Dissertation

The role of isoprene emission on the performance of different genera of Arundineae

by

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Summary

Isoprene is a highly reactive volatile organic compound (VOC) affecting the oxidative capacity of atmosphere and it is emitted by many plant species. The protective role of isoprene in plants against high temperature and oxidative stresses has been suggested. *Arundo donax* is a drought tolerant species of the Arundineae tribe and is known as a promising biofuel crop in Mediterranean regions. To further elucidate the role of isoprene in plant protection, this work was focused on the Arundineae tribe (Poaceae) as a case study, as several members of this tribe are fast-growing species known as strong isoprene emitters (e.g., *A. donax* and *P. australis*, as well as closely related species). Through measurements of photosynthetic and isoprene emission capacity in six different species from this tribe, I provide the first comparative characterization of isoprene emission in a clade of monocotyledons, which until now received less attention than isoprene-emitting dicotyledons. Based on the results of these analyses, two isoprene emitting (*A.collina, A.donax*) and one non-isoprene emitting (*H.macra*) species were selected to further study the function and structure of photosynthetic apparatus with the respect to the possible photoprotective role of isoprene emission (Chapter 2). Then, the effect of drought on plant performance in relation to its isoprene emission capacity was investigated, as aspect of abiotic stress tolerance which is still poorly characterized. To this end, the photosynthesis and secondary metabolism (isoprenoids and phenylpropanoids) in the isoprene emitting (*A.donax*) and non isoprene emitting (*H.macra*) species were compared at control conditions, and under different levels of drought as well as recovery (Chapter 3). Finally, to better understand the effect of isoprene emission on the drought tolerance of *A.donax*, the physiological characteristics of two *A.donax* ecotypes from Italy (IT) and Bulgaria (BG) associated with habitats characterized by different xericity were examined (Chapter 4). The results of these analyses revealed that all the studied members of the Arundineae tribe except *H.macra* are isoprene emitters, indicating the relevance of this trait in this clade based on its evolutionary conservation. The quantum yield and efficiency of PSII and chloroplast ultra structure of the species were positively correlated to their isoprene emission capacity under control conditions. Drought negatively affected photosynthetic performance regardless of isoprene emission capacity of the species studied. However, the damage was more severe in the absence of isoprene emission. Isoprene emission remarkably enhanced the ability of the photosynthetic machinery to recover its structure and function after re-watering. Based on these results, it is suggested that isoprene emission in *A.donax* triggers the MEP pathway and the production of non-volatile isoprenoids (carotenoids) under drought and this effect is intensified by the higher isoprene emission during the earlier stages of drought. Additionally, it is proposed that the high phenylpropanoids content in *H.macra* under drought was due to the high level of oxidative damage experienced by this species. However, in *A.donax* the ecotype which presented higher

phenylpropanoids under drought experienced less photosynthetic impairment. Therefore, it is suggested that the protective role of phenylpropanoids and/or the strength of their protection under drought could vary between different plant species. The fast adjustment of stomatal opening and the induction of isoprenoids and phenylpropanoids in response to drought and eventually the resilience of the photosynthetic apparatus were identified as the special characteristics of *A.donax* leading to its drought tolerance.

Key words: Isoprene, Arundineae, Photosynthesis, Drought, *A.donax*, Phenylpropanoids, ecotype

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Chapter 1

Introduction

1.1 Definition of Isoprenoids

Isoprenoids are the largest group of natural products in plants, amounting to around 25,000 different compounds (Zwenger and Basu, 2008). There are few isoprenoids involved in primary metabolism such as gibberellins, abscisic acid, brassinosteroid phytohormones, phytosterols and carotenoid pigments (Bohlmann and Keeling, 2008; Moses et al., 2013; Vranová et al., 2012). However,the majority of isoprenoids is associated with secondary metabolism and plays a relevant role in plant interactions with the environment, such as e.g. pollinator attraction and protection against biotic and abiotic stresses (Gershenzon and Dudareva, 2007).

The term isoprenoids refers to the group of organic compounds which is constituted of a $(C_5H_8)_n$ backbone as well as of its oxygenated, hydrated, dehydrogenated derivatives (Dewick, 1995). Different classes of isoprenoids are classified depending on the number of carbon atoms in their chemical structure, consisting of hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}), tetraterpenes (C_{40}) and polyterpenes ($>C_{40}$). Isoprenoids are largely used in pharmaceutical and food industry as medicine and food additions. However they also have a wide application in commercial products including solvents, detergents, antialergenic agents, cleaners, as well as industrial polymers (e.g. rubber, chice) and agrochemicals (e.g. pyrethrins, azadirachtin) (Pollier et al., 2011; Singh and Sharma, 2014; Zwenger and Basu, 2008).

The low amount of isoprenoids accumulating in plants is the major hurdle for the commercial exploitation of plant isoprenoids. Production of new cultivars and varieties through breeding and genetic engineering with enhanced capacity of isoprenoid synthesis, using either engineered microbial hosts, or plant cells/ root cultures are potential approaches to increase the production of plant isoprenoids. Therefore, understanding the biosynthetic pathways of isoprenoids and deepening the knowledge about genes and enzymes involved in their biosynthesis is essential. Despite the large variability of plant isoprenoids, the basic biosynthetic pathway leading to their production is similar (Moses et al., 2013).

1.2 Definition of BVOC

Biogenic volatile organic compounds (BVOCs) constitute trace gases emitted from terrestrial ecosystems to the atmosphere excluding carbon dioxide and monoxide (Kesselmeier and Staudt, 1999). The most abundant biogenic VOCs belong to the isoprenoids (isoprene (C_5), monoterpenes (C_{10}) and sesquiterpenes (C_{15})), followed by other groups including alcohols, carbonyls, alkanes, alkenes, esters, ethers, and acids (Kesselmeier and Staudt, 1999; Penuelas and Llusià, 2001). The atmospheric concentrations of BVOCs differ from few ppt to several ppb (Table 1). Different factors such as source and sink strength, meteorological conditions, chemical reactivity can determine the VOC concentration in the atmosphere (Kesselmeier and Staudt, 1999). BVOC

emissions from plants were first reported in the 1950s and 1960s (Rasmussen and Went, 1964; Sanadze and Kalandaze, 1966) and are considered as a major source of hydrocarbons in the atmosphere (Peñuelas and Staudt, 2010; Sharkey et al., 2008). Basically, the emission of VOCs from vegetation results from a physical process of diffusion along a vapor pressure gradient since the BVOCs concentration inside the cell compartments is considerably higher than in the ambient air (Fall, 1999). Beyond this physical process, the emission rate of BVOCs from plants is mainly regulated by internal (genetic and biochemical) and external (biotic and abiotic) factors, as well as VOC volatilization (Penuelas and Llusià, 2001).

Table 1: Comparison of non-methane volatile organic compound (NMVOC) categories (Goldan et al., 1993; Guenther et al., 1995; Singh et al., 1994)

Name	Chemical lifetimes		Example	Atmospheric concentration
	Day	Night		
Isoprene	3 hrs	1.5 hrs	isoprene	ppt to several ppb
Monoterpenes	2-3 hrs	5-30 min	α –pinene; β-pinene, sabinene	
	40-80 min	5-20 min	Limonene, t-β-ocimene,myrcene	
	15-20 min	< 1min	Terpinolene, α-phellandrene	
	< 5 min	< 2 min	α-terpinene	
Sesquiterpenes	< 4 min	< 2 min	β- caryophyllene	Not detectable due to high reactivity
ORVOC	< 1 day		2-methyl-3-buten-2-ol	1-3 ppb
OVOC	>1day		Methanol, acetone	2-30 ppb

[a] Lifetimes are estimated in relation to NO_3 10ppt, O_3= 20 ppb for night; and to OH= 10^6 molecules/cm^3, O_3 = 20 ppb for daylight conditions. ORVOC: other reactive VOC; OVOC: other VOC. Table adopted from (Kesselmeier and Staudt, 1999)

Isoprene and monoterpenes are the predominant source of biogenic hydrocarbons in the atmosphere (Kesselmeier and Staudt, 1999). The large quantity, high volatility and chemical reactivity of these compounds make them a significant factor affecting atmospheric chemistry. On other hand, they also have important physiological and ecological functions (Niinemets and Monson, 2013).

Isoprene (C_5H_8), is an acyclic hydrocarbon. Isoprene is not stored in the plant tissues, but released to the atmosphere as soon as it is synthesized (Guenther, 2013; Kesselmeier and Staudt, 1999; Penuelas and Llusià, 2001). The heavier isoprenoids are built by fusion of isoprene units. Monoterpenes ($C_{10}H_{16}$) can be acyclic, mono-, di-, tri-cyclic, without insertion of O_2 (menthol, camphor, linalool, and geraniol) or with O_2 insertion, which are called in general monoterpenoids (Kesselmeier and Staudt, 1999). Monoterpenes mainly constitute the essential oils of plant and are stored in plant organs such a glandular trichomes or resin ducts and are normally released after a mechanical damage or herbivore attack. There might be some correlation between storage and

emission rate of monoterpenes. However, in some oak species the monoterpenes are emitted immediately after their synthesis (Ciccioli et al., 1999; Dudareva et al., 2006).

1.3 Atmospheric impact of BVOCs

In the past, the climate effect of BVOCs was not consider carefully, since it was believed that due to their short life span (fractions of a day to months), these compounds cannot have a significant effect on atmospheric quality. However, this trend changed after discovering the significant role of BVOCs in secondary aerosol formation and greenhouse effect. In addition the magnitude of BVOC emission and chemical reactivity of these compounds intensify the significant role of them in atmospheric quality (Peñuelas and Staudt, 2010). BVOC emission represents 1150 Tg C per year, which is 10 times higher than anthropogenic VOC emission. Isoprene and monoterpenes, with respectively 44% and 11% contribution to the annual BVOC emission, are the most abundant and reactive compounds among BVOCs (Guenther et al., 2012; Guenther et al., 1995). The high reactivity of BVOCs with the major atmospheric oxidants, e.g. ozone, hydroxyl and nitrate radicals, leads to their huge impact on the oxidative capacity of the troposphere (Peñuelas and Staudt, 2010). Contribution to ozone production is one of the direct atmospheric impacts of BVOCs on greenhouse gases. In the remote areas with low NO_x concentrations, the increase of BVOCs reduce the ozone level, while in polluted ones with high NO_x levels, the elevation of BVOC emissions induce ozone formation (Rosenkranz et al., 2015). Even in non-polluted environments, BVOCs can indirectly affect the atmospheric concentration of methane, another important greenhouse gas. High oxidation rates of BVOCs results in depletion of atmospheric OH radicals. This eventually decreases the oxidation of methane by OH and increases its lifetime in the atmosphere (Dicke and Loreto, 2010; Peñuelas and Staudt, 2010). Products of BVOC oxidation have low volatility. Therefore, these compounds may form condensable vapors and aerosol particles, which can grow to cloud condensation nuclei (CCN) sizes in short time and increase the number of CCN (Carslaw et al., 2013; Kurtén et al., 2003). Higher CCN numbers result in increased cloud thickness, which causes more reflection of irradiance to the space and corresponding cooling of the earth surface. In addition, the increase of aerosols decreases the solar radiation quantity reaching the earth surface and thus has a cooling effect. On the other hand, BVOC emissions positively affect global warming by increasing the lifetime of methane, by the contribution to ozone production and also the direct greenhouse effect of some BVOCs (Goldstein et al., 2009; Peñuelas and Staudt, 2010).

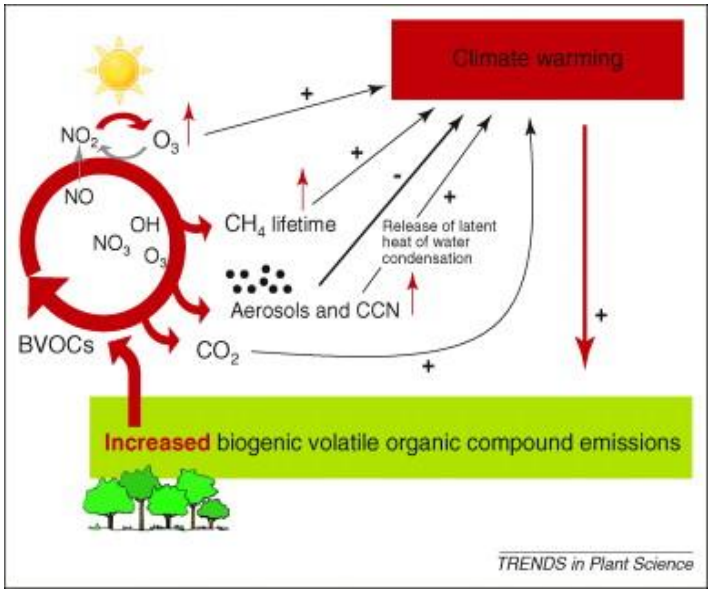

Figure 1. Effects of increased BVOC emissions on atmospheric chemistry and climate. Schematic figure of coupling of enhanced BVOC emissions and atmospheric and climatic changes: increased temperature will enhance BVOC emissions (+). Increased BVOC emissions will enhance aerosol formation and growth and therefore also enhance aerosol and CCN concentrations. Enhanced aerosol and CCN concentrations will decrease temperature (–) as a result of increased reflection of sunlight from low clouds back to space. However, other positive feedbacks (direct greenhouse effect of BVOCs, indirect greenhouse effect through ozone formation and methane lengthening lifetime, CO_2 production and release of latent heat of water condensation) are also present and require further research.

