micrographs of representative chloroplast cross sections taken from intact leaves of isoprene-emitting **(B)** and non-emitting **(D)** poplar. GT – granal thylakoids, P – plastoglobuli, ST – stroma, S – starch grain. Bars = 1µm at 6300X magnification (Velikova et al. – Plant Physiology 168:859-870, 2015).

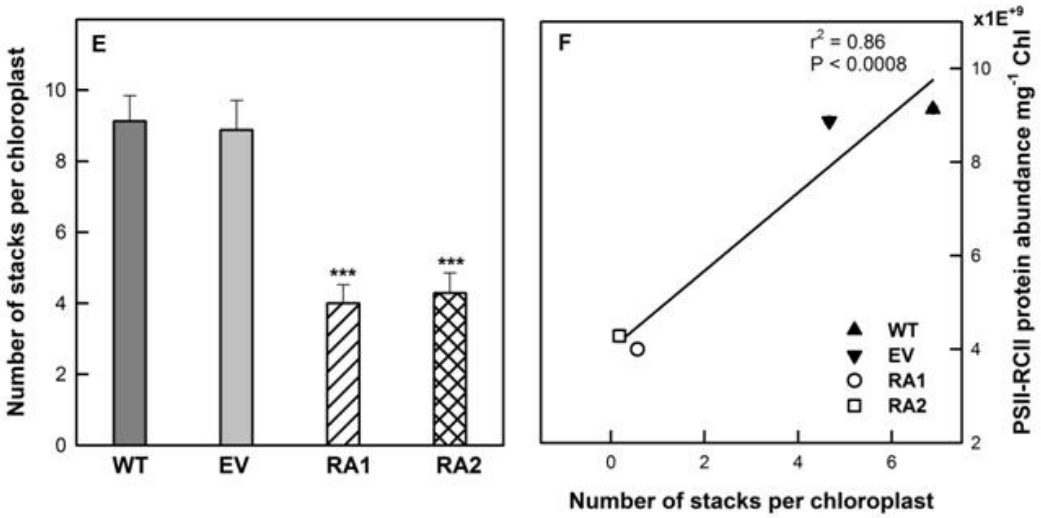


Fig. XVII.4. Average number of stacks per chloroplast **(E),** and correlation between PSII-RCII protein abundance and number of stacks **(F)** in isoprene-emitting (WT, EV) and non-emitting (RA1, RA2) poplar genotypes. Asterisks indicate significant differences from the wild-type (WT) (Velikova et al. – Plant Physiology 168:859-870, 2015).

A significantly lower amount of unsaturated fatty acids, particularly linolenic acid (18:3) in non-emitting chloroplasts (Supplemental **Fig. XVII.S1**), was associated with the reduced fluidity of thylakoid membranes, which in turn negatively affects photosystem II photochemical efficiency. The low photosystem II photochemical efficiency (Φ_{PSII}) in non-emitting plants was negatively correlated with nonphotochemical quenching (NPQ) (**Fig. XVII.6A,C**).

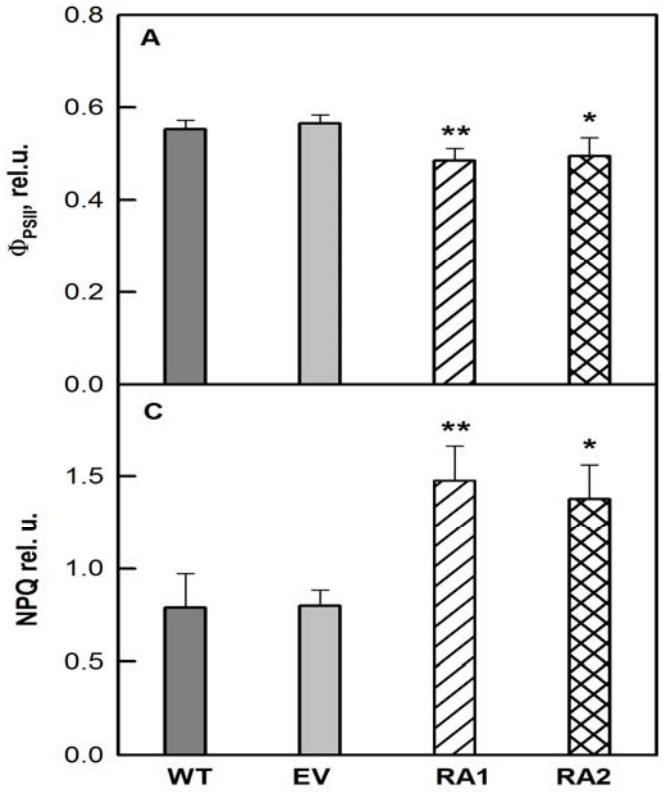


Fig. XVII.6. Photosystem II photochemical efficiency (Φ_{PSII}) **(A)** and nonphotochemical quenching (NPQ) **(C)** of isoprene-emitting (WT, EV) and non-emitting (RA1, RA2) poplar plants at growth conditions. Asterisks indicate significant differences from the wild type (WT): *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (Velikova et al. – Plant Physiology 168:859-870, 2015).

For the first time, we provided direct evidence of the relationship between isoprene emission and the level of main lipid classes and their fatty acid composition, and we characterized the structural organization of the photosynthetic machinery in isoprene-emitting and non-emitting poplar genotypes. The suppressed isoprene production in non-emitting plastids was associated with the reduced amount of galactolipids and PLs, the lower level of the major fatty acid (18:3), and the altered chloroplast ultrastructure. The suppression of isoprene biosynthesis causes considerable metabolic changes, particularly in lipid biosynthesis (Way et al. 2013; paper XIV. Velikova et al. 2014; Kaling et al. 2015). It was suggested retrograde signaling (Pfannschmidt 2010) in non-emitting chloroplasts. However, the precise mechanisms for the transmission of the changes in chloroplast to the nucleus in non-emitting plant cells remain elusive.

4.6. Relationship between isoprenoids and phenylpropanoids under stress conditions (papers **XVI. Tattini et al. - New Phytologist 207: 613-626, 2015, XIX. Velikova et al. - Plant, Cell and Environment 39: 2185-2197, 2016, XX. Ahrar et al. - Journal of Experimental Botany 68(9): 2439-2451, 2017**)

4.6.A. Isoprenoids and phenylpropanoids are orchestrated daily by drought-stressed Platanus x *acerifolia* plants (paper XVI. Tattini et al. - New Phytologist 207: 613-626, 2015)

Our experiment reveals a sequence of antioxidants that were used daily by plants to orchestrate defense against oxidative stress induced by drought and associated high light and high temperature. Plants invest in the biosynthesis of isoprenoids and phenylpropanoids to compensate for the reduction of primary antioxidants when photosynthesis is strongly inhibited by drought. We offered **new evidence** of a daily orchestration of antioxidant defenses, and unveil an unanticipated integration of volatile, nonvolatile isoprenoid, and flavonoid biosynthesis in plants challenged by a combination of high temperature, high light and drought. Antioxidant enzymes clearly help leaves to cope with drought stress conditions mostly during the morning hours, whereas they do not seem to operate effectively during the central and hottest hours of the day. Carotenoids, in particular zeaxanthin, are the main antioxidant molecules during the sunniest and hottest hours of the day, which is consistent with the long-known sunlight-driven violaxanthin de-epoxidation and the consequent scavenging of ROS. β-carotene might also operate to counter photooxidative stress in all plants under these conditions.

Overall, the antioxidant enzymes (with the exception of guaiacol peroxidases) were mostly associated with drought during the morning hours (8:00–11:00 h; upper panels of **Fig. XVI.10**), whereas volatile and nonvolatile isoprenoids (isoprene and zeaxanthin) were clearly associated with drought early in the afternoon (15:00 h). Changes in phenylpropanoids, nonchloroplastic antioxidant enzymes, and antioxidant metabolites (quercetin, guaiacol peroxidases and ascorbic acid) were observed late in the afternoon. When the drought was severe, the largest differences in component functions were observed for quercetin - guaiacol peroxidases. Isoprene, whose biosynthesis is primarily temperature-dependent (Loreto & Sharkey 1990), might complement the "antioxidant" functions attributable to zeaxanthin (i.e. improving the thermal stability of thylakoid membranes; Peñuelas et al. 2005; paper XII. Velikova et al. 2011), particularly when declines in sunlight irradiance are coupled with an increasing air temperature, for example, early in the afternoon. An antioxidant system that probably operates in the vacuole, constituted by quercetin 3-*O*-glycosides, guaiacol peroxidases, and ascorbic acid, mainly reduces H_2O_2 following long exposure to high sunlight irradiance and heat stress, that is, late in the afternoons, in both unstressed control and drought-stressed leaves. The significance of this "secondary" H_2O_2-detoxifying system appears to be very important in severely drought-stressed leaves, following the severe depletion of antioxidant enzyme activity.