(Peñuelas and Staudt, 2010)

1.4 Inventories of VOC emission

Due to the important role of BVOC emissions for ozone formation and the predicted increase of temperature in future (Guenther et al., 2000; Scott and Benjamin, 2003), the quantification of BVOC emission from vegetation should be considered for air quality and climate assessments. On the global scale, the VOC emission from natural sources is almost 10 times higher than from anthropogenic sources (Guenther et al., 1995; Simon et al., 2006; Watson et al., 1991). The inventories to determine VOC emission based on the emitting species in each ecosystem or per unit biomass are performed on the global (Guenther et al., 1995), continental (Simeonidis et al., 1999) and regional (Schaab et al., 2000; Simeonidis et al., 1999) scale and the results represent important input for atmospheric chemistry models. Due to the multiple abiotic and biotic parameters affecting the BVOC emissions, there is a high level of uncertainty on the magnitude of BVOC emissions reported by different inventories (Monks et al., 2009; Oderbolz et al., 2013; Stewart, 2003). The inventories of BVOC emission vary through time because of ecological changes. For example, soil fertility influences VOC emission through its effect on allocated carbon for VOC emission per unit of biomass and also the biomass production per unit of land area (Monson et al., 1995). The positive relation between N level of the soil and isoprene emission per unit of biomass was reported first in velvet bean (Harely, 1994). The nutrient availability of soil is continuously changing and therefore its effect on BVOC emission changes (Monson et al., 1995). Also the land cover change resulting from human activities, herbivory, invasive plant species as well as climate change can significantly affect the regional VOC emission (Monks et al., 2009; Monson et al., 1995; Stewart, 2003). For example, the predicted increase in CO_2 and temperature would result in replacement of C_3 with C_4 species, which emit less isoprene. Also the migration of isoprene emitting woody plants toward north as a result of global warming will shift the VOC emission due to the replacement of coniferous monoterpene emitting species with woody isoprene emitting species (Monson et al.,

1995). There are also other reasons that decrease the accuracy of the inventories such as: focusing on singlespecies, performing measurements one time during the year despite circadian, environmental and ontogeny effects on emission over time, measuring few individuals and ignoring the intra- and inter-population variability, the sampling method and detection sensitivity (Fineschi et al., 2013). Considering the mentioned variables, BVOC emissions can change by one or two orders of magnitude over a period of years or decades (Monks et al., 2009). Therefore, more accurate and frequent estimation of BVOC emissions through field measurements (Bäck et al., 2012) and optimization of models for BVOC emission (Karl et al., 2009) are necessary to improve the validity of the BVOC inventories (Oderbolz et al., 2013).

1.5 Biological diversity of Isoprene emission

The global terrestrial vegetation is composed of temperate, tropical and boreal ecosystems (each 25-35 %) and the remaining area is covered by croplands (15%) (Guenther, 2013) . About 80 % of the global BVOC emission is from tropical ecosystems. The global vegetation cover is composed of needle leaf trees, broadleaf trees, shrubs, grass and crops which each have a contribution of 10-30 % to the total vegetation. Broadleaf trees have the largest contribution (80%) to the global VOC emission. According to the inventories so far, broadleaf trees are mostly isoprene emitters and can be divided in two groups with low (<1 $\mu g\ g^{-1}\ h^{-1}$) and high emission rates (about 90 $\mu g\ g^{-1}\ h^{-1}$) (Guenther, 2013). The presence of isoprene emission in plants could be explained by the protective role of isoprene for plant under transient rise of temperature. In small leaf plants, the boundary layer is not thick, therefore the air can easily circulate between leaf and ambient air so the leaf temperature does not exceed the plant threshold to trigger the isoprene biosynthesis as a thermo-protective mechanism. As a result isoprene emission is rarely observed among desert plants (Sharkey and Yeh, 2001). On the other hand when the leaves are exposed to high temperatures, the stomatal opening and transpiration can help the plant to cool down. The large stomatal size and high transpiration rate in crop plants might be one of the reasons for their low isoprene emission (Sharkey and Yeh, 2001). While in humid environments like tropical forests, where the high humidity slows down the transpiration, the leaf is heated up more often. In order to cope with the adverse effect of high temperature, the biosynthesis of isoprene is heat-stimulated, which is a common trait in tropical plants. This explanation is in line with the hypothesis that isoprene emission first appeared when the mosses evolved. Moss species which are growing far from the water level are experiencing large temperature variation compared with those close to the water. Therefore isoprene emission could play a buffering role and prevent the damage to the plant cell due to the large fluctuation of temperature in the populations of mosses which were growing distant from water surface (Loreto and Fineschi, 2015; Sharkey and Yeh, 2001). In addition, the isoprene emission capacity is associated with fast growing habit and perennial life form. It is suggested that

isoprene emission enables plants to maintain a fast growth rate under transient periods of stress. However monoterpenes, which are less volatile compounds, are associated with more stress resistance (Llusià et al., 2010). For example hydrophyte fast growing Oak species such as *Q.rubra* and *Q.robur* are isoprene emitters, while the trait disappears and is substituted by monoterpene emission in slow growing oaks such as *Q.ilex* and *Q.suber*, which endure longer periods of stress or drought. It is suggested that isoprene emission is replaced by monoterpene emission in the plant adapted to more xeric environment (Loreto and Fineschi, 2015).

1.6 Biosynthesis of isoprenoids

Isoprene in eubacteria, chloroplast of plants and green algae is produced through the Methylerythritol 4- phosphate (MEP) pathway in chloroplasts, however the immediate precursor of isoprene, dimethylallyl diphosphate (DMAPP), and its isomer isopentenyl diphosphate (IPP) can also be imported from the cytosol, where they are produced through the Mevalonate pathway (MVA) (Sharkey et al., 2005). Glyceraldehyde-3-phosphate (G3P) and pyruvate are the first intermediates of the isoprene pathway which are produced through photosynthesis (Fig 2). G3P is the main product of the Calvin cycle, but it also could be imported from the cytosol, where it is the end product of glycolysis (Sharkey et al., 2008). Pyruvate can be produced inside the chloroplast during glycolysis or as a byproduct of the Ribulose-1,5-bisphosphate (RuBP) carboxylase activity. It also can be imported from the cytosol in the form of pyruvate or phosphoenol pyruvate (PEP), which is converted to pyruvate by pyruvate kinase (Funk et al., 2004). Within the MEP pathway, isoprene is synthesized over 5 intermediate steps, which are each controlled by a specific gene. The primary genes in the isoprene biosynthesis pathway have regulatory effects on isoprene biosynthesis and conserved between different plant species. However, isoprene emission rate shows a stronger correlation with the activity of Isoprene synthase (*IspS*), which is the last enzyme in the pathway to convert the DMAPP to isoprene (Sharkey et al., 2008, 2005).

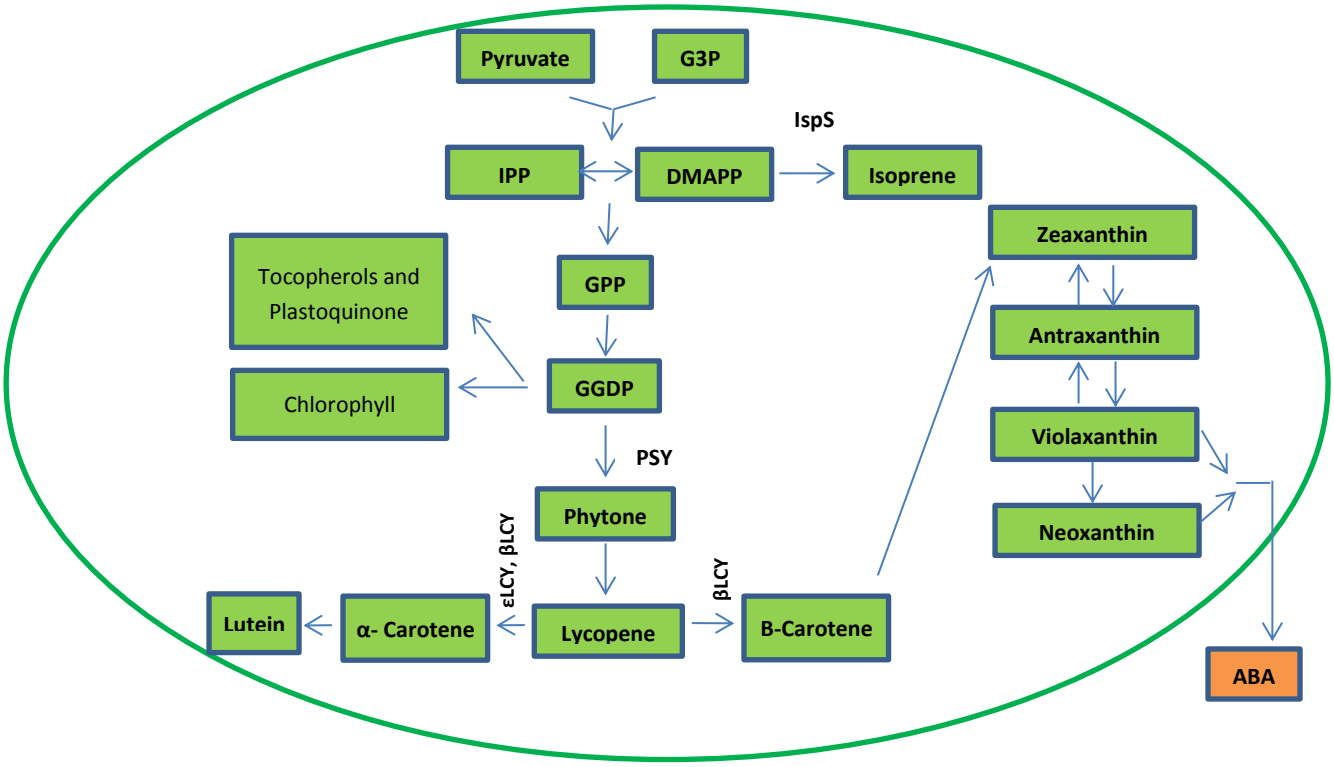

Figure 2. The isoprenoids biosynthetic pathway in plants. G3P; Glyceraldehyde-3-phosphate, IPP; isopentenyl diphosphate, DMAPP; dimethylallyl diphosphate , GPP; geranyl diphosphate, GGPP ;geranylgeranyldiphosphate, IspS; Isoprene synthase, εLCY, βLCY; lycopene cyclase, ABA, Abscisic acid. Adopted from Laule et al., 2003; Rodríguez-Concepción et al., 2004; Cazzonelli et al., 2011

In the plastid the sequential head-to-tail addition of IPP units to DMAPP by prenyltranferase yields geranyl diphosphate (GPP, C_{10}) which is the universal precursor of monoterpenes and it undergoes the subsequent steps to produce the cyclic or acyclic monoterpenes (Mahmoud and Croteau, 2002).

Further addition of IPP to DMAPP results in production of geranylgeranyldiphosphate (GGPP, C_{20}) which is the precursor of different classes of diterpenes including chlorophyll, tocopherols, plastoquinone, gibberellic acid and carotenoids (Hsieh and Goodman, 2005). Phytone is the first carotenoid in the MEP pathway, which is produced by condensation of two GGPP molecules. The reaction is catalyzed by the phytoene synthase (PSY) enzyme. PSY activity is a rate limiting step in carotenoids biosynthesis. The expression of PSY is co-regulated with the genes related to photosynthesis and isoprenoid biosynthesis (Meier et al., 2011). The PSY gene is highly regulated at transcriptional level by developmental, environmental and metabolic factors (Cazzonelli and Pogson, 2010). Lycopene is produced from phytone following 4 steps catalyzed by different enzymes. After Lycopene, the carotenoid biosynthesis is divided into two branches catalyzed by two lycopene cyclases, εLCY and β LCY. The εLCY activity is animportant factor determining the ratio of the most abundant carotenoid, lutein, to the β-carotenoids. The interaction between εLCY

and β LCY at the molecular level is the main factor determining the flux through the biosynthesis of lutein, β carotene and other xanthophylls (Bai et al., 2009; Cazzonelli, 2011; Yu et al., 2008) (Fig 2) . The oxidative functionalization of α and β carotene results in generation of xanthophyll carotenoids, which are significantly involved in the photoprotection of photosynthesis, membrane stability and plant response to stress (Dall'Osto et al., 2006; Pogson et al., 1998). Dissipation of excess energy under stress conditions through the expoxiation/de-epoxidation cycle of xanthyphyll carotenoids is well known. The epoxidation of zeaxanthin produces antheraxanthin and violaxanthin. The high pH across the thylakoid membranes (e.g. under high light stress) results in de-epoxidation of violaxanthin and the reverse reaction regenerates antheraxanthin and zeaxanthin (Fig 2) . Violaxanthin is found in the light harvesting complex of PS1 and PS2 and its cycle is present in both photosystems (Havaux, 1998). Violaxanthin is converted to neoxanthin, which is the final carotenoid of this branch in the classical biosynthesis pathway. However, other xanthophyll carotenoids are detected in other plant tissues which are not discussed here (Cazzonelli, 2011). Xanthophylls also are the precursors of Abscisic acid (ABA), which is an important signaling molecule in plants under stress condition. The MEP pathway partly contributes to the pool of foliar ABA in both isoprene emitting and non-emitting plants. However there are several ABA pools in plants which have different biosynthesis and regulatory steps. The ABA generated through the MEP pathway controls stomatal opening in response to rapid changes of the water status (Barta and Loreto, 2006).

1.7 Regulation of isoprene emission capacity

1.7.1 Environmental factors

1.7.1.1 Temperature

BVOCs have generally a high Henry law constant (e.g. k_H ~7500 Pa m^3 mol^{-1} at 25 °C for isoprene). Therefore, they are mainly partitioned in the gas phase rather than liquid phase (Niinemets et al., 2004). Rising the temperature can exponentially increase the emission rate of a variety of volatile isoprenoids in different plant species. The increase of emission is partly due to the elevated vapor pressure of these compounds at higher temperature. However the rise of emission in response to the increased temperature is far beyond the physical process and it could be explained by the higher activity of enzymes which are responsible for their biosynthesis (Holopainen and Gershenzon, 2010; Loreto and Schnitzler, 2010). For example the Q_{10} (the increased rate of emission for 10 °C rise of temperature) of isoprene biosynthesis varied between 3 and 8 depending on the plant species and the measured temperature (Harley et al., 1997; Monson et al., 1992; Sharkey and Loreto 1993). The optimum temperature for many enzymes in MEP pathway and most of isoprenoids synthases is around 40-45 °C. Above the optimal temperature, the biosynthesis of these compounds is suppressed due to the impairment of the primary metabolism and carbon supply to the MEP

pathway (Monson et al., 1994; Sharkey and Loreto, 1993; Singsaas and Sharkey, 2000). It is estimated that the global average temperature will increase around 1.8-4 °C during the twenty first century (IPCC, 2007). In addition to the direct stimulation of BVOC biosynthesis and vapor pressure, rising the global temperature can also elongate the growing season and change the land cover, therefore indirectly affecting the total annual BVOC emission including volatile isoprenoids. However, the anticipated effect of global warming on BVOC emission can be different according to the geographical regions, being more significant in cold regions, such as arctic ecosystems, compared to the Mediterranean regions (Peñuelas and Staudt, 2010).

1.7.1.2 Light

Isoprene emission is dependent on light as photosynthesis, but it has a higher light saturation point. In darkness, isoprene biosynthesis is suppressed and it is induced within 30 minutes after illumination. It has a very fast response to photosynthetically active radiation. In the long term, the light intensity over time spansof days affects the basal isoprene emission rate through isoprene biosynthesis and leaf specific mass. Plants grown under high light intensities showed more carbon lost as isoprene (Alves et al., 2014; Harley et al., 1996; Litvak et al., 1996). The light response of isoprene emission could be the result of the larger supply of substrate because of higher photosynthesis, stimulated *IspS* activity and/or increased amount of reducing power/ energy available for isoprene biosynthesis (Sharkey and Loreto, 1993). Circadian regulation of isoprene emission is reported in some plant species such as cottonwood, oak (Funk et al., 2003) and oil palm (Wilkinson et al., 2006). The underlying process was explored in oil palm at the molecular level by measuring the abundance of the transcription factors (Circadian Clock Associated 1, (*CCA1*)/Late Elongated Hypocotyl (*LHY*)) which are strongly clock regulated. The rhythmic function of the circadian clock was maintained even at a temperature of 38°C, which is above the normal threshold for circadian clock functioning. This observation strengthens the possibility of the circadian control of the observed rhythm of isoprene emission. The presence of many copies of the circadian-associated cis elements in the promoter region of the isoprene synthesis suggest the circadian regulation of isoprene biosynthesis at the transcriptional level. The circadian control of isoprene biosynthesis can be also imposed in the upstream genes in the MEP pathway (Wilkinson et al., 2006). For example portioning of the PEP between cytosol and chloroplast is a limiting stage for isoprene biosynthesis. In addition, nitrate assimilation in the cytosol is a strong PEP sink which is clock regulated (Yang and Midmore, 2005). As it was reported for oil palm, the activation of nitrate metabolism in the afternoon limited the PEP availability for isoprene biosynthesis in the chloroplast and consequently reduced the isoprene emission (Wilkinson et al., 2006). Considering this phenomenon, the inclusion of the circadian control of isoprene emission in local, regional and

global scale models of atmospheric chemistry is necessary for a more reliable estimation of the isoprene emission and ozone levels (Hewitt et al., 2011; Keenan and Niinemets, 2012).

1.7.1.3 Water stress

Is it expected that isoprene emission is decreased under drought as a result of lower stomatal conductance and less availability of photosynthetic carbon. However, the available reports indicate that isoprene emission is resistant to water deficit (Brilli et al., 2007; Funk et al., 2004; Pegoraro et al., 2004b; Sharkey and Loreto, 1993). This effect is explained by contribution of the alternative carbon sources in MEP pathway such as xylem-transported glucose (Kreuzwieser et al., 2002; Schnitzler et al., 2004), chloroplastic starch (Karl et al., 2002) or re-fixation of CO_2 generated by light respiration (Anderson et al., 1998; Loreto et al., 2004), which maintain the isoprene emission at a constant rate. In some case the isoprene emission rate was stimulated in response to drought. This could be the result of less cytosolic carboxylation of PEP, which leads to increased import of PEP to the chloroplast, where it is converted to dimethylallyl-diphosphate (DMAPP), the immediate precursor of isoprene (Pegoraro et al., 2004a; Pegoraro et al., 2004b). In addition, the reduced photosynthesis due to the low Ci could also increase the availability of electrons/energy for isoprene biosynthesis in stressed leaves (Dani et al., 2014; Morfopoulos et al., 2014). In some plant species, the induced emission of isoprene under drought was regulated at transcriptional and posttranscriptional level (Brilli et al., 2007; Fortunati et al., 2008). Generally the reduced stomatal conductance under drought is not considered a limiting factor for isoprene emission because of its high partial pressure within the cell which drives the emission despite the diffusion resistance (Loreto and Schnitzler, 2010). The rise of leaf temperature as a consequence of lowered transpiration under prolonged drought could also induce the emission (Sharkey and Loreto, 1993). Isoprene and monoterpene emission drop under severe drought when the photosynthesis is highly inhibited and alternative carbon sources are depleted (Brilli et al., 2007; Jardine et al., 2014).

1.7.1.4 CO_2 concentration

In the short term, rising the atmospheric CO_2 could increase the productivity and standing biomass and therefore favor the BVOC production. But the effect of long-term elevated CO_2 on BVOC emission is not yet fully clear and it depends on different factors such as plant species, environmental and phenological conditions. It was shown that the isoprene emission rate was reduced by elevation of CO_2 , but some reports do not support this observation (Peñuelas and Staudt, 2010). The highest isoprene emission was measured at the intercellular CO_2 concentration (Ci) of 150-200 ppm which is in the range found in the tree leaves under natural conditions. By increasing the Ci, the isoprene emission progressively decreased (Loreto and Schnitzler, 2010). Under high CO_2 concentration the competition for PEP is shifted toward carboxylation in the cytosol rather than the MEP pathway in the chloroplast. Consequently, the DMADP pool size for

isoprene production in the chloroplast is decreased (Karl et al., 2002; Possell and Hewiitt, 2011; Rosenstiel et al., 2003; Trowbridge et al., 2012). On the other hand, increase of CO_2 fixation with increasing Ci reduces the oversupply of electrons directed to the isoprene pathway (Morfopoulos et al., 2013). In contrast to isoprene, there are few studies about the effect of CO_2 concentration on monoterpene emission. In holm oak (*Quercus ilex*) grown under elevated CO_2 the monoterpene and terpene synthase (TPS) activities decreased and consequently reduced the emission. However,in some studies the monoterpene emission was not inhibited by high CO_2. Currently we don't know whether CO_2 has a general impact on isoprene and monoterpene biosynthesis. However if the emission is regulated by low CO_2 concentration, then the stress condition which reduces Ci could stimulate the emission (Loreto and Schnitzler, 2010).

1.7.1.5 Other oxidative stresses

Other oxidative conditions such as high ozone concentration also stimulate the biosynthesis of terpenes. However since the BVOC are reacting with ozone, the emission rate could be underestimated. The overall trend shows more increase than decrease in BVOC emission in plants exposed to ozone (Peñuelas and Staudt, 2010). Plants respond to acute and heavy doses of ozone (150 -300 ppb) by increasing the emission of isoprene and monoterpenes, which protect the photosynthetic machinery from damage. The stimulation of volatile isoprenoids happens only when the ozone concentration passes a threshold and causes cellular damage, while photosynthesis is not yet impaired. Therefore, it can provide the carbon supply for the MEP pathway. The leaves grown in high ozone concentration had higher capacity to increase the isoprene synthesis than the ones grown under normal conditions, which could indicate more oxidative resistance in leaves adapted to high ozone levels (Fares et al., 2006). High UV radiation also triggered the antioxidant pathway and isoprenoids biosynthesis, but there are few studies on the effect of UV radiation on BVOC emissions and the effect might vary according to the type of BVOC and plant species as well as stress level (Peñuelas and Staudt, 2010).

1.7.2 Developmental factors

In addition to environmental factors, developmental stages also control the isoprene emission capacity of the leaves. It was shown in dicot species that generally the isoprene emission is associated to late developmental stages. For instance, the newly emerged leaves of poplar are characterized by monoterpene emissions. By further development of the leaves, the monoterpene is replaced by isoprene emission. The onset of isoprene emission in *Populus trichocarpa* was observed in the 5^{th} open leaf (Brilli et al., 2009; Sharkey et al., 2008). However, in *A.donax* it was reported that the isoprene emission started earlier, and immediately after unrolling the new leaves and the developing leaves did not emit monoterpenes. These findings suggest the difference in transcriptional regulation of *IspS* and monoterpene biosynthesis genes or the size of DMADP pool

between monocots and dicots (Ahrar et al., 2015). However, the environmental conditions, specifically growth temperature, can delay or accelerate the isoprene emission capacity of leaves (Monson et al., 1994; Wiberley et al., 2005).

1.8 The physiological role of isoprene in plants

Since isoprenoids and more specifically isoprene biosynthesis demand considerable amounts of carbon and energy, it is believed that the biosynthesis of these molecules should confer an advantage to plants (Sharkey et al., 2008; Vickers et al., 2009a). Under physiological conditions plants re-emit around 0.5 % of the recently fixed carbon as volatile isoprenoids into the atmosphere and this percentage can increase up to 10% in stressed leaves. Several studies have indicated the protective role of volatile isoprenoids under biotic and abiotic stress. So far three major approaches have been used to determine the role of volatile isoprenoids in plants: 1) chemical inhibition of the MEP pathway by using fosmidomycin, which is an antibiotic/herbicide that inhibits one of the key enzymes of MEP pathway, 2) fumigation of leaves with exogenous isoprenoids, like e.g. isoprene and monoterpenes, 3) genetically modified plants with stimulated or suppressed isoprenoid biosynthesis. Each of these approaches has some drawbacks that may result in over/underestimation of the effect of volatile isoprenoids. For example fosmidomycin inhibits the biosynthesis of essential non-volatile isoprenoids such as carotenoids, chlorophylls, plastoquinone, abscisic acid and gibberellins. Also in genetically modified plants, sometimes no difference was found in stress tolerance between transgenic and wild type plants. This might be due to the adverse impacts of transformation and tissues culture, which can be transferred through several transgenic generations before the transgene stabilized. Consequently this stabilization period can hamper the study of stress response in transgenic plants using species with long generation times. However, in the majority of the studies, regardless of the selected approach, the protective role of volatile isoprenoids under stress conditions was verified (Velikova, 2008; Vickers et al., 2009a).

There are different hypotheses regarding the protective role of isoprenoids and specifically isoprene. The metabolic hypothesis suggests isoprene biosynthesis as a safety valve to release the oversupply of energy or metabolites. The residual of reducing power/ energy produced through light reactions and not taken up by other electron sinks (e.g. assimilation and photorespiration) is directed to isoprene biosynthesis (Dani et al., 2014; Morfopoulos et al., 2014, 2013). In addition, it is suggested that the excess of chloroplastic DMAPP which is not used for production of essential isoprenoids, is used instead for biosynthesis of volatile isoprenoids (Owen and Peñuelas, 2013) .

Thermo-protection is another hypothesis about the role of isoprene and monoterpenes. The physiological aspects of isoprene biosynthesis have a tight relation with temperature (activating the emission after acclimation to high temperature, high Q_{10} values of emission, localization of isoprene synthase in the chloroplast) (Monson et al., 1994; Wildermuth and Fall, 1996). Sharkey and

Singsaas (1995) first observed that the stability of the photosynthetic apparatus under high temperature was better maintained in isoprene emitting leaves of Kudzu (Sharkey and Singsaas, 1995). Also the photosynthetic capacity of plants after high temperature stress recovered better in plants which were emitting or fumigated with isoprene or monoterpenes (Sharkey and Yeh, 2001; Velikova and Loreto, 2005). Siwko et al. (2007) by using the phospholipid membrane model showed that isoprene is located in the center of membranes and enhances the order of membrane lipids without changing the dynamics of the membrane. This study proved the positive role of isoprene on membrane stability under transient high temperature (Siwko et al., 2007). Partitioning of lipophilic isoprene into the thylakoid membrane prevents formation of water channels in the membrane which cause membrane leakage under high temperature. In addition, isoprene increases the hydrophobic properties within thylakoids and, therefore, strengthens the interaction between lipids and proteins of the membrane during high temperature stresses (Loreto and Schnitzler, 2010). The thermo-protective role of isoprene was confirmed in several studies (Hanson et al., 1999; Sharkey and Yeh, 2001; Velikova et al., 2006).

The other hypothesis is the antioxidant role of isoprenoids. Environmental factors such as high temperature and light, drought, salinity, heavy metal contamination and air pollution can cause oxidative stress in plants. Under these circumstances, reactive oxygen species (ROS), e.g. O_2, OH^+, H_2O_2, and O_2^+, are formed and accumulated in the plant cell, which results in oxidation of biological membranes and organelles (Grassmann et al., 2002; Mittler, 2002). In the thylakoid membrane, lipid peroxidation affects membrane fluidity and impairs the photochemical functioning. As it is reported in several studies, the oxidative effect of ozone and other ROS was reduced in isoprene and monoterpene emitting plants (Velikova, 2008). Engineered tobacco plants with isoprene emission capacity were more resistant to ozone (Vickers et al., 2009b). Also, in poplar clones the ozone sensitivity was inversely related to the isoprene emission capacity (Calfapietra et al., 2008). Production of new compounds and disappearing of reagents as a result of interaction between isoprene/ monoterpenes with ozone and other ROS reveal the oxidation of isoprenoids (Pinto et al., 2007; Ryan et al., 2009). The presence of conjugated double bonds in the structure of isoprenoids eases the transfer of electrons, energy and heat dissipation, therefore increases the antioxidant capacity of the cell (Sharkey and Yeh, 2001; Vickers et al., 2009b). The thermoprotective role of volatile isoprenoids could be also partly due to their antioxidant effect - under high temperature the hydrogen peroxide is produced due to the higher photorespiration. In *Platanus orientalis* isoprene-fumigated leaves showed less accumulation of H_2O_2 in the leaves under high temperature stress (Velikova et al., 2006) . The higher concentration of catalase and peroxidases in the isoprene inhibited leaves under heat stress also indicate the formation of more oxidative species in the absence of isoprene (Velikova and Loreto, 2005). In *Quercus ilex* applying

exogenous isoprene delayed the antioxidant response to high temperature, which could support ROS scavenging by isoprene (Peñuelas et al., 2005). However, this effect is not enough to protect the thylakoid membrane from denaturation under heavy heat stress and it should be accompanied with the effect of isoprene on enhanced integrity of the membrane. In addition under oxidative stress, isoprene can quench nitrogen reactive species which function as a messenger for hyper-sensitive response to stress (Loreto and Schnitzler, 2010). Sesquiterpenes might also have a scavenging role for ROS, however due to their high reactivity there are limited studies about their effect on stressed plants (Helmig et al., 2004; Vickers et al., 2009b).