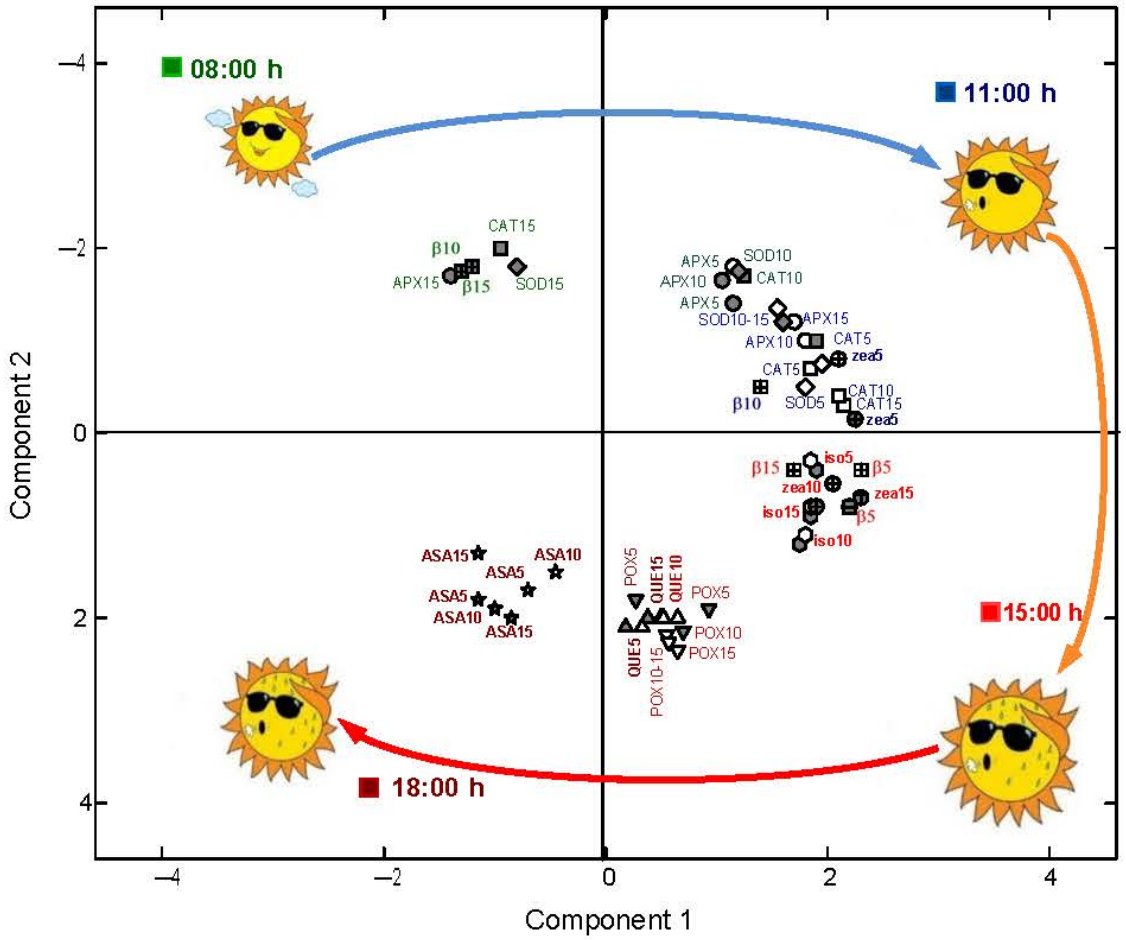


Fig. XVI.10. Principal component analysis (PCA) describing the daily orchestration on single components of the antioxidant machinery operating in leaves of well-watered (open symbols) or drought-stressed (closed symbols) *Platanus* x *acerifolia* concomitantly exposed to combined high solar irradiance and air temperature. *APX*, ascorbate peroxidase; ASA, ascorbic acid; β, β-carotene; *CAT*, catalase; iso, isoprene; *POX*, guaiacol peroxidases; QUE, quercetin derivatives; *SOD*, superoxide dismutase; zea, zeaxanthin (Tattini et al. – New Phytologist 207:613-626, 2015).

4.6.B. Physiological significance of isoprenoids and phenylpropanoids in drought response of Arundinoideae species with contrasting metabolism (paper **XIX. Velikova et al. - Plant, Cell and Environment 39: 2185-2197, 2016**)

The physiological role of isoprenoids and phenylpropanoids has been studied in species of the Arundinoideae subfamily, with contrasting capacity to emit isoprene - *Arundo donax* (a strong isoprene emitter) and *Hakonechloa macra* (does not emit any isoprenoids at detectable level) (Ahrar et al. 2015). We **hypothesize** that *A. donax* and *H. macra* respond to drought activating an alternative network of antioxidant defenses, with isoprenoids contributing to confer additional resistance to transient drought to the chloroplasts of *A. donax*, and phenylpropanoids offering antioxidant protection that sustain slow growth of *H. macra*.

Our study provided functional (**Fig. XIX.1**) and metabolic (**Fig. XIX.4**; **Fig. XIX.5; Fig. XIX.6**) evidences for *A. donax* resistance to drought, better plasticity and larger distribution than other Arundinoideae species, namely, *H. macra*. In particular, we show that *A. donax* (1) is able to efficiently limit water losses through a coordinated reduction of mesophyll (g_m) and stomatal (g_s) conductances, thus achieving a remarkable WUE under

stress; (2) displays an inherent and versatile antioxidant system to protect chloroplast structure and photo/biochemistry; and (3) is fully resilient after drought stress, achieving soon photosynthetic performances and structural features similar to unstressed leaves.

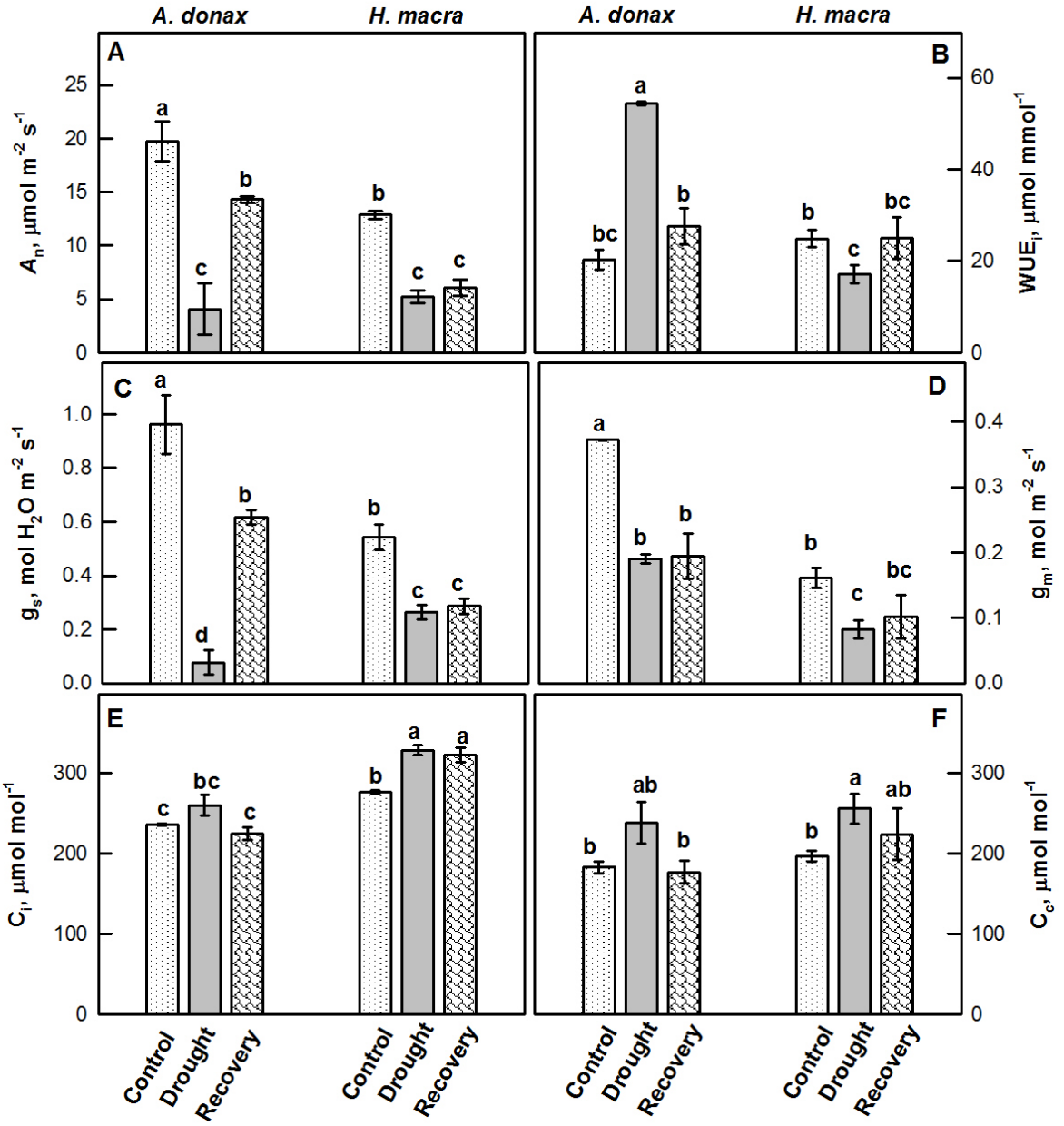


Fig. XIX.1. Photosynthesis (A_n) **(A)**, intrinsic water-use efficiency (iWUE) **(B)**, stomatal conductance (g_s) **(C)**, mesophyll conductance (g_m) **(D)**, and internal (C_i) **(E)** and chloroplast (C_c) **(F)** concentration of CO_2 in the control, drought stressed and recovered plants of *Arundo donax* and *Hackonechloa macra* (Velikova et al. – Plant, Cell and Environment 39:2185-2197, 2016).