Considering that all the environmental stresses eventually change the oxidative status of the cell, the protective mechanism of volatile isoprenoids could be defined as a single biochemical mechanism for multiple physiological stresses, which lead to enhanced antioxidant capacity of the cell. This mechanism includes direct interactions with oxidizing species, alteration of ROS signaling and membrane stabilization, which consequently decrease the lipid peroxidation (Vickers et al., 2009a).

1.9 Biofuel crops

Combustion of fossil fuels contributes significantly to the amount of CO_2 released into the atmosphere (more than 15 billion tons yearly) and, therefore, is directly associated to global warming (Kamm et al., 2006).Therefore, fossil fuel is not considered ecologically and environmentally sustainable (Chandra et al., 2012). The limitation of fossil fuels and their adverse environmental impact create a need to search for renewable and more sustainable sources of energy. Biomass is the most suitable replacement of fossil fuels, which can be used as an alternative fuel in the transport section (Chandra et al., 2012; Naik et al., 2010). First-generation biofuels are those produced from food crops, which mainly constitute production of ethanol from maize and sugar cane and production of biodiesel from oil palm or rapeseed. 98% of the total biofuels belong to the first generation of biofuel. The second generation of biofuels are those from ligno-cellulosic crops, whose lignin and celluloseare converted to the ethanol. A third generation of biofuels exploit the high biomass production from micro-algae. On the global level, biofuels are aimed to partially replace fossil fuels. For example in EU, the replacement should reach to 10% by2020. The recent rise in biofuel cultivation around the world will increase the land (Müller et al., 2007; Ravindranath et al., 2009) and water demand (King et al., 2013) of this sector, which might result in competition with food/feed crops (Naylor et al., 2007; Pilu et al., 2013; Pimentel et al., 2010). In addition, replacing the vegetation with biofuel crops specially in the areas like tropical forests with high biodiversity could lead to a significant loss of biodiversity (Campbell and Doswald, 2009). The most common positive effect of biofuel crops is the reduction of greenhouse gas emission into the atmosphere. These crops have a neutral carbon balance since the amount of CO_2 they release during the combustion of their biomass is almost equal to the amount they take up during their growth. The

contribution of the biofuel crops to the energy supply depends on the net energy ratio (NER), which is an indicator of the biofuel energy content/ energy consumption for their production. The biofuels which have NER higher than 1 are acceptable in terms of the energy balance (Petrou and Pappis, 2009). In addition to the positive effect of biofuel in mitigation of climate change, the large scale cultivation of these crops could have some adverse environmental impacts. The high requirement for pesticides and fertilizer application in the intense cultivation of these crops could contribute to water resources contamination and consequently eutrophication and eco-toxicity. In addition, most of the biofuel crops are strong BVOC emitters. Therefore, concerns about the possible detrimental impacts that large scale cultivation of these crops may have on air quality have been raised and the characterization of BVOC emissions in sustainability assessment of candidate bioenergy crops advocated (Ashworth et al., 2013, 2012; Porter et al., 2012).

1.10 The promising features of *A.donax* as a biofuel crop

Arundo donax is a tall, perennial C_3 grass from the Poaceae family, Arundineae tribe. It originated most likely in Asia and spread in the Mediterranean areas, US , China, Australia and southern Africa (Angelini et al., 2009; Hardion et al., 2014; Pilu et al., 2012). The interest for application of perennial grasses as energy crops increased since the mid 1980ies because of their high biomass, high lignin and cellulose content in their biomass and potential positive environmental impact. Based on the studies done so far, *A. donax* is considered as a promising crop for biofuel production in Mediterranean regions (Lewandowski et al., 2003; Pilu et al., 2013). Compared to other perennial grasses like panicum, *A. donax* has a very efficient biomass energy production with high Eroi (energy returned on energy invested) parameters, , due to its high yield and low input cultivation condition (Angelini et al., 2005; Pilu et al., 2012). *A.donax* biomass is transformed into energy through different methods, including direct combustion as biofuel for heat production, gasification and pyrolysis, anaerobic digestion to produce biogas and alcoholic fermentation to produce bioethanol (Jeon et al., 2010; Pilu et al., 2012). Considering the predicted competition for land and water resources between biofuel and food/feed crops, the cultivation of the low input (water, nutrients) species like, *Arundo donax,* which can grow in marginal lands with low water availability (Jeon et al., 2010; Nassi o Di Nasso et al., 2013; Pilu et al., 2013, 2012) is more environmentally favorable (Lewandowski et al., 2003). Water use efficiency, which may have severe hydrological consequences at local scales especially in a changing climate where freshwater resources may become more limited, is an important factor to assess the long-term sustainability of bioenergy species. Several studies addressed the WUE of *A. donax* (Nassi o Di Nasso et al., 2011; Triana et al., 2015). A direct comparison of *A.donax* with *Miscanthus* indicated similar WUE of established plantations of these two perennial grasses (Triana et al., 2015). This result is somehow surprising as C_3 species like *A. donax* are generally expected to have lower WUE than C_4 species like

Miscanthus. On the other hand, the leaves of *A.donax* are emitting considerable amounts of isoprene. The results of a literature survey on the main features of some common biofuel crops are presented in Table 2. However, these data are gathered from independent experiments carried out under different environmental conditions, thus making the comparison between them difficult. Among the species presented in Table 2, the two perennial rhizomatous grasses, *Miscanthus giganteus* and *A.donax*, have very good performance. Therefore, they could be considered as the best candidates for biofuel production due to their high biomass and WUE. Among isoprene emitters, woody perennials (oak, poplar and sweetgum) have the highest emission of isoprene per biomass unit, which is two/three times higher than emission from grasses. The most notable exception to this trend is *Eucalyptus*, which emits less isoprene per unit of biomass than *A. donax* and *P. australis*. According to this data, *A. donax* presents an excellent combination of productive features with high yields and WUE (Table 2). Compared to largely established woody biomass species like poplar and oak, *A. donax* has significantly lower isoprene emission per unit of produced biomass and higher absolute yields per unit of cultivated surface. *A. donax*, therefore, seems to be a promising addition/alternative to traditional bioenergy crops with a proper balance between environmental costs and benefits. *A.donax* is able to grow in wide range of environmental conditions from very arid to humid areas. The studies done so far revealed the drought tolerance of *A.donax*. In comparison with other biofuel crops e.g. *Miscanthus*, *A.donax* is better adapted to drought and is able to survive and grow under prolonged drought periods (Angelini et al., 2009; Mann et al., 2013). Morphological characteristics of *A.donax*, e.g. a deep rooting system, high root biomass and well developed canopy, significantly contribute to its drought tolerance (Cosentino et al., 2014; Erickson et al., 2012; Monti and Zatta, 2009). Recent studies were focused on the effect of water deficit on the physiological characteristics of *A.donax*, especially its photosynthetic response. These studies demonstrated the good ability of *A.donax* to conserve the photosynthetic capacity under drought (Cosentino et al., 2016; Haworth et al., 2016a, 2016b; Nackley et al., 2014; Sánchez et al., 2015).

Table 2: Literature survey of plant photosynthesis, WUE, isoprene emission and biomass production for plant species used for bioethanol production.

Biofuel crop	Isoprene emission rate ($nmol/ m^2s^1$)	Photosynthesis [a] ($\mu mol/ m^2s^1$)	Carbon lost as isoprene [b] (%)	WUE [c] ($\mu molCO_2/mmolH_2O$)	Biomass [d] (Mgr_{DM}/ha year)	Ratio (Iso/Bio)[e]
Miscanthus gigantus	0.003 - 0.009 [f]	26	0	3- 4.2	21.7	0.04
Panicum virgatum	0.003 - 0.009 [g]	17.5	0	2.3	10.1	0.09
Eucalyptus spp	20-30 [h]	16.8	0.74	1.6	17.5	171.43
Phragmites spp	20-30 [i]	15.2	0.8	1.3	10.1	297.03
A.donax	28-68 [j]	22.8	1.05	4	25.6	265.63
Liquidambar styraciflua	10-20 [k]	8.6	1.16	0.9	4.4	454.55
Populus hybrids	30-40 [l]	12.7	1.38	1.8	8.1	493.83
Quercus	40- 50 [m]	10.4	2.40	2.4	7.2	694.44

a & d: King et al., 2013; b: Calculated from the presented data (IE*5*100)/(A*1000); c : Calculated from King et al., 2013; e: Ratio of maximum isoprene emission rate/biomass production , the values are calculated from the presented data in the table; f: Crespo et al., 2013; g: Eller et al., 2011; h :Loreto & Delfine, 2000; i: Scholefield et al., 2004; j : Hewitt et al., 1990; k: Ninnement et al., 1999; l: Behnke et al., 2007; m: Sharkey et al., 1996

1.11 Aims of the study

The objectives of this thesis are: a) to determine the physiological characteristics of Arundineae species in relation to their isoprene emission capacity, b) to investigate the role of isoprene emission in the plasticity of *Arundo donax* under stress condition, and c) to study the ecotypic differences in isoprene emission of *A.donax* and its possible link to plant response to water stress.

More specifically:

In the **second chapter** we characterise different genera of Arundineae including *A.donax* and other closely related species to

- Investigate whether isoprene emission is conserved among selected species from the Arundineae tribe,

- Determine interspecific differences in the amount of emitted isoprene and its relation to photosynthetic capacity,

- Study the effect of isoprene on leaf anatomy and chloroplast ultrastructure, and

- Understand whether the impact of isoprene emission on physiological and structural parameters could explain differences in biomass productivity among Arundineae species.

To this end we investigated the isoprene emission from 6 different species of Arundineae. We revealed that the variation in isoprene emission capacity within the tribe was directly correlated to the photosynthetic capacity. The capacity to emit isoprene was lower in the species adapted to more xeric habitats. Isoprene emission enhanced the structural organization of the thylakoid membrane and improved the photosynthetic performance. However, this did not result in higher biomass production in higher isoprene emitting species.

Chapter 2

Ahrar M., Doneva D., Koleva D., Romano A., Rodeghiero M., Tsonev T., Biasioli F., Stefanova M., Peeva V., Wohlfahrt G., Loreto F., Varotto C., Velikova V. (2015) Isoprene emission in the monocot Arundineae tribe in relation to functional and structural organization of the photosynthetic apparatus. *Environmental and Experimental Botany* **119**, 87-95, doi: 10.1016/j.envexpbot.2015.04.010.

Contribution: The PhD candidate conducted the experimental work (90 %), analysed the data (100%) and wrote the paper (80 %).

In the **third chapter** we compare isoprene emitting and non-isoprene emitting species of Arundineae (*A. donax* and *H.macra*) under water stress to

- Assess the drought impact on chloroplast ultra-structure and photosynthetic efficiency of *A.donax* and *H.macra* in relation to their isoprene emission capacity,

- Compare the significant effect of isoprenoids and phenylpropanoids as antioxidant defence under drought between *A.donax* and *H.macra,* and

- Identify the physiological characteristics of *A.donax* associated to its drought tolerance.

Two Arundineae species with contrasting habitats, growth traits and metabolism namely *A.donax,* a fast growing strong isoprene emitter, and *H.macra,* a slow growing and non-isoprene emitting species, were exposed to drought stress. The drought caused a significant damage to the chloroplast structure only in *H.macra*. The photosynthesis was similarly inhibited in both species under drought, but it recovered after re-watering only in *A.donax*. Drought induced the secondary antioxidant metabolism (isoprenoids and phenylpropanoids) in both species. However, the response was different between *A.donax* and *H.macra*. According to these results we suggest that in *A.donax,* the volatile and non-volatile isoprenoids had a significant contribution to conserve the chloroplast structure and protect the photosynthetic machinery under water deficit, while in *H.macra* the phenylpropanoids were more stimulated under drought and likely were not as efficient as isoprenoids to prevent drought damage.

Chapter 3

Velikova V., Brunetti C., Tattini M., Doneva D., **Ahrar M.**, Tsonev T., Stefanova M., Ganeva T., Gori A., Ferrini F., Varotto C., Loreto F. (2016) Physiological significance of isoprenoids and phenylpropanoids in drought response of Arundinoideae species with contrasting habitats and metabolism. *Plant, Cell and Environment*, doi: 10.1111/pce.12785.

Contribution: The PhD candidate conducted the experimental work (30 %), analysed the data (25%) and wrote the paper (20%).

Doneva. D, **Ahrar. M**, Tsonev. T, Loreto. F, Varotto. C & Velikova.V. (2016). The role of isoprene in two Arundineae species exposed to progressive drought. Proceeding of the Bulgarian Academy of Science. In press.

Contribution: The PhD candidate conducted the experimental work (40 %), analysed the data (30%) and wrote the paper (35%).

In the **fourth chapter** we examine the physiological performance of two different ecotypes of *A.donax* (Italian and Bulgarian) under water stress to

- Quantify the differences in isoprene emission, photosynthetic and stomatal responses to water deficit,

- Identify any traits confirming plant resistance to drought associated with ecotypic differences, and

- Investigate the role of isoprene for stimulating the biosynthesis of secondary metabolites with antioxidant activity.

During this study two ecotypes of *A.donax* isolated from Srebarna in Bulgarian (500 mm annual precipitation) and Sesto Fiorentino in Italy (800 mm annual precipitation) were exposed to drought stress. The photosynthesis of both ecotypes was limited by diffusional limitation to CO_2 under mild drought, while the metabolic limitation developed during the course of the drought. BG showed stimulation of isoprene emission under mild drought and represented stronger induction of non-volatile isoprenoids and phenylpropanoids under severe drought, which could be associated to its enhanced photosynthetic performance under drought and better recovery of Calvin cycle after re-watering. Our results suggest the signaling role of isoprene emission to stimulate the antioxidant response and also provide more evidence on co regulation of isoprenoids and phenylpropanoids metabolism.

Chapter 4

Ahrar M., Doneva D., Brunetti C., Rodeghiero M., Biasioli F., Loreto F., Wohlfahrt G., Varotto C., Velikova V. (2016) Isoprene emission enhances the photosynthetic performance of *Arundo donax* under drought stress. In preparation.

Contribution: The PhD candidate conducted the experimental work (70 %), analysed the data (90%) and wrote the paper (90%).

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Chapter 2

Isoprene emission in the monocot Arundineae tribe in relation to functional and structural organization of the photosynthetic apparatus

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Isoprene emission in the monocot Arundineae tribe in relation to functional and structural organization of the photosynthetic apparatus

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ABSTRACT

Several plant species emit isoprene, a compound able to protect plants against high temperatures and oxidative stresses, and to affect the oxidative capacity of the atmosphere. The emission of isoprene in monocots is much less investigated than in dicots. We explored the emission of isoprene among members of the Poaceae tribe Arundineae, and its potential impact on plant performance. Our results confirm, also in monocots, the existence of a significant correlation between photosynthesis and isoprene emission and further suggest that isoprene inversely correlates to habitat xericity in unstressed Arundineae. Isoprene emission capacity developed rapidly in unfolding leaves, implying some developmental differences in the control of this biosynthetic pathway with respect to dicots. Among Arundineae, marked species-specific differences in several parameters related to plant productivity (photosynthesis, carbon lost as isoprene, water use efficiency, biomass and chloroplast ulrastructure) were observed. Isoprene presence improved structural organization of thylakoid membrane system and decreased the heat dissipation at physiological temperature. Our results demonstrate that, as in dicots, also in monocots isoprene could contribute to improve photosynthesis performance, although this was not necessarily reflected in higher yields, at least in unstressed conditions.

1. Introduction

Plants emit a wide range of volatile organic compounds (VOC), of which isoprene is the single most abundant compound (Guenther et al., 2006, 2012). Once synthesized, isoprene is not stored in the plant but is emitted through the stomata into the atmosphere. Since isoprene is a highly reactive VOC, it may impact air quality especially over urbanized areas, contributing to the formation of ozone and secondary organic aerosol particles (Claeys et al., 2004). In addition, isoprene has a higher affinity for oxidation by hydroxyl radicals compared to methane and thus indirectly increases the atmospheric lifetime of methane (Folberth et al., 2006).

Besides trying to elucidate isoprene impacts on atmospheric chemistry, many studies have focused on understanding the role of isoprene in plant biology. Isoprene seems to be implicated in protection against different environmental stresses (reviewed in Vickers et al., 2009; Loreto and Schnitzler, 2010; Niinemets, 2010). It has been hypothesized that isoprene preserves thylakoid membrane stability and chloroplasts ultrastructure, and/or exerts antioxidant properties (Loreto and Velikova, 2001; Velikova et al., 2009, 2011; Loreto et al., 2014a).

Many prokaryotic and eukaryotic organisms are able to emit isoprene. However, a limited number of plant families synthesize isoprene, and systematic studies aiming to uncover taxonomic or

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phylogeographic patterns have not delivered clear results (Sharkey et al., 2005; Monson et al., 2013; Loreto et al., 2014b; Loreto and Fineschi, 2015). Few plant genera only include isoprene-emitting species (e.g., *Populus*, *Salix*) (Loreto et al., 2014b). However, in many other cases, emitting and non-emitting species can be found in the same genus (e.g., various genera of Fabaceae) (Harley et al., 1999; Logan et al., 2000). Fast-growing plants are often strong isoprene emitters (Loreto and Fineschi, 2014) and the trait is often associated to perenniality (Dani et al., 2014) and with hydrophily (Loreto et al., 2014b). The nuclear *isoprene synthase* (*IspS*) gene codes for the only enzyme specific of the isoprene biosynthetic pathway. In poplar, the IspS protein is found in nearly equal amounts in the stroma and on the stromal side of thylakoid membranes (Schnitzler et al., 2005). The scattered phylogenetic occurrence and low protein homology across lineages indicate that IspS function possibly evolved independently more than once during the evolution of land plants (Sharkey et al., 2008; Monson et al., 2013).

Being a metabolically expensive trait, with high energy and carbon requirement compared to other metabolic pathways (Sharkey and Yeh, 2001), isoprene emission is tightly regulated and influenced by multiple factors, both during plant development and in response to stress. During development, the leaf age dependence of isoprene emission is mainly linked to the transcriptional regulation of the *IspS* gene (Mayrhofer et al., 2005; Wiberley et al., 2005). Isoprene emission capacity (i.e., the capacity to synthesize IspS) develops with a delay between few days and a month, compared to photosynthesis, and expanding leaves are generally low emitters. The time span of the delay depends on environmental conditions, mainly temperature (Centritto et al., 2004).

Many studies on isoprene emission from dicots are available, however to date the information about the capacity of monocots to emit VOC is scarce. Up to now isoprene emission from monocots has been detected e.g., among Cyclanthaceae, Arecaceae and Poaceae (Harley et al., 1999). Only one species from Cyclanthaceae, and 21 out of 25 species from Arecaceae were found to emit isoprene (Harley et al., 1999). Based on the information currently available, isoprene emission is a rare trait within Poaceae and only 8 out of 26 screened genera of this family (Harley et al., 1999), which mainly belong to the tribes Bambuseae, are isoprene emitters (Wiedinmyer et al., 2004). However, information about isoprene emission as a trait is not available for the majority of the species from Arundineae tribe in the Poaceae family. In the present study, we have focused on plant species belonging to four genera of the Arundineae tribe (Harley et al., 1999), namely *Arundo*, *Phragmites*, *Hakonechloa* and *Molinia* (Linder et al., 1997; Mathews et al., 2000). *Arundo donax* (giant reed) is a well-studied Arundineae species because of its importance as an energy crop in the US and Europe (Lewandowski et al., 2003). *Phragmites australis* (common reed) is a common wetland plant frequently used for phytoremediation of soil or contaminated water (Lee and Scholz, 2007) and, like *A. donax*, has been proposed as possible biofuel crop (Sathitsuksanoh et al., 2009). Isoprene emission from leaves of *A. donax* and *P. australis* was reported in former studies (Hewitt et al., 1990; Scholefield et al., 2004). *Hakonechloa* is a monospecific genus used in landscape design as ornamental grass (Harvey et al., 2004). *Molinia* is a genus of moorland grasses grown in different parts of Europe such as England, Scotland and the Netherlands mainly for livestock feeding (Marrs et al., 2004). *Hackonechloa, Molinia*, and *Phragmites* form a relatively well-supported phylogenetic clade, while *Arundo* belongs to the second sub-clade of the Arundineae (Barker et al., 1995; Linder et al., 1997; Mathews et al., 2000).

In this study, we investigated whether: (1) isoprene emission is conserved among selected species from the Arundineae tribe; (2) interspecific differences in the amount of emitted isoprene are related to photosynthesis; and (3) isoprene impacts on foliar anatomy and chloroplast ultrastructure. Further, we aimed at understanding whether the impact of isoprene emission on physiological and structural parameters could also explain differences in biomass productivity among Arundineae species.

2. Materials and methods

2.1. Plant material and growing conditions

Six different plant species from the Arundineae tribe were investigated: *A. donax*, *A. plinii*, *A. collina*, *P. australis*, *H. macra* and *Molinia caerulea*. Recently, *A. collina* and *A. plinii* have been proposed to be a single species, part of the *A. plinii* s.l. aggregate (Hardion et al., 2012), but in this work they are still treated separately. The species chosen belong to the two major Arundineae clades identified in previous phylogenetic reconstructions (Hsiao et al., 1998; Mathews et al., 2000; Christin et al., 2013). All plants were propagated by rhizomes. The rhizomes were potted in 15 L pots, filled with soil mixture consisting of decomposed white sphagnum peat and clay (30% V/V), fully decomposed raised bog peat (30% V/V), Knauf perlite (20% V/V), and sand (20% V/V). Plants were grown in a greenhouse under environmental conditions controlled to favor optimal isoprene biosynthesis (day/night temperature $30/20 \pm 5\,^{\circ}C$, day/night relative humidity 60/50%). When the solar light intensity was lower than 400 $\mu mol\ m^{-2}\ s^{-1}$ of photosynthetic photon flux density (PPFD), supplemental light was provided by high pressure sodium lamps (Hortilux Schréder, Holland) to provide at least 600 $\mu mol\ m^{-2}\ s^{-1}$ during the 16-h day length. Pots were regularly watered to keep the optimal water regime and to avoid anoxia or drought stress. Plants were fertilized with liquid fertilizer (Cifo, Italy) once a week in order to supply mineral nutrients at free access rates.

2.2. Experimental design

Measurements of photosynthesis and isoprene emission started on fully expanded leaves (4th node from the apical meristem) of 4-week-old plants. The same leaf was used during the whole experiment. The measurements were repeated weekly for five consecutive weeks, carrying out one measurement for all plant species within the same day. The order of sampled species was randomized every day. To relate the capacity of biomass production to isoprene emission rate, leaves and stems were harvested at the end of the experiment and their dry weight was determined.

Three plant species (*A. donax*, *A. collina* and *H. macra*), characterized by different isoprene emission capacities, were selected for additional analyses (chlorophyll fluorescence, leaf anatomy and chloroplast ultrastructure, thermoluminescence). To test for the effect of leaf developmental stage on isoprene emission, a full plant profile was performed in *A. donax* starting from the first top unfolding leaf to the last bottom green leaf in the stem. Ten leaves per plant profile were measured on four different plants of *A. donax*. One profile was measured per day.

2.3. Gas exchange, chlorophyll fluorescence and thermoluminescence measurements

Carbon dioxide and water vapor gas exchange were measured with a portable leaf gas exchange system (LI-6400, Li-Cor Lincoln, NE, USA). The middle part of the leaf was clamped into the 6 cm^2 gas exchange cuvette and exposed to a flow (300 $\mu mol\ s^{-1}$) of synthetic air (21% O_2, 79% N_2, and 400 $\mu mol\ mol^{-1}$ CO_2). During all measurements, the cuvette was maintained at $30\,^{\circ}C$, 45–50%

relative humidity and the leaf was illuminated with 1000 μmol m^{-2} s^{-1} PPFD. The gas exchange parameters were recorded after reaching steady state photosynthesis. The carbon cost of isoprene emission (%) was calculated as the ratio of isoprene emission to photosynthesis.

Chlorophyll fluorescence was measured by a Fluorescence Monitoring System (FMS, Hansathech Instruments, UK) simultaneously to gas exchange measurements. Leaves were dark adapted for 20 min prior to the determination of minimum (F_o) and maximum (F_m) fluorescence. The maximum quantum yield of PSII photochemistry (F_v/F_m) was determined as $(F_m - F_o)/F_m$. Then leaves were adapted to the growth light intensity and a saturating pulse of 0.8 s with over 6000 μmol photons m^{-2} s^{-1} was applied in order to determine the maximum (F_m'). The quantum yield of PSII (Φ_{PSII}) was calculated according to Genty et al. (1989): $\Phi_{PSII} = (F_m' - F_s)/F_m'$, where F_s is the steady-state fluorescence during the

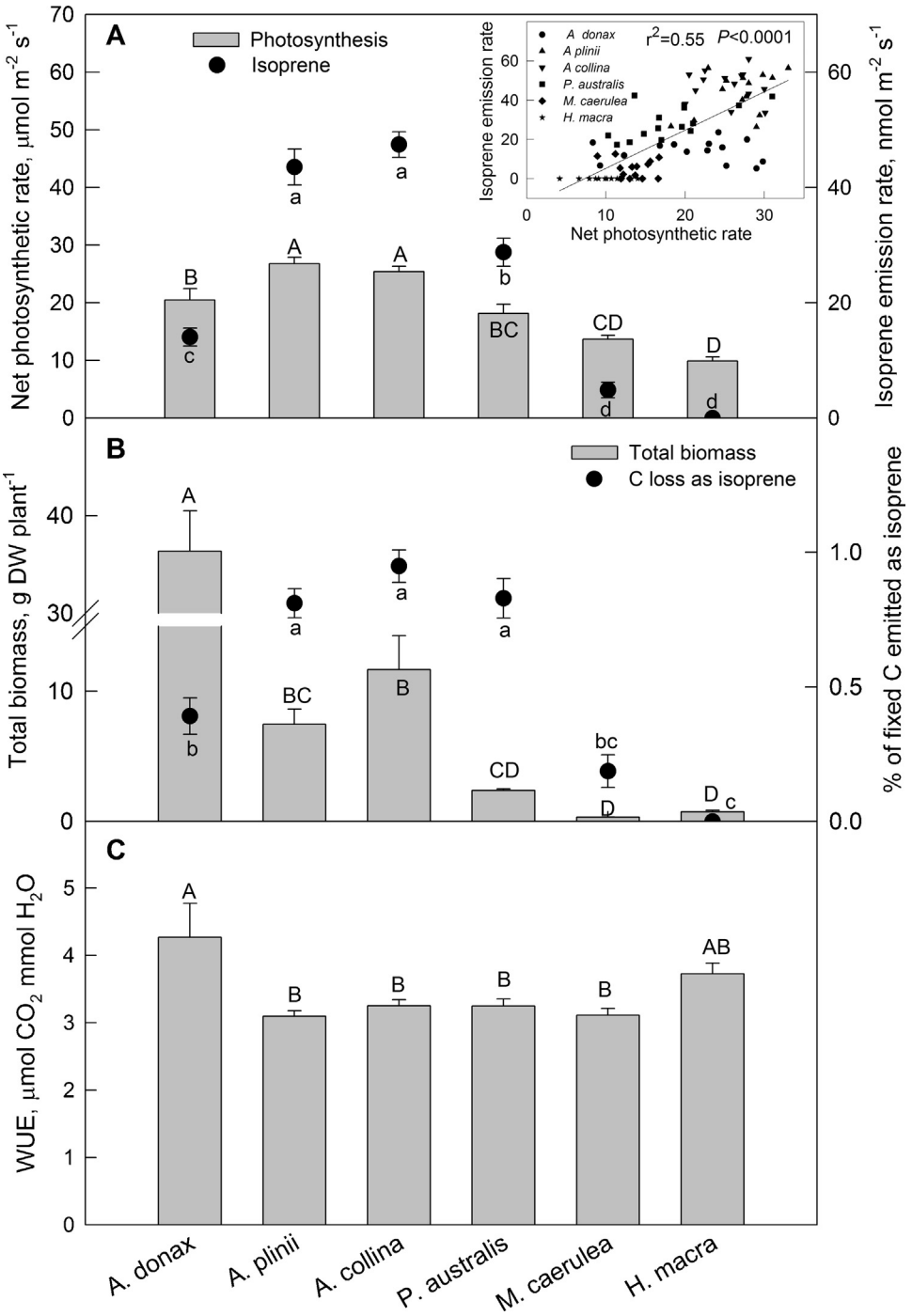

Fig. 1. (A) Net photosynthetic (grey bars) and isoprene emission rates (closed circles), total above ground biomass per plant (grey bars) and percentage of fixed carbon lost as isoprene (closed circles), and (C) water use efficiency for different species of Arundineae; (inset) correlation between photosynthetic rate and isoprene emission rate of six different species of Arundineae. $r^2 = 0.55$, $P < 0.0001$. Each dot represents one measurement. Measurements of net photosynthetic and isoprene emission rates were performed at 1000 μmol m^{-2} s^{-1} PPFD, 30 °C leaf temperature and 400 μmol mol^{-1} [CO_2]. Biomass was measured at the end of the growth period ($n = 3$). Results are means ± SD of three biological replicates which were measured weekly during five consecutive weeks ($n = 15$ measurements in total per plant species). Different letters indicate statistically different means based on Tukey's test with $^*P < 0.05$.