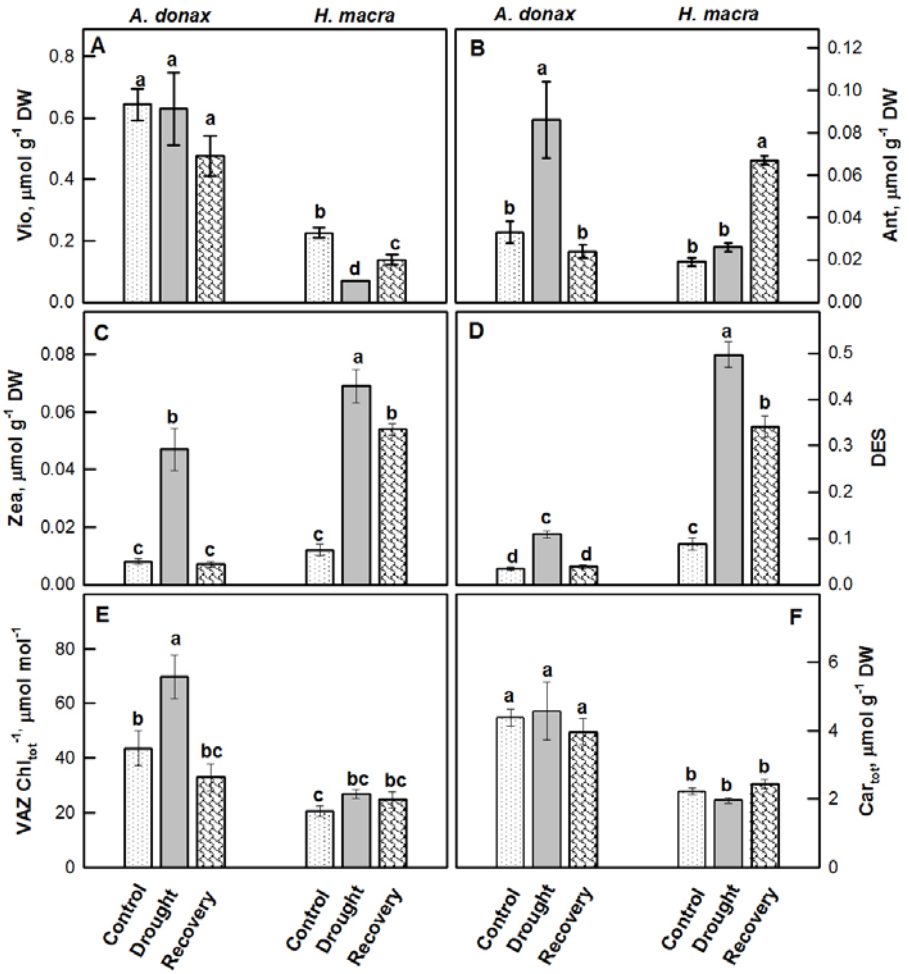


Fig. XIX.4. Violaxanthin (Vio) **(A)**, antheraxanthin (Ant) **(B)**, zeaxanthin (Zea) **(C)** and de-epoxidation state (DES) **(D)**, xanthophyll to chlorophyll ratio (VAZ Chl^{-1}) **(E)**, and total concentration of carotenoids (Car_{tot}) **(F)** in the control, drought stressed and recovered plants of *Arundo donax* and *Hackonechloa macra* (Velikova et al. – Plant, Cell and Environment 39:2185-2197, 2016).

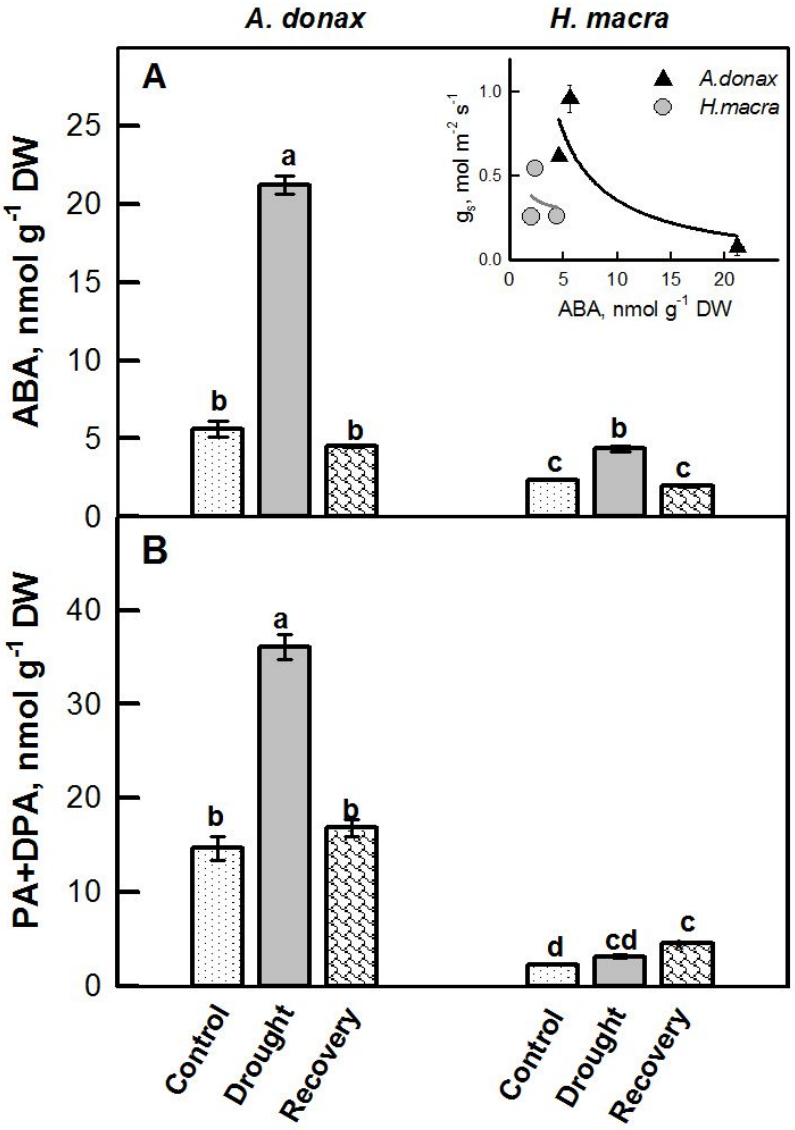


Fig. XIX.5. Levels of abcisic acid (ABA) **(A)** and sum of phaseic and di-hydrophaseic acids (PA-DPA) **(B)** in the control, drought stressed and recovered plants of *Arundo donax* and *Hackonechloa macra*. (**insert**) correlation between ABA content and stomatal conductance in *A. donax* (r^2=0.659) and *H. macra* (r^2=0.046) (Velikova et al. – Plant, Cell and Environment 39:2185-2197, 2016).