actinic illumination. Non-photochemical quenching (NPQ) due to dissipation of excess light energy was calculated as NPQ = ($F_m - F_m'$)/F_m' (Bilger and Björkman, 1991).

Thermoluminescence (TL) was detected with a photomultiplier tube (Hamamatsu R943-02, Hamamatsu Photonics) linked to an amplifier in a home-built apparatus. A complete description of the TL equipment is provided in Zeinalov and Maslenkova (1996). TL measurements were performed as detailed in Velikova et al. (2011). Briefly, intact plants were dark adapted for 6 h at room temperature. TL emission from leaf segments was excited by one or two saturating single-turnover flashes at 1 °C. After the flash exposure, TL was recorded during warming of the samples to 70 °C in darkness with a ramp of temperature at a constant rate of 0.6 °C s^{-1}. Dark adapted leaf segments were illuminated with far-red (FR) light in order to measure the recombination/deactivation decay time of the "after glow" (AG) emission, which has been related to the cyclic electron transport around PSI (Ducruet, 2003). TL measurements were repeated on 5 leaves from different individuals of each plant species.

2.4. Isoprene measurements

Isoprene emission was determined by connecting the outflow of the Li-Cor cuvette to a high sensitivity proton transfer reaction mass spectrometer (PTR-MS; Ionicon Analytik GmbH, Innsbruck, Austria). The drift tube of PTR-MS was heated up to 80 °C and operated at 550 V drift voltage and 2 mbar drift tube pressure, corresponding to an E/N ratio of about 140 Td (E being the electric field strength and N the gas number density; 1 Td = 10–17 V cm^{-2}). The operating parameters of the PTR-MS were held constant during measurements. When photosynthesis reached steady-state the PTR-MS was connected to the leaf cuvette via a t-junction at the cuvette outlet. The air leaving the cuvette headspace was sampled for isoprene at a flow rate of 0.1 L min^{-1}. Isoprene was detected as a parent ion at protonated m/z = 69. Calibration for isoprene was performed with a standard of a gas calibration unit (GCU; Ionicon Analytik GmbH, Innsbruck, Austria). Isoprene emission rate was calculated as IE = (E/26)(F/LA). E is the mole fraction of the air leaving the cuvette corrected for the blank (E − Eo; nmol/mol) was multiplied with the molar density of air (1/26; mol/m^3), multiplied with the flow rate through the cuvette (F, m^3/s) and divided by the leaf area (LA; m^2) of the leaf clamped in the leaf cuvette. Using this equation, the units of isoprene emission were nmol m^{-2} s^{-1}.

2.5. Water-use efficiency (WUE) and aboveground biomass

WUE refers to the ratio of assimilated carbon to water lost by the plant through transpiration, which is also called photosynthetic (or instantaneous) WUE. It was calculated by dividing the rate of photosynthesis by the rate of transpiration (T) (A/T = WUE; μmol CO_2 $mmol^{-1}$ H_2O).

At the end of the experiment, the aboveground parts of the investigated plants were harvested and divided into stems and leaves. Fresh and dry weights (oven dried at 80 °C) were recorded. The biomass accumulation per plant was calculated.

2.6. Light microscopy (LM) and transmission electron microscopy (TEM)

For LM, segments (4–5 mm^2) from the middle part of fully expanded leaves, taken from the 4th nodes, were fixed in 3% (m/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 12 h at 4 °C and used in microscopy studies. At least 30 handmade transversal sections per species were mounted on slides in glycerol. Observations were carried out and micrographs were taken using a light microscope and a camera (Eclipse 50i, Nikon, Tokyo, Japan).

For TEM, leaf segments (1–2 mm^2) were fixed in 3% (m/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and post-fixed in 1% (m/v) $KMnO_4$ in the same buffer for 2 h at room temperature. After dehydration by increasing concentrations of ethylalcohol (from 25 to 100%), the samples were embedded in Durcupan (Fluka, Buchs, Switzerland) and cross-sectioned using an ultramicrotome (Reichert-Jung, Wien, Austria). Observations were performed with an electron microscope (JEOL 1200 EX, Tokyo, Japan). Minimum 15 photos for each species were analyzed.

2.7. Statistical analyses

Data points are reported as means of biological replicates, which consisted of three to five leaves from different individual plants. The statistical analyses were carried out using one-way analysis of variance (ANOVA) tests. The mean differences were statistically separated by Tukey's test and considered to be significant at the 5% level.

3. Results

3.1. Physiological characteristics of different Arundineae species

Photosynthesis and isoprene emission rates from different Arundineae species are shown in Fig. 1A. Photosynthesis rates of *A. collina* and *A. plinii* were significantly higher than in the other investigated species. *M. caerulea* and *H. macra* were characterized by the lowest photosynthesis rates.

A. collina and *A. plinii* were the strongest isoprene emitters (~47.5 and 43.6 nmol m^{-2} s^{-1}, respectively), followed by *P. australis* (~28.8 nmol m^{-2} s^{-1}). *A. donax* emitted only ~14.0 nmol m^{-2} s^{-1} of isoprene, and *M. caerula* showed the lowest emission compared with the other emitting species (~4.9 nmol m^{-2} s^{-1}). *H. macra* did not emit isoprene or other volatile isoprenoids at detectable levels. A significant correlation between isoprene emission and photosynthesis was observed within the tribe (Fig. 1A inset). Plant species characterized by higher photosynthesis also showed higher isoprene emission (R^2 = 0.55, $P < 0.0001$). This trend was confirmed when expressing isoprene emission on a leaf dry weight basis in order to account for possible interspecific differences in leaf thickness (data not shown).

Total aboveground biomass accumulation was greatest in *A. donax*, followed by *A. collina* and *A. plinii*, whereas the lowest biomass accumulation was observed in *M. caerulea* and *H. macra* (Fig. 1B). A larger percentage of carbon was lost as isoprene in leaves of *A. collina*, *A. plinii* and *P. australis* as compared to *A. donax* (Fig. 1B).

The WUE was highest in *A. donax* and *H. macra* (respectively 4.27 and 3.73 μmol CO_2 $mmol^{-1}$ H_2O), but the difference with the other investigated plant species was statistically significant only for *A. donax* (Fig. 1C).

3.2. Chlorophyll fluorescence

The chlorophyll fluorescence parameters were determined in *A. donax*, *A. collina* and *H. macra*. The maximum efficiency of PSII in dark-adapted leaves (F_v/F_m) was similar in *A. donax* and *A. collina*, whereas this parameter was statistically lower in *H. macra* (Fig. 2). The actual quantum yield, i.e. the true efficiency of PSII (Φ_{PSII}) in illuminated leaves, was higher in *A. donax* followed by *A. collina*. *H. macra* had the lowest Φ_{PSII}. Lower F_v/F_m and Φ_{PSII} corresponded to lower photosynthesis and no isoprene emission in *H. macra* (see Fig. 1A). Interestingly, NPQ, the parameter reflecting the mechanism whereby plants convert excess energy to heat and thus

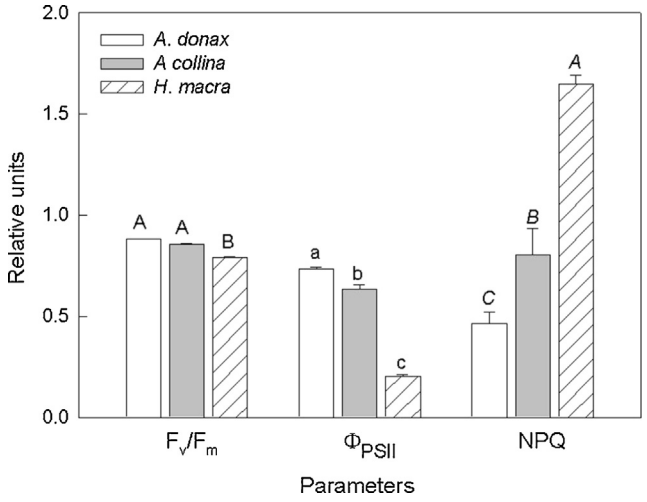

Fig. 2. Chlorophyll fluorescence parameters for three species with different isoprene emission capacity. Groups of bars represent different fluorescence parameters for *A. donax*, low isoprene emitter, (white bars), *A. collina*, high isoprene emitter, (grey bars) and *H. macra*, non-isoprene emitter, (striped bars). Results are means ± SD of three biological replicates which were measured weekly during five consecutive weeks ($n = 15$ measurements in total per plant species). Means were separated by Tukey's test and different letters indicate means statistically different with $^*P < 0.05$.

minimize subcellular damage in stressful conditions, was remarkably higher in *H. macra* than in the isoprene-emitting species.

3.3. Thermoluminescence

Thermoluminescence measurements were performed in two plant species, *A. donax* and *H. macra*, contrasting in their ability to emit isoprene. Similarly to our previous study with isoprene emitting and non-emitting plant species (Velikova et al., 2011), the peak position of the B band, which is formed as a result of $S_{2/3}Q_B^-$ charge recombination, was found at 27 ± 3 °C in *A. donax*, and at 19 ± 2 °C in *H. macra* (data not shown). Luminescence emission obtained after a far-red irradiation of intact leaves revealed the so-called "afterglow" (AG) band ($S_{2/3}Q_B^- + e$) at around 46 °C (Ducruet, 2003). Thermoluminescence curves after far-red excitation showed differences of the ratio between the intensity of B and AG bands, namely the AG/B ratio was significantly higher in *H. macra* compared to *A. donax* (Fig. 3).

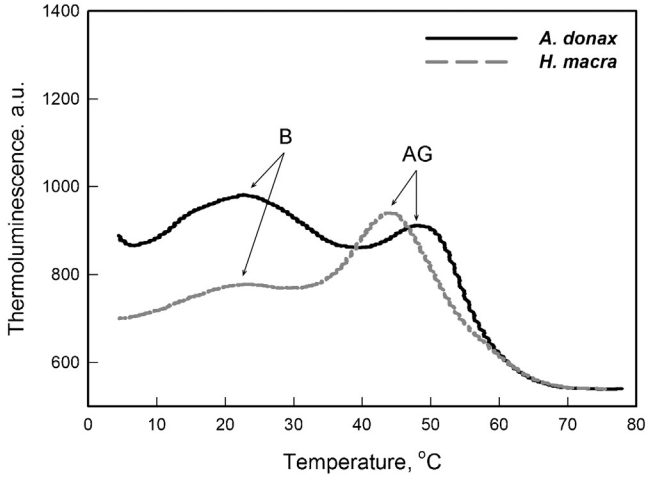

Fig. 3. Representative TL curves of *A. donax* (black solid line) and *H. macra* (grey dash line) after illumination with far-red. Curves are representative of measurements on five leaves from different plants. a.u. – arbitary units.

3.4. Photosynthetic and isoprene emission capacity at different leaf developmental stages in A. donax

Photosynthesis and isoprene emission capacity in relation to leaf development stages are shown in Fig. 4. The first node was assigned to the terminal leaflet, which was unfolded. Photosynthesis was similar in 1st–9th leaf nodes, whereas in the oldest leaf (10th) it was significantly lower, at least compared to the 2nd leaf node. Although there was no significant difference between 1 and 9 leaf nodes, the overall trend showed a reduction of photosynthetic capacity during leaf aging.

Isoprene emission gradually increased along the profile. The emission was highest at the 6th leaf node, with a tendency to decrease in older leaves (Fig. 4).

3.5. Leaf anatomy and chloroplast ultrastructure

A. donax, *A. collina*, and *H. macra* are characterized by a leaf anatomy which is typical for Poaceae (Fig. 5A–C). The organization of the mesophyll was similar in the different species, consisting of closely situated and irregularly arranged photosynthetic parenchyma cells. The vascular bundles were closed, collateral, with double bundle-sheaths and prominent sclerenchyma girders above and beneath the bundle-sheaths. Structural differences between the three examined species were only observed in the number and arrangement of the adaxial epidermis bulliform cells.