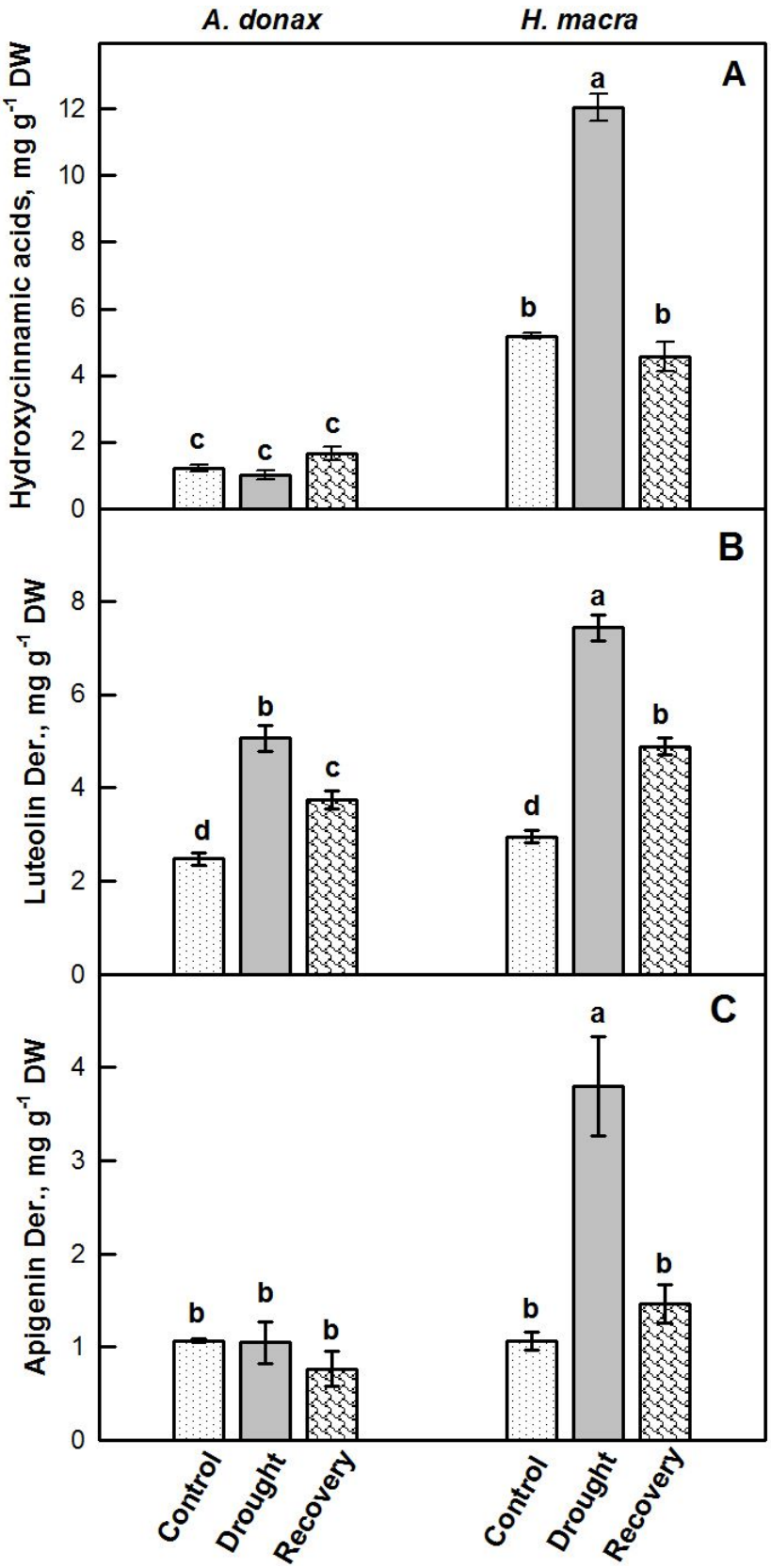


Fig. XIX.6. Hydroxycinamic acid **(A)**, luteolin **(B)** and apigenin **(C)** derivatives in the control, drought stressed and recovered plants of *Arundo donax* and *Hackonechloa macra* (Velikova et al. – Plant, Cell and Environment 39:2185-2197, 2016).

The results clearly indicate that isoprene emitting species have a large flux of carbon in the MEP pathway, and a better capacity to activate the overall pathway in response to

stress Volatile and non-volatile isoprenoids produced by *A. donax* efficiently preserve the chloroplasts from transient drought damage. Species that do not emit isoprene and produce low MEP pathway metabolites, produce higher levels of de-epoxidized xanthophylls (**Fig. XIX.4D**), which likely defend plants when photosynthesis is inhibited by the stress, but further decrease the substrate available for ABA synthesis (**Fig. XIX.5**) (Lichtenthaler 2007). *H. macra* invests on phenylpropanoids that are less efficient in preserving photosynthesis but likely offer better antioxidant protection under prolonged stress.

4.6.C. Phenotypic differences determine drought stress responses in ecotypes of Arundo donax adapted to different environments (paper XX. Ahrar et al. - Journal of Experimental Botany 68(9): 2439-2451, 2017)

In order to further extend our knowledge of the complementary actions of volatile and non-volatile isoprenoids and phenylpropanoids under stress, the responses to drought of two *Arundo donax* ecotypes adapted to contrasting environmental conditions were examined. We **hypothesized** that the Bulgarian (BG) ecotype, adapted to drier conditions, exhibits greater drought tolerance than the Italian (IT) ecotype, adapted to a more mesic environment, and that this is associated with phenotypic adjustments.

Under well-watered conditions the BG ecotype was characterized by higher photosynthesis (**Fig. XX.1a**), mesophyll conductance (**Fig. XX.1c**), intrinsic water use efficiency (**Fig. XX.1d**), PSII efficiency, isoprene emission rate (**Fig. XX.3a**) and carotenoids (**Fig. XX.4**), whereas the IT ecotype showed higher levels of hydroxycinnamates (**Fig. XX.5**).

Photosynthesis of water-stressed plants was mainly limited by diffusional resistance to CO_2 in BG, and by biochemistry in IT (**Fig. XX.1**). Recovery of photosynthesis was more rapid and complete in BG than in IT, which may indicate better stability of the photosynthetic apparatus associated to enhanced induction of volatile (**Fig. XX.3**) and non-volatile (**Fig. XX.4**) isoprenoids and phenylpropanoid (**Fig. XX.5**) biosynthesis.

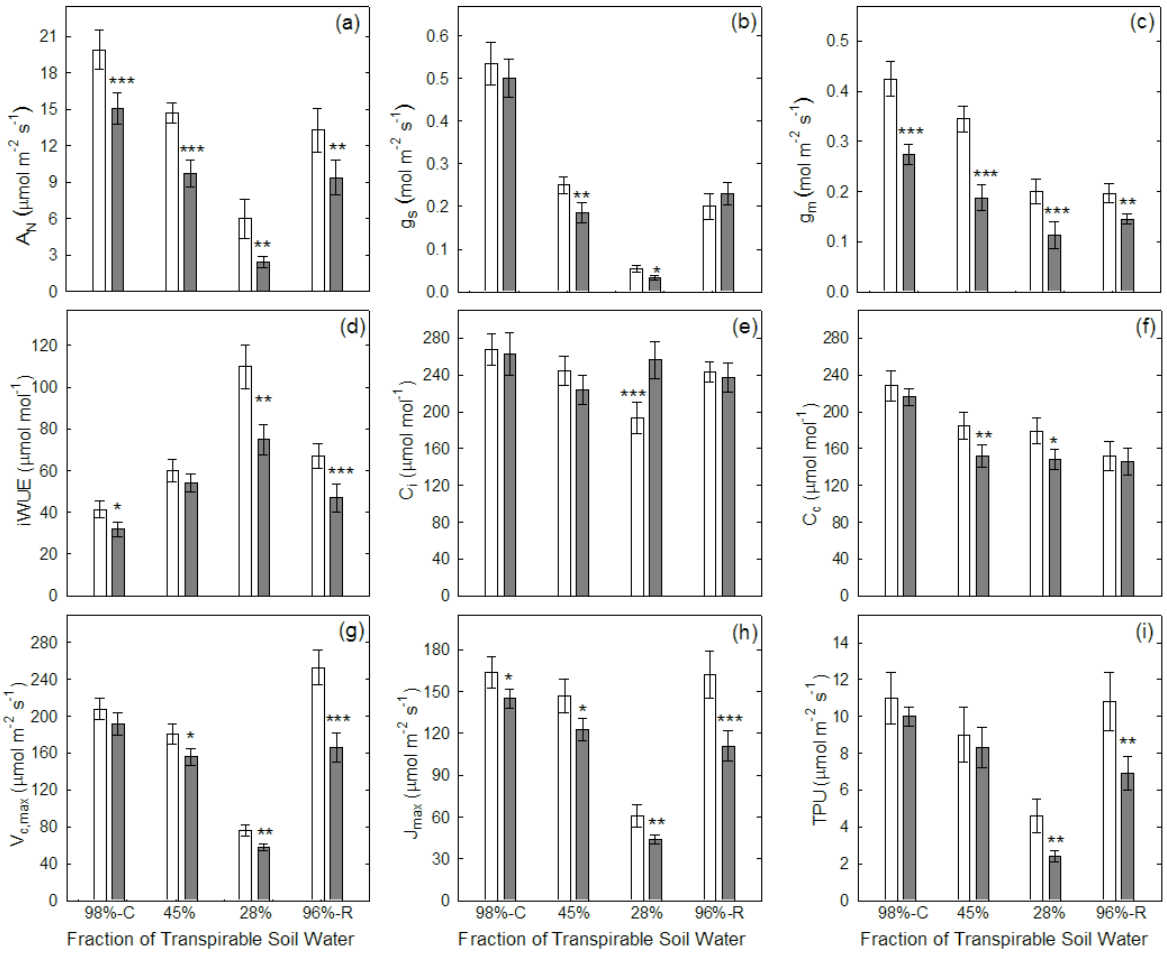


Fig. XX.1. Photosynthesis (A_N) **(a)**, stomatal conductance (g_s) **(b)**, mesophyll conductance to CO_2 (g_m) **(c)**, intrinsic water use efficiency (iWUE) **(d)**, intercellular CO_2 concentration (C_i) **(e)**, chloroplast CO_2 concentration (C_c) **(f)**, apparent maximum carboxylation rate (V_{cmax}) **(g)**, maximum rate of photosynthetic electron transport (J_{max}) **(h)**, and limitation of photosynthesis by triose phosphate use (TPU) **(i)** in BG (white bars) and IT (grey bars) ecotypes of *Arundo donax* before drought (FTSW=98%-C), during drought stress (FTSW=45 and 28%), and after re-watering (FTSW=96%-R) (Ahrar et al. – Journal of Experimental Botany 68:2439-2451, 2017).

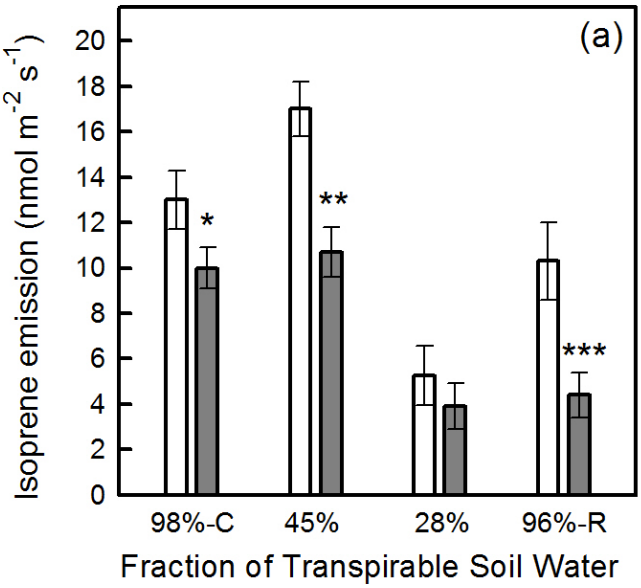


Fig. XX.3a. The isoprene emission rate in BG (white bars) and IT (grey bars) ecotypes of *Arundo donax* before drought (FTSW=98%-C), during drought stress (FTSW=45 and 28%), and after re-watering (FTSW=96%-R) (Ahrar et al. – Journal of Experimental Botany 68:2439-2451, 2017).

In conclusion, our data show that photosynthetic inhibition of drought-stressed *A. donax* is largely due to a strong reduction of stomatal and mesophyll conductance, which limits CO_2 entry, down-regulates Rubisco and the electron transport rate, but allows iWUE to increase and resistance to prolonged stress to occur. Analysis of ecotypes adapted to different climate suggests that the metabolic impairment of photosynthesis only occurs in the ecotype adapted to mesic conditions (IT). In the BG ecotype, which was adapted to harsher conditions, enhanced biosynthesis of isoprenoids might have contributed to protecting photosynthetic membranes, avoiding the metabolic damage under severe drought and allowing for a more complete and rapid recovery of parameters determining potential photosynthesis after re-watering

This study shows that a large phenotypic plasticity among *A. donax* ecotypes exists, and may be exploited to compensate for the low genetic variability of this species when selecting plant productivity in constrained environments.

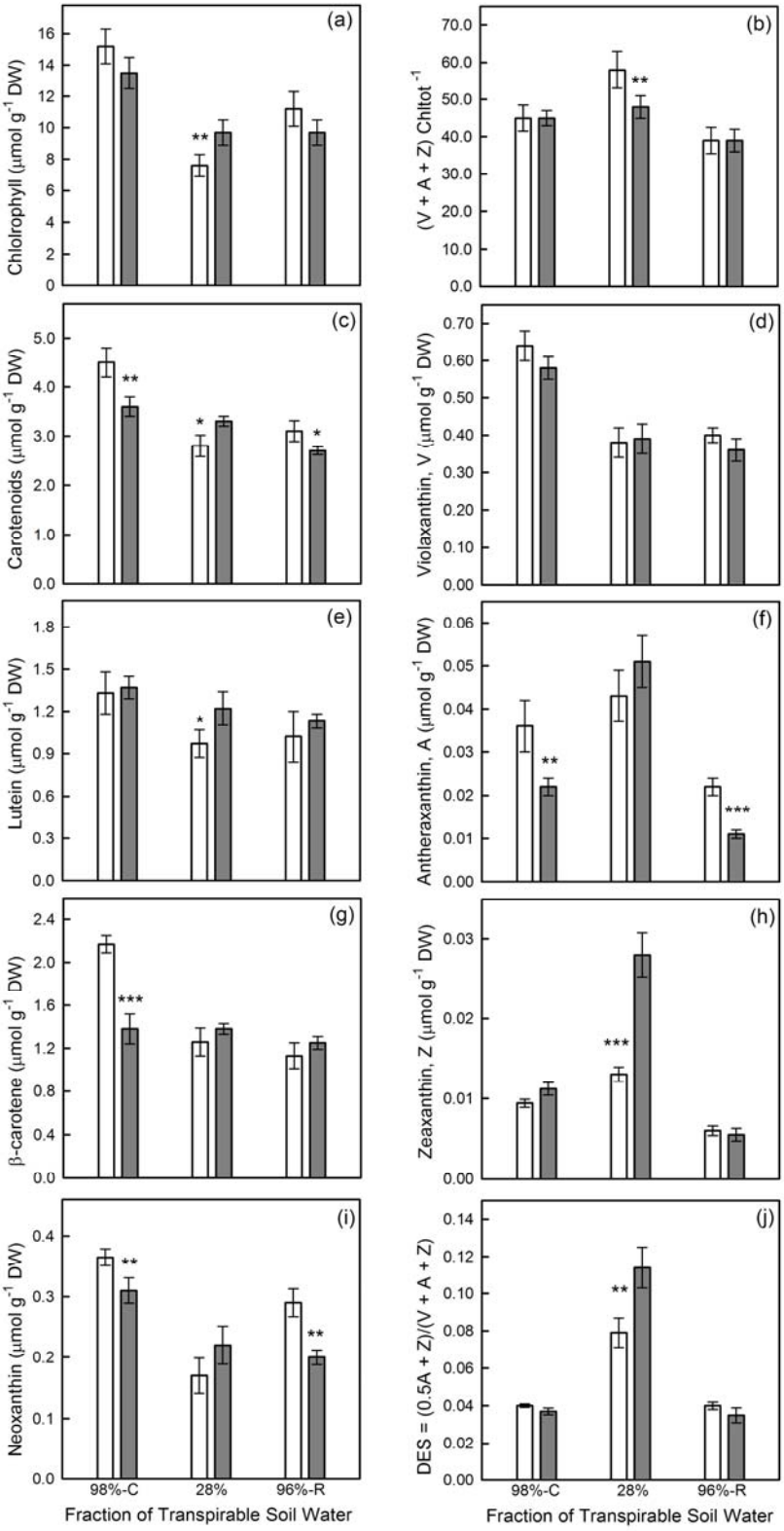


Fig. XX.4. The concentrations of total chlorophyll **(a)**, the concentration of xanthophyll pigments relative to total chlorophyll **(b)**, total **(c)** and individual carotenoids **(d - i)**, and the xanthophyll de-epoxidation state **(j)** in BG (white bars) and IT (grey bars) ecotypes of *Arundo donax* before drought (FTSW=98%-C), during drought stress (FTSW=45 and 28%), and after re-watering (FTSW=96%-R) (Ahrar et al. – Journal of Experimental Botany 68:2439-2451, 2017).

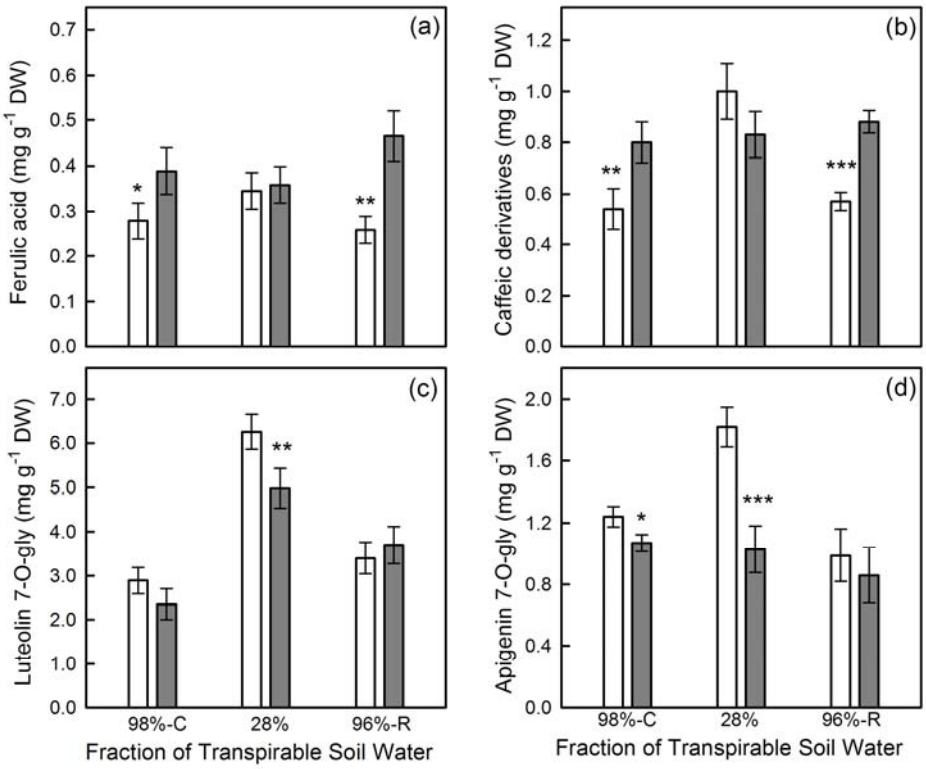


Fig. XX.5. The concentrations of hydroxycinnamic acid **(a,b)** and flavonoid **(c,d)** derivatives in BG (white bars) and IT (grey bars) ecotypes of *Arundo donax* before drought (FTSW=98%-C), during drought stress (FTSW=45 and 28%), and after re-watering (FTSW=96%-R) (Ahrar et al. – Journal of Experimental Botany 68:2439-2451, 2017).