The chloroplasts in the mesophyll of *A. donax* leaves were highly elongated, flattened, with a well-structured internal membrane system (Fig. 5D). Chloroplasts were composed of evenly distributed grana of different height (15–30 thylakoids), connected with relatively short stroma thylakoids. Some of the grana took up the whole transverse section of the chloroplasts. In *A. collina* the mesophyll chloroplasts showed a typical elliptical shape (Fig. 5E). The internal membrane system was composed of a large number of middle height grana (around 20) evenly distributed in the stroma, and of relatively densely situated stroma thylakoids. The chloroplasts in the mesophyll cells of *H. macra* were more round (Fig. 5F). Their membrane system consisted of relatively smaller number of grana thylakoids uniformly occupying the entire stroma space. No starch and plastoglobuli were observed in all three investigated species.

4. Discussion

Isoprene emission has been studied in the last decades for its importance in plant interactions with the environment and with other organisms (Loreto and Schnitzler 2010). However, focus has predominantly been on dicotyledonous trees, whereas only scant information is available for monocots (Harley et al., 1999; Sharkey et al., 2005). The present study is the first to cover isoprene emission from a tribe of monocotyledonous plants. We used the Arundineae tribe as a case study, as this clade includes fast-growing plants which are known as strong isoprene emitters, e.g., *A. donax* and *P. australis* (Loreto et al., 2014b), as well as closely related species.

4.1. Evolution of isoprene emission in Arundineae may follow the same patterns as in dicots

The ability to produce isoprene over evolutionary times was probably gained and lost repeatedly, even among closely related species (Sharkey et al., 2005; Monson et al., 2013). In the case of the studied species from the Arundineae tribe, isoprene emission was detected in all species, with the exception of *H. macra*. The conflicting reconstructions of the Arundineae phylogeny (Linder et al., 1997; Barker, 1997; Hsaio et al., 1998; Mathews et al., 2000;

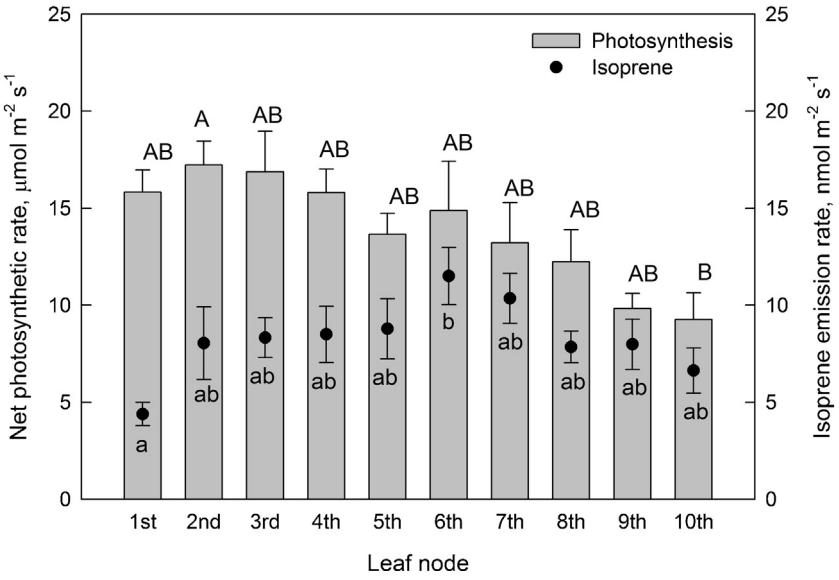

Fig. 4. Effect of leaf developmental stage on isoprene emission and photosynthesis in *A. donax*. Isoprene emission (closed circles) and net photosynthetic rates (grey bars) were measured at 1000 μmol m^{-2} s^{-1} PPFD, 30 °C leaf temperature and 400 μmol mol^{-1} [CO_2]. Results are means ± SD of four biological replicates ($n = 4$). Means were separated by Tukey's test and different letters indicate means statistically different with $^*P < 0.05$.

Christin et al., 2013) do not allow a conclusive placement of *H. macra* compared to the other species characterized in this study. However, given that the two most recent phylogenies agree on placing *P. australis*, *H. macra* and *M. caerulea* in a monophyletic clade (Mathews et al., 2000; Christin et al., 2013), the most parsimonious hypothesis is that in *H. macra* a secondary loss of the *IspS* gene function has occurred.

The lower isoprene emission in *M. caerulea* is also worth noting, as it may indicate ongoing subfunctionalization for a second taxon in the same clade. Taken together, these results indicate that also in Arundineae (monocots) isoprene biosynthesis is associated with selective tradeoffs analogous to those hypothesized to drive the evolution of this trait in dicots (Harley et al., 1999). A correlation between perenniality and the retention of isoprene trait over evolutionary time has been recently proposed (Dani et al., 2014; Loreto and Finesch, 2014). As all Arundineae species considered in this study are perennial, additional life history traits may play a role in determining isoprene emission. It has also been proposed that monoterpene emission replaces isoprene emission in species associated to xeric environments (Loreto et al., 2014b). Our results are in line with this notion, as the Arundineae species that were examined in this study are all riparian, with the possible exception of *H. macra*, the only species that does not emit isoprene.

4.2. Isoprene emission has different developmental pattern in Arundineae and in dicots

A strong developmental control on isoprene emission has been reported in dicots. In *Populus trichocarpa* the onset of isoprene emission occurs at the 5th open leaf, which has been related to delayed biosynthesis of isoprene synthase (Sharkey et al., 2008). In *A. donax*, the emission of isoprene started earlier, immediately after unrolling of the new leaves, indicating *IspS* transcriptional regulation differences compared to dicots. A further difference is that *A. donax* developing leaves did not emit monoterpenes (data not shown), while in hybrid poplars monoterpenes are emitted at early developmental stages in the place of isoprene (Brilli et al., 2009). These findings also suggest differences in the transcriptional regulation of monoterpene biosynthetic genes and/or in the size of the dimethylallyl diphosphate (DMADP) pool, consistent with recent results from poplar (Ghirardo et al., 2014; Rasulov et al., 2014). Mining of the recently published giant reed *de novo* transcriptome may help answer this question in the future, by allowing the identification and expression level quantification of candidate *IspS* and monoterpene synthases biosynthetic genes (Sablok et al., 2014).

4.3. Possible involvement of isoprene in photosynthetic optimization in Arundineae

A positive correlation between photosynthesis and isoprene emission was observed in some dicot plants (Harley et al., 1994; Litvak et al., 1996), but not in poplars (Guidolotti et al., 2011). The relationship between isoprene and photosynthesis was also clear in our experiment with the monocot Arundineae (Fig. 1A). In non-stressed plants, isoprene almost completely originates from carbon freshly fixed by photosynthesis (Delwiche and Sharkey, 1993; Brilli et al., 2007) and a relationship between isoprene and photosynthesis is therefore expected. This, in turn, implies that either photosynthetic precursors, or energy and reducing power produced by photosynthesis, limit isoprene biosynthesis (Loreto and Sharkey, 1993; Sharkey and Yeh, 2001). Along this line, Morfopoulos et al. (2013) have suggested a modeling mechanism by which isoprene emission rate is directly proportional to the excess of reducing power (NADPH) generated by linear electron flow and unused by photosynthesis. However, *A. donax* has higher Φ_{PSII}, a parameter which is related to linear electron flow (Genty et al., 1989), but lower photosynthesis and isoprene emission with respect to *A. collina* (Figs. 1A, 2). Probably photorespiration accounts for the extra electron transport monitored by Φ_{PSII}, and maintains the excess of NADPH available for isoprene biosynthesis. Isoprene emission seems to be stimulated when both photosynthesis and photorespiration are active, being instead inhibited when photorespiration is suppressed, e.g., by elevated intercellular CO_2 (Scholefield et al., 2004; Guidolotti et al., 2011). However, in *A. donax* decreasing photorespiration was reported to enhance isoprene emission and photosynthesis (Hewitt et al., 1990).

Interestingly, we found that isoprene-emitting *Arundo* species are characterized by much lower NPQ with respect to the non-emitting species *H. macra* (Fig. 2). Recent reports have shown that the NPQ is lower in isoprene-emitting dicots, both in presence and

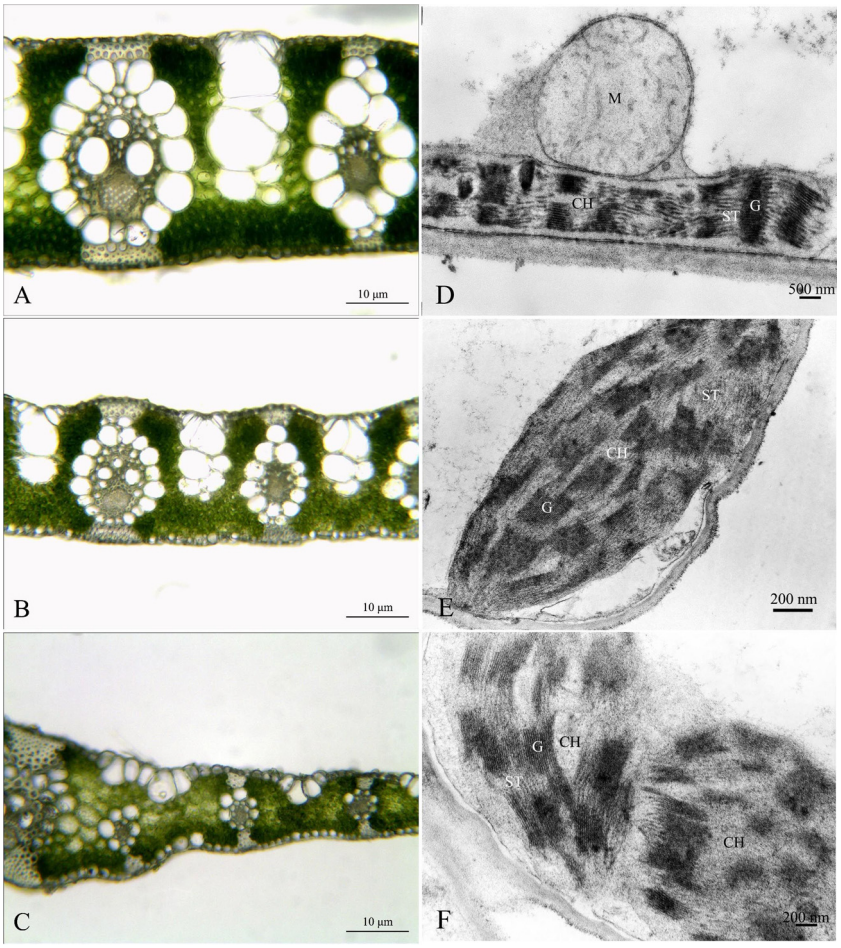

Fig. 5. Leaf structural organizations. Leaf lamina transverse section of *A. donax* (A), *A. collina* (B), and *H. macra* (C). Chloroplast ultrastructure of *A. donax* (D), *A. collina* (E), and *H. macra* (F). CH – chloroplast, G – grana tylakoids, ST – stroma tylakoids, M – mitochondria.

in absence of stresses that quench photosynthesis and stimulate mechanisms of heat dissipation (Behnke et al., 2007; Pollastri et al., 2014). Our data support the idea that isoprene facilitates the flow of electrons through photosystems, probably strengthening and tightening the assemblage of photosynthetic membranes (Pollastri et al., 2014). More specifically, isoprene was shown to improve the stability of ordered arrays of the light-harvesting complex II of photosystem II in the stacked region of the thylakoid grana (Velikova et al., 2011). On the other hand, the high NPQ of *H. macra* could be due, at least in part, to its adaptation to low-light environments (Harvey et al., 2004), analogously to what was previously suggested in the case of woody perennials and ferns (Ishida et al., 1999; Danin, 2004; Saldaña et al., 2009).

Considering the possible role of isoprene to enhance the photosynthetic performances of chloroplast membranes, additional differences in the photochemistry of isoprene-emitting and non-emitting Arundineae are to be expected. TL measurements were performed to obtain rapid information about the redox potential changes on the primary (Q_A) and secondary (Q_B) quinone acceptors of PII. Illumination of dark-adapted samples with single-turnover flashes generates charge pairs within PSII reaction centers that are energetically stabilized on the donor and acceptor side of PSII. Even small changes in the redox properties of radical pairs affect the intensity and the peak position of TL bands and TL emission curves provide important information for structural changes in both the donor and acceptor side of PSII (Ducruet and Vass, 2009). Indeed, a higher intensity and up-shift of the main TL B-band was observed in *A. donax* in comparison to *H. macra* at physiological temperatures, similarly to what has been recently demonstrated in isoprene-emitting *Arabidopsis* and *P. orientalis* plants (Velikova et al., 2011). This confirms that isoprene enables plants to perform more efficient primary photochemistry of PSII. We also observed a higher cyclic electron flow around PSI of the non-emitting *H. macra* (Fig. 3), as indicated by the changes in afterglow luminescence emission (Ducruet, 2013). This may reflect a growing necessity to dissipate energy in absence of isoprene.

High WUE is an important trait for crop sustainability, especially in a warming and more arid climate (Nicotra and Davidson, 2010). Here we show that the WUE of *A. donax* is higher than in most Arundineae species (Fig. 1C). This observation is not related to the capacity of emitting isoprene, or accumulating biomass (Fig. 1A and B). As an example, the WUE was similar in *A. donax* and *H. macra*, the species that did not emit isoprene and had the lowest biomass accumulation. The mechanisms underlying the high WUE in these two contrasting species are still unknown and need to be further explored.

4.4. Anatomy and ultrastructure of isoprene-emitting Arundineae assist efficient photosynthesis

It is also worth noting that the potentially higher efficiency of photochemistry is accompanied in emitting species by anatomical and ultrastructural differences, especially at chloroplast level (Fig. 5). We found that *A. donax* and *A. collina* (isoprene-emitting) have thicker leaf lamina than *H. macra* (non-emitter). The mesophyll adjacent to the bulliform cells was also thicker in

Arundo species than in *H. macra*. A larger number of bulliform cells arranged in columns in *Arundo* species may enhance the effectiveness of light absorption (Lambers et al., 2008; Vogelmann and Gorton, 2014), which is related to the higher photosynthesis measured in these plants.