4.7. Effect of anthropogenic pollution on isoprene emission

4.7.A. Changes in photosynthesis, mesophyll conductance to CO_2, and isoprenoid emissions in Populus nigra plants exposed to excess nickel (paper **XI. Velikova et al. – Environmental Pollution 159:1058-1066, 2011**)

Heavy metal pollution of soil and water is a problem of increasingly importance, because of the contamination of large areas worldwide due to the anthropogenic activity. Because of the different solubility most heavy metals may be available for living cells and are of some importance for organism and ecosystems under physiological conditions (Weast 1984). Nickel (Ni), which is the 24th element in order of natural abundance in the Earth's crust, is an essential mineral nutrient found in most natural soils at trace concentrations (Bai et al. 2006). Naturally elevated Ni concentrations can be observed in soils formed from serpentine (ultramafic) minerals (Kopittke et al. 2007). Several studies have shown that Ni excess inhibits photosynthesis (Clijsters & Van Assche 1985; Seregin & Kozhevnikova 2006; Ahmed & Häder 2010) and it is due to reduced stomatal conductance and the enzymatic capacity of the photosynthetic apparatus (Bertrand & Poirier 2005; Seregin & Kozhevnikova 2006). Diffusional limitations to photosynthesis include also mesophyll conductance (g_m) (Loreto et al. 1992; Evans et al. 2009). To the best of our knowledge only the effect of zink on mesophyll conductance to CO_2 was described (Sagardoy et al. 2010). The **first aim of this study was to investigate the effects of Ni stress on g_m and its relationship with photosynthetic capacity**. Understanding how anthropogenic stresses alter the constitutive emissions of isoprenoids while simultaneously inducing new emissions is crucial for quantitative prediction of isoprenoid emissions form stressed plants. This determines our **second aim to investigate isoprenoid emissions in**

Ni-treated poplar saplings. Our **hypothesis** was that exposure to heavy metal stress, as in the cases of other stresses, would affect the diffusional limitations to photosynthesis as well as constitutive and induced isoprenoid emissions. We focused on the response of mature and developing leaves during Ni exposure, because these leaves are known to emit isoprene at different rates in poplar (Centritto et al. 2004).

Ni stress significantly decreased photosynthesis, and this effect depended on the leaf Ni content, which was lower in mature than in developing leaves (**Fig. XI.3a**). Photosynthesis limitations include stomatal and mesophyll conductance to CO_2 and the carboxylation capacity. Because C_i was slightly, although significantly, reduced only in developing leaves in Ni_{200} (**Fig. XI.4b**), g_s played a very minor role in limiting photosynthesis. Whereas the large decline in g_m caused by leaf Ni concentration resulting in dramatic decrease in C_c, especially in developing leaves (**Fig. XI.4d**), constituted a major photosynthesis limitation (**Fig. XI.3a**).

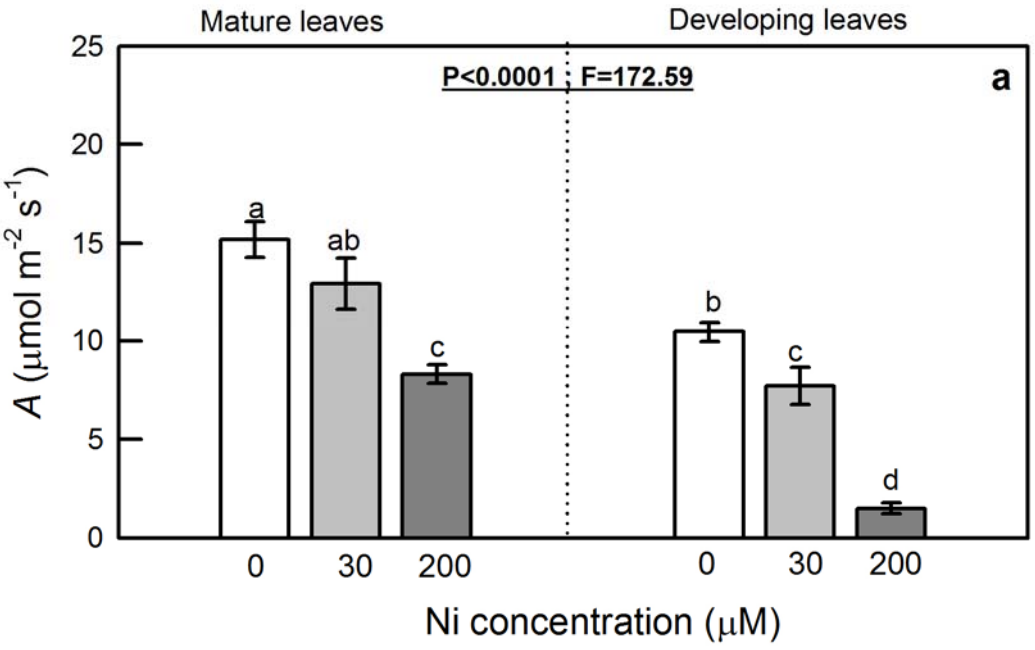


Fig. XI.3a. Net photosynthetic rate in mature and developing leaves of *Populus nigra* plants exposed to different concentrations of Ni for 14 days. (0 µM, white bars; 30 µM, light grey; 200 µM, dark grey) (Velikova et al. – Environmental Pollution159:1058-1066).

Ni treatment did not affect isoprene emission from mature poplar leaves (**Fig. XI.6a**). However, the emission of isoprene by developing leaves considerably increased with leaf Ni concentration despite it remained significantly lower than the emission form mature leaves. This stimulation of isoprene emission was not associated to higher photosynthesis rates, confirming that environmental stresses may increase the fraction of the photosynthetic carbon budget allocated to isoprene, or may stimulate a cross-talk with carbon sources for isoprene formation alternative to photosynthesis intermediates (Fares et al. 2006; Brilli et al. 2007). In our experiment no other isprenoid emission was detected under control conditions in both mature and developing leaves. However, our results demonstrate that Ni stress elicits the release of other inducible isoprenoids. An induction of cis-b-ocimene in mature leaves and linalool in both mature and developing leaves was

detected after exposure to Ni (**Fig. XI.6b,c**). Linalool emission was induced in mature leaves at both Ni concentrations, while in developing leaves this emission was elicited only at Ni_{200}.

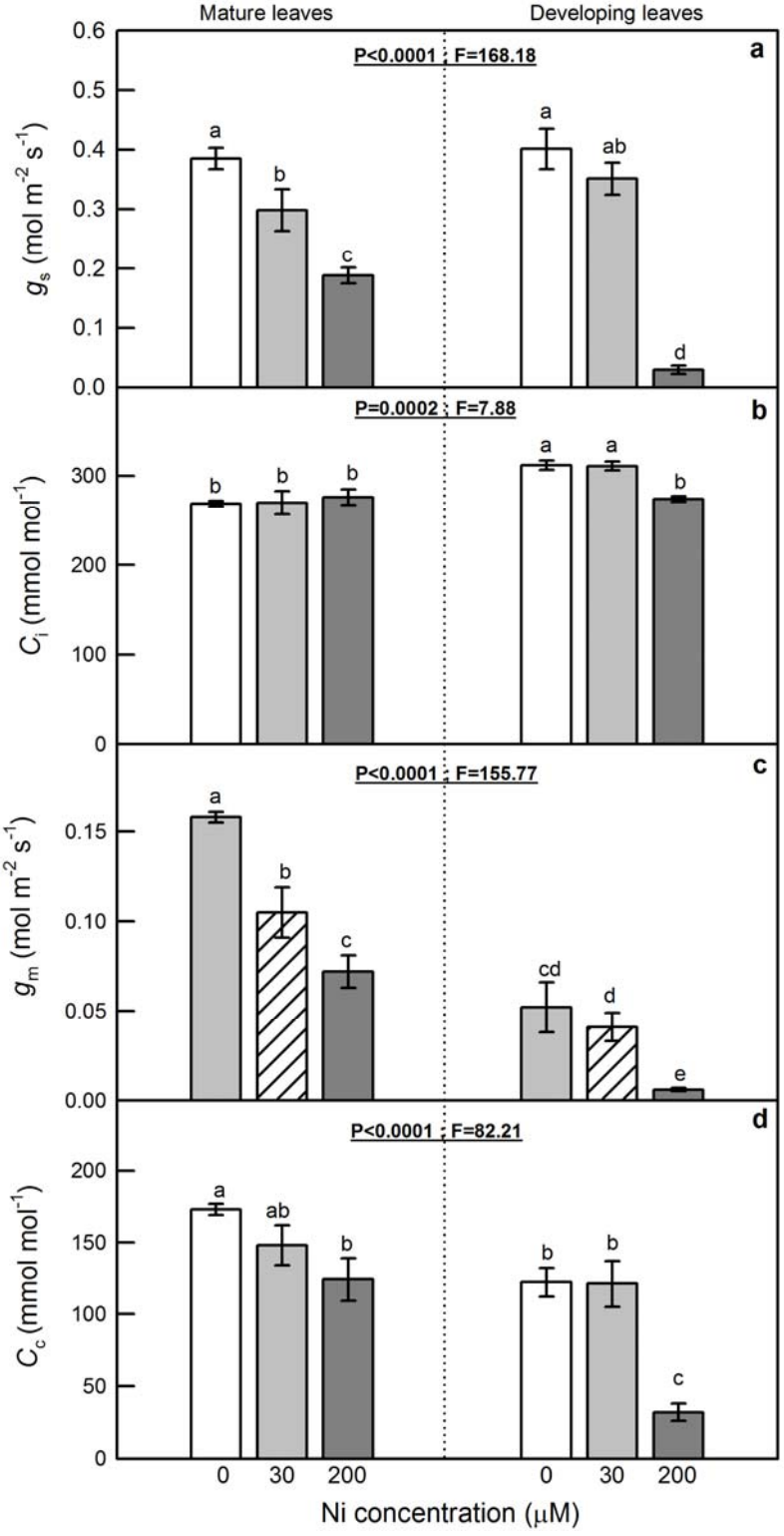


Fig. XI.4. Stomatal conductance (g_s) **(a)**, intracellular CO_2 concentration (C_i) **(b)**, mesophyll conductance (g_m) **(c)** and CO_2 concentration at carboxylation site (C_c) **(d)** in mature and developing leaves of *Populus nigra* plants exposed to different concentrations of Ni for 14 days. (0 μM, white

bars; 30 μM, light grey; 200 μM, dark grey) (Velikova et al. – Environmental Pollution159:1058-1066).

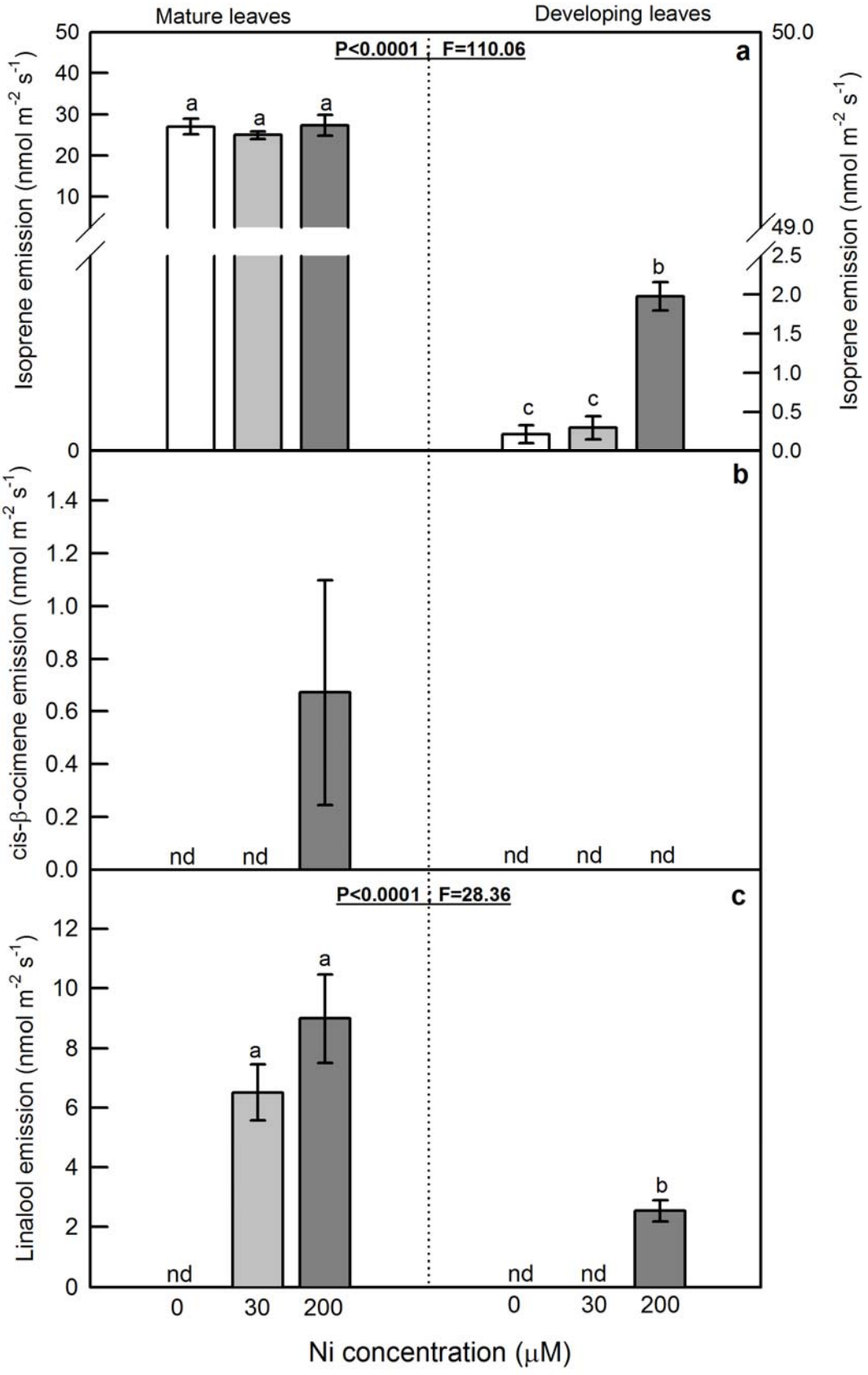


Fig. XI.6. Isoprene **(a)**, cis-β-ocimene **(b)** and linalool **(c)** emission rates from mature and developing leaves of *Populus nigra* plants exposed to different concentrations of Ni for 14 days. (0

μM, white bars; 30 μM, light grey; 200 μM, dark grey) (Velikova et al. – Environmental Pollution159:1058-1066).

In conclusion, we observed that poplar leaves at two different ontogenetic steps differ in their response to Ni stress in terms of mesophyll diffusional limitation and of emission of constitutive and induced isoprenoids. The impact of Ni was mainly attributed to the different uptake of Ni by developing and mature leaves. Induced emission of isoprenoids is thought to indicate the onset of a stress response that activates antioxidants and may feed-forward on associated plant responses against abiotic or biotic stresses. Our finding may be useful for predicting emissions of both constitutive and induced isoprenoids under stress conditions.

5. CONCLUSSION

Isoprene is the most abundant biogenic volatile hydrocarbon compound naturally emitted by many plants and it plays a major role in atmospheric chemistry and air quality. In plants, isoprene emission is associated with considerable metabolic cost, both in terms of energy and carbon, thus it is assumed that isoprene plays fundamental roles in protecting plants from environmental stresses.

Through a multidisciplinary approach (physiological, biophysical, biochemical and structural studies) this dissertation provides evidences on the ability of endogenous isoprene to confer resistance to oxidative stress caused by ozone, singlet oxygen, high temperature, drought, heavy metal (Ni) anthropogenic pollution. Isoprene protective role has been associated with the ability of this molecule to affect thylakoid membrane organization and to regulate the formation of reactive oxygen and nitrogen species, conferring tolerance to heat and oxidative stress. As a part of the plant defense system isoprene plays complementary “antioxidant” roles together with other volatile and non-volatile isoprenoids and phenylpropanoids providing better protection under stress conditions.

New information has been provided that biogenic isoprene contributes to the higher resistance of plants to adverse environmental factors and its effect is manifested at different levels. The experimental results clearly show that in the presence of isoprene the photosynthetic activity of the plants is less impaired under different stresses, unlike the plants with inhibited isoprene emission. (**papers I, II, IV, V, VI, VIII, IX, X, XI, XII, XIII, XIV, XV, XVII, XVIII, XIX, XX**)

Isoprene improves the thermal stability of thylakoid membranes (**paper XII**) by affecting the membrane lipid composition (**paper XVII**). Direct evidences are provided on the relationship between isoprene emission and the level of main lipid classes and their fatty acid composition, and structural organization of the photosynthetic machinery in isoprene-emitting and non-emitting poplar genotypes. The suppressed isoprene production in non-emitting plastids was associated with the reduced amount of galactolipids and phospholipids, the lower level of the major fatty acid (18:3), and the altered chloroplast ultrastructure. The suppression of isoprene biosynthesis causes significant alterations in the chloroplast proteome (**paper XIV**).

These results support the first hypothesis that isoprene is essential for increased plant tolerance, and it is related to functional, proteomic, metabolic and structural modifications.

Endogenous isoprene has an important protective role in plants by regulating the level of reactive oxygen and nitrogen species (**papers V, VIII, XIII**) via the control of the S-nitrosylation levels of ROS metabolizing enzymes. (**paper XVIII**) These results support the second hypothesis that isoprene modulates plant response to stress indirectly through the regulated generation of reactive oxygen species and nitrogen. (**paper XIII**)

Experimental evidences are presented to support the third hypothesis that biogenic isoprene is a part of the plant antioxidant system and acts in synchronization with other protective metabolites, providing better protection for plant under stress conditions. (**papers XIX, XX**)

6. ACHIEVEMENTS:

- For the first time we demonstrate that endogenous isoprene has an important protective role in *P. australis* plants, namely isoprene quenches the amount of hydrogen peroxide formed in leaves expose to ozone and reduces lipid peroxidation of cellular membranes. (**paper I**)

- Genetically modified tobacco plants capable to produce isoprene as a natural metabolite have been shown to be better protected against oxidative stress due to ozone than non-emitting isoprene wild-type tobacco. (**paper IX**)

- The isoprene conjugated double bond structure may facilite energy transfer and heat dissipation. The protective role of endogenous isoprene against singlet oxygen was also demonstrated (**paper II**), suggesting that the protection mechanisms may involve a direct reaction of isoprene with this harmful reactive oxygen species.

- The phenomenon of enhanced heat resistance has been demonstrated in *Phragmites australis* leaves with chemically inhibited isoprene emission, as well as in *Platanus orientalis* plants of different ages. Isoprene not only protects against heat stress, but it also helps plants to quickly recover when the stress is allevaited. (**papers IV, VI**) The significantly higher electron transport rate detected in isoprene-emitting leaves than in isoprene-inhibited leaves exposed to heat suggests that isoprene may facilitate electron flow through photosynthetic / photorespiration cycles. (**paper III**)

- For the first time it is directly demonstrated by using different biophysical techniques that isoprene has crucial role in preserving the intactness of thylakoid membranes and their functionality. Isoprene has been found (1) to enhance the thermal stability of the light-harvesting complex of PSII in the stacked regions of granal thylakoids; (2) reduces the fluidity of the thylakoid membranes at high temperatures; (3) shifts about 10°C to the high-temperature range of the main B peak of the thermoluminescent spectra, suggesting modification changes in the lipid bilayer of the thylakoid membranes. (**paper XII**)

- For the first time it is proven that the absence of isoprene emission modifies the lipid matrix of thylakoid membranes and influences the chloroplast ultrastructure in poplar (**paper XVII**), strongly supporting the idea that isoprene is closely associated with structural organization and function of plastidic membranes. (**papers I, XII**)

- For the first time new approach for quantitative and qualitative proteomics based on stable isotope-coded protein labeling (ICPL) is used to demonstrate that the suppression of isoprene synthase by RNA interference in poplar remodels the chloroplast proteome affecting the structural organization of thylakoid membranes and increases plant sensitivity to oxidative stress. It was demonstrated that in non isoprene-emitting poplar genotypes the levels of chloroplast proteins involved in photosynthesis decrease, especially proteins related to light reactions of photosynthesis, redox

regulation and oxidative stress defense, and the levels of histones and ribosomal proteins increase. (**paper XIV**)

- For the first time, isoprene has been shown to limit the accumulation of nitric oxide (NO) in the leaves of Phragmites australis that experience ozone stress. NO diffusely colonized the mesophyll cells of ozonated isoprene inhibited leaves. NO was not detected in ozonated isoprene emitting leaves. It is suggested that isoprene may be an effective mechanism to control dangerous compounds formed under abiotic stress conditions, while simultaneously attenuating the induction of the hypersensitive response leading to cellular damage and death. (**papers V, VIII**)

- For the first time have been shown that genetically modified Arabidopsis thaliana plants emitting isoprene as a natural metabolite have a smaller pool of reactive oxygen and nitrogen species compared to the wild type that does not emit isoprene. (**paper XIII**)

- The earlier proposed molecular dialogue between isoprene and NO (**papers V, VIII**) was demonstrated in non-emitting isoprene poplar genotypes (**paper XVIII**). Isoprene influences rapid stress-induced changes in NO emission and thus in the pattern of the in vivo S-nitrosoproteome. The main target sites of NO action in non-emitting isoprene poplar are proteins related to the light and dark reactions of photosynthesis, the tricarboxylic acid cycle, protein metabolism, and redox regulation. The results indicate that isoprene indirectly regulates ROS formation via the control of the S-nitrosylation levels of ROS metabolizing enzymes. (**paper XVIII**)

- Transgenic tobacco plants that emit isoprene better tolerate severe drought stress. Defensive mechanisms triggered by isoprene, unveil cooperation with multiple metabolic pathways (non-volatile isoprenoids and phenylpropanoids) in protection from stress events, and highlight the central role of isoprene in stress-induced metabolic tuning. (**paper XV**)

- New evidence of a daily orchestration of antioxidant defenses is provided, and it unveils an unanticipated integration of volatile, nonvolatile isoprenoid, and flavonoid biosynthesis in plants challenged by a combination of high temperature, high light and drought. (**paper XVI**)

- It is demonstrated that, under drought stress conditions, plants, e.g. *Arundo donax*, investing on isoprenoids (isoprene, carotenoids, abscisic acid and its catadolites – phaseic and dihydrophaseic acids), activate protection that allows better and faster resilience from transient stress. Whereas, plants, e.g. *Hakonechloa macra*, investing on phenylpropanoids (hydroxycinamic acids, and luteolin and apigenin derivatives) are not able to avoid damage to the photosynthetic apparatus but are able to cope with prolonged exposure to oxidative species. Moreover, *A. donax* is able to efficiently limit water losses through a coordinated reduction of mesophyll and stomatal conductance, thus achieving a remarkable water-use-efficiency under stress. (**paper XIX**)

- It is shown that a large phenotypic plasticity among *A. donax* ecotypes exists, and may be exploited to compensate for the low genetic variability of this species when selecting plant productivity in constrained environments. In the ecotype, adapted to harsher conditions, the enhanced biosynthesis of isoprenoids might have contributed to protecting photosynthetic membranes, avoiding the metabolic damage under severe drought and allowing for a more complete and rapid recovery of parameters determining potential photosynthesis after re-watering. (**paper XX**)

- Original experimental evidence is provided demonstrainig that nickel (Ni) stress inhibits photosynthesis and the main limitations are attributed to reduced mesophyll conductance and metabolic impairment. The negative effect is stronger in developing leaves, that emit isoprene at lower rate and accumulate higher amount of Ni, compare to mature leaves. It is shown that not only isoprene, but other, higher molecular weight isoprenoids, such as cis-β-ocimene and linalool, may play an important role in plant resistance mechanisms against heavy metal stress. (**paper XI**)

List of scientific publications summarized the dissertation

Assistant Professor (I - V), Professor (VI - XIII)

I. Loreto F, **Velikova V** - 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, **2001. IF 5.105 (Q1); 561** quotations.

II. Velikova V, Edreva A, Loreto F - Endogenous isoprene protects *Phragmites australis* leaves against singlet oxygen. ***Physiologia Plantarum*** 122: 219-225, **2004. IF 2.017 (Q1); 55** quotations.

III. Velikova V, Pinelli P, Loreto F - Consequences of inhibition of isoprene synthesis in *Phragmites australis* leaves exposed to elevated temperatures. ***Agriculture, Ecosystems & Environment*** 106 (2-3): 209-217, **2005. IF 1.495 (Q1); 19** quotations.

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No		IF*	Q	Qn**
I	*Plant Physiology* 127: 1781-1787, **2001**	5.105	Q1	561
II.	*Physiologia Plantarum* 122, 219-225, **2004**	2.017	Q1	55
III	*Agriculture, Ecosystems & Environment* 106 (2-3): 209-217, **2005**	1.495	Q1	19
IV	*Plant Cell and Environment* 28, 318-327, **2005**	3.601	Q1	119
V	*New Phytologist* 166: 419-426, **2005**	4.285	Q1	81
VI	*Functional Plant Biology*, 33: 931-940, **2006**	2.272	Q1	17
VII	*Plant Biology* 10: 38-43, **2008**	1.944	Q1	16
VIII	*Plant Cell and Environment* 31: 1882-1894, **2008**	4.666	Q1	35

IX	*Plant Cell and Environment* 32: 520-531, **2009**	**5.081**	**Q1**	**125**
X	*Environmental Pollution* 157: 2629-2637, **2009**	**3.426**	**Q1**	**31**
XI	*Environmental Pollution* 159, 1058-1066, **2011**	**3.746**	**Q1**	**58**
XII	*Plant Physiology* 157, 905-916, **2011**,	**6.535**	**Q1**	**67**
XIII	*Plant Signaling & Behavior* 7(1), 139-141, **2012 IF(2017)**	**1.395**	**Q2**	**47**
XIV	*Journal of Proteome Research* 13 (4), 2005-2018, **2014**	**4.245**	**Q1**	**17**
XV	*Plant, Cell and Environment* 37 (8), 1950-1964, **2014**	**6.960**	**Q1**	**14**
XVI	*New Phytologist* 207, 613-626, **2015**	**7.210**	**Q1**	**30**
XVII	*Plant Physiology* 168: 859-870, **2015**	**6.280**	**Q1**	**13**
XVIII	*Plant Physiology* 170 (4), 1945-1961, **2016**	**6.456**	**Q1**	**8**
XIX	*Plant, Cell and Environment* 39, 2185-2197, **2016**	**6.173**	**Q1**	**8**
XX	*Journal of Experimental Botany* 68(9): 2439-2451, **2017**	**5.354**	**Q1**	**5**
		86.851		**1326**

* - Impact factor in the year of publication
** - quotations up to 01.11.2019 г.

Quotations

The **total number of citations** of the publications included in the dissertation up to 01.11.2019 is **1326**, of which 1159 are in scientific publications refered and indexed in Web of Scienece and Scopus, 75 - in other scientific publications, and 92 - in foreign PhD theses.

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