The shape of the chloroplasts affects the orientation of the grana, as well as orientation and length of the stroma thylakoids, in turn possibly altering the efficiency of light capture by light harvesting complexes (Lambers et al., 2008). The chloroplasts of the high isoprene-emitter *A. collina* have the typical elliptical shape that allows efficient distribution of thylakoids and high net photosynthesis. On the other hand, the chloroplasts of the non-emitting *H. macra* were round, and TEM analysis also showed a more disordered and less abundant grana stacking in the thylakoid membrane system of *H. macra* with respect to the two isoprene-emitting *Arundo* species. Thin section immunolabeling and biochemical fractionation studies suggest that about 85% of the PSII complexes is concentrated in the stacked regions of grana thylakoids, whereas the PSI complexes are associated with stroma thylakoids (Staehelin, 2003). Thus, the lower efficiency of PSII in *H. macra* could be attributed to less PSII functional units than in isoprene emitting *Arundo* species. A broader sampling in the tribe, however, will be necessary to further elucidate the relationship between isoprene emission and the anatomical/ultrastructural differences existing among species.

In conclusion, this study on Arundineae has for the first time gathered information on the interspecific differences in isoprene emission in a monocot tribe. It has also highlighted similarities and differences of isoprene emission patterns in monocots compared to the much better investigated dicots. Our results indicate similar evolutionary tradeoffs associated to isoprene in dicots and in monocots, but possible regulatory differences (especially those affecting the developmental pattern of isoprene emission) deserving further investigation. As for dicots, also in monocots isoprene emission seems to be associated with better photosynthetic performance, and with optimal chloroplast ultrastructure. However, the level of isoprene emission was not associated to biomass production, possibly due to additional limitations downstream of primary carbon fixation, or to altered partitioning of carbon allocation between aboveground and belowground sinks.

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Chapter 3

3.1. Physiological significance of isoprenoids and phenylpropanoids in drought response of Arundinoideae species with contrasting habitats and metabolism

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Original Article

Physiological significance of isoprenoids and phenylpropanoids in drought response of Arundinoideae species with contrasting habitats and metabolism

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ABSTRACT

Physiological, biochemical and morpho-anatomical traits that determine the phenotypic plasticity of plants under drought were tested in two Arundinoideae with contrasting habitats, growth traits and metabolism: the fast-growing *Arundo donax*, which also is a strong isoprene emitter, and the slow-growing *Hakonechloa macra* that does not invest on isoprene biosynthesis. In control conditions, *A. donax* displayed not only higher photosynthesis but also higher concentration of carotenoids and lower phenylpropanoid content than *H. macra*. In drought-stressed plants, photosynthesis was similarly inhibited in both species, but substantially recovered only in *A. donax* after rewatering. Decline of photochemical and biochemical parameters, increased concentration of CO_2 inside leaves, and impairment of chloroplast ultrastructure were only observed in *H. macra* indicating damage of photosynthetic machinery under drought. It is suggested that volatile and non-volatile isoprenoids produced by *A. donax* efficiently preserve the chloroplasts from transient drought damage, while *H. macra* invests on phenylpropanoids that are less efficient in preserving photosynthesis but likely offer better antioxidant protection under prolonged stress.

Key-words: *Arundo donax*; *Hakonechloa macra*; abscisic acid; chloroplast ultrastructure; isoprene; leaf anatomy; xanthophylls.

INTRODUCTION

Drought severely constrains photosynthesis and consequently impairs plant growth and yield (Lawlor & Cornic 2002). The detrimental impact of drought on plant performances will likely increase in the next future, because the frequency and severity of drought stress events are rapidly increasing worldwide (IPCC 2013). The effect of drought stress on key physiological and metabolic processes may be particularly heavy for plant species that evolved in hygrophilous habitats (Tattini *et al.* 2015). Indeed, hygrophilous mesophytes do not possess mechanisms for efficient water conservation, characterizing species that evolved in drier habitats (Valladares *et al.* 2007), e.g. sclerophyllous plants (Cowling *et al.* 1996).

Plants with wide geographical distribution usually display a high-phenotypic plasticity when subjected to drought stress, which involves morpho-anatomical, physiological and biochemical changes (for review Ingram & Bartels 1996; Ludlow & Muchow 1990; Matesanz & Valladares 2014). The activation of secondary metabolism is of particular significance when drought stress becomes severe (Selmar & Kleinwächter 2013; Tattini *et al.* 2004, 2015; Brunetti *et al.* 2015). The main driver for enhanced synthesis of secondary metabolites under drought stress is likely photosynthesis inhibition and increased generation of reactive oxygen species (ROS) because of oversupply of reducing equivalents (Tattini *et al.* 2004; Selmar & Kleinwächter 2013). Secondary metabolites (volatile and non-volatile) are often efficient scavengers of excessive reducing power under stressful conditions (Agati *et al.* 2012; Harrison *et al.* 2013; Esteban *et al.* 2015). It has been suggested that, among volatile isoprenoids, isoprene regulates both reactive oxygen and nitrogen species formation (Behnke *et al.* 2010a, 2010b; Velikova *et al.* 2012; Vanzo *et al.* 2016), thereby indirectly providing a general antioxidant action (for review, see Vickers *et al.* 2009; Loreto & Schnitzler 2010). It is also shown that isoprene stabilizes thylakoid membrane structure and its function is closely associated with structural organization and functioning of plastidial membranes (Velikova *et al.* 2011, 2015; Pollastri *et al.* 2014). Furthermore, non-volatile isoprenoids, such as carotenoids, and phenylpropanoids play a recognized antioxidant function in plant response to different environmental constrains, including drought stress (reviewed in Brunetti *et al.* 2015). The concerted relevance of isoprenoids and

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phenylpropanoids in the network of antioxidant defences results from their specific sub-cellular locations (Brunetti *et al.* 2015) and from their temporally sequential activation during the day (Tattini *et al.* 2015). For example, preservation of thylakoid membranes from denaturation is mainly attributed to volatile and non-volatile isoprenoids (Velikova *et al.* 2011; Cazzonelli 2011; Esteban *et al.* 2015). However, phenylpropanoids, which largely accumulate in the vacuoles of epidermal and mesophyll cells, are preferential substrates for vacuolar peroxidases, thus reducing H_2O_2 that freely diffuses from the chloroplast to the vacuole under severe drought (Tattini *et al.* 2004; Agati *et al.* 2012; Brunetti *et al.* 2015).

In a previous research, we compared six species from the Arundinoideae subfamily, which are adapted to different environmental conditions, for their emission of isoprene (Ahrar *et al.* 2015). It was suggested that isoprene production in monocots, similarly to dicots, is associated with better photosynthetic performance (Ahrar *et al.* 2015). In this study, we focus on two species (*Arundo donax* and *Hakonechloa macra*) one of which (*A. donax*) is a strong isoprene emitter, while *H. macra* does not emit any isoprenoids at detectable level (Ahrar *et al.* 2015). *A. donax* is associated with riparian and wetland systems (Jain *et al.* 2015), whereas *H. macra* grows in moist, mountainous areas, on cliffs and hillsides (Darke 2007). Several studies clearly demonstrate the general positive effect of isoprene on plant resistance to different environmental stresses (reviewed in Vickers *et al.* 2009 and Loreto & Schnitzler 2010). We now expand our investigation to the effect of drought stress on the whole secondary metabolism of Arundinoideae and investigate the impact of isoprenoids and phenylropanoids in plant response to drought. 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*.

MATERIAL AND METHODS

Plant material and growth conditions

Rhizomes of *A. donax* were collected from single plants in a field close to Sesto Fiorentino, Florence, Italy. *H. macra* plants were purchased from Bowdens Nurseries (Norfolk, UK). All plants were propagated by rhizomes. Rhizomes were planted in 12 L and 2 L pots for *A. donax* and *H. macra*, respectively. Pots were filled with commercial soil mixture (peat mixture blond-black – 90–95%, perlite – 5–10%, calcium carbonate – 3–5 kg m^{-3}) with addition of 5–10% sand. Plants were grown in a climatic chamber under controlled environmental conditions (day/night temperature of 27/20 °C, relative air humidity of 60/70 ± 10%, photosynthetic photon flux density (PPFD) of 300 µmol m^{-2} s^{-1}). Plants were regularly watered until beginning of the drought experiment to keep the optimal water regime and to avoid anoxia or drought stress and were fertilized with half strength Hoagland solution every 2 weeks in order to supply mineral nutrients at free access rates.

Experimental design

Plants were 4–5 weeks old and 70–80 cm high in case of *A. donax*, and 7–8 weeks old and 15–20 cm high in case of *H. macra* when the experiments started. The first set of measurements was performed under optimal water conditions. The drought treatment was then initiated by stopping watering, and the water content of the pots was daily determined as the fraction of transpirable soil water (FTSW, %) as shown elsewhere (Brilli *et al.* 2007). The second set of measurements was performed when FTSW reached 30 ± 3%, that is, after 2–3 weeks in *A. donax* and 3–4 weeks in *H. macra*. The different time course at which the drought stress occurred reflects smaller leaf area and more closed stomata in *H. macra*. Then plants were irrigated again to pot capacity, and a third set of measurements was performed 3 weeks after rewatering, when FTSW was 90%. During drought stress, pots were closed in plastic bags to avoid water loss by evaporation from the soil. Three plants for each species were kept at optimal soil water content throughout the experimental period, and subjected to the first and third set of measurements, to assess possible age effects.

Gas exchange and isoprene measurements

Steady-state values of photosynthesis (A_n), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were measured by a LCpro + portable photosynthesis system (ADC BioScientific Ltd., Hoddesdon, Herts, UK). Measurements were performed on individual leaves enclosed into a 6.25 cm^2 broad leaf chamber. Leaves were exposed to a flow rate of 200 µmol s^{-1} of air containing natural concentration (0.4 mg g^{-1}) of CO_2. Measurements were performed under controlled relative humidity (55–60%), leaf temperature (30 ± 1 °C) and PPFD (500 µmol m^{-2} s^{-1}). The intrinsic water-use efficiency (WUE_i) was calculated as A_n/g_s ratio. Responses of A_n to C_i were generated by changing CO_2 concentration between 50 and 1400 µmol mol^{-1} after removing stomatal limitation (Centritto *et al.* 2003). These measurements were made at saturating PPFD (500 µmol photons m^{-2} s^{-1}, based on light-response curves conducted prior to measurements) and at a leaf temperature of 30 °C. The method of Sharkey *et al.* (2007) was used for fitting A_n/C_i responses and for calculating maximum carboxylation rate by Rubisco (V_{cmax}), photosynthetic electron transport rate based on NADPH requirement (J_{max}), triose phosphate utilization (TPU), day respiration (R_d) and mesophyll conductance (g_m).

Volatile isoprenoids were collected under the same conditions used for measuring other gas exchange parameters. Part of the air flow exiting the leaf cuvette was diverted into a silcosteel cartridge packed with 200 mg of Tenax (Markes International Limited, Llantrisant, UK). A volume of 2 L of air was passed through the trap at a rate of 200 mL min^{-1}. The cartridges were analysed by gas chromatography-mass spectrometry (GC-MS) with a Perkin Elmer Clarus 580 gas chromatograph coupled with a Clarus 560 Mass-Selective-Detector and a thermal desorber TurboMatrix (Perkin Elmer Inc., Waltham, MA, USA). A 30 m Elite-5 MS capillary column

was used to separate the desorbed compounds. Column temperature was first maintained at 40 °C for 5 min, then increased with a 5 °C min^{-1} ramp to 250 °C and maintained at 250 °C for 2 min. The NIST library was used for identification of the separated compounds. GC was calibrated using standard of isoprene at several dilutions from the initial concentration of 1000 ng g^{-1} (Rivoira, Milan, Italy). GC peak retention time was substantiated by analysis of parent ions and main fragments on the spectra. Isoprene concentration inside the leaf (I_i, nmol mol^{-1}) was calculated by the following equation $I_i = I_a + 1.94 \times I_e/g_{sH2O}$ according to Sharkey *et al.* (1996), where I_a is the isoprene concentration in the air outside the leaf; 1.94 is the square root of the ratio of molecular weights of isoprene to water; I_e (nmol m^{-2} s^{-1}) is the isoprene emission rate; g_{sH2O} (mol m^{-2} s^{-1}) is the stomatal conductance to water vapor. The ratio $(I_e/A_n) \times 100$ represents the carbon loss (%) as isoprene.

Chlorophyll fluorescence measurements

Modulated Chl fluorescence was measured by a FMS-1 fluorimeter (Hansatech Instruments, Norfolk, UK) in dark and light adapted leaves. The analysis was performed on the same leaf portions used for gas exchange measurements. Samples were dark adapted for 30 min and then a saturating pulse of 0.8 s with 6000 µmol m^{-2} s^{-1} PPFD was applied to calculate the maximum photosystem II (PSII) quantum yield [$F_v/F_m = (F_m - F_o)/F_m$]. Leaves were then adapted to actinic light, and a second saturating pulse was applied. This allowed us to determine the maximum fluorescence in light-adapted state (F_m') and the steady-state fluorescence (F_s) during the actinic illumination, and to calculate the quantum efficiency of PSII in illuminated leaves ($\Phi_{PSII} = (F'_m - F_s)/F'_m$) (Genty *et al.* 1989) and the non-photochemical quenching (NPQ = $(F_m - F_m')/F_m'$) (Bilger & Björkman 1991).

Analyses of abcisic acid, Phaseic and dihydrophaseic acid

Fresh leaf tissue (300–350 mg) was grinded in liquid nitrogen and added with 40 ng of deuterium-labeled internal standards (d_6-ABA, d_3-PA and d_3-DPA, all from the National Research Council of Canada). The extraction solvent was CH_3OH/H_2O (50/50) adjusted to pH 2.5 with formic acid. Samples were extracted with 3 × 3 mL of extraction solvent, and the supernatant was decolorized by normal hexane extraction twice. The aqueous-methanolic phase was purified through Sep-Pak C18 cartridges (Waters, Massachusetts, USA) eluting with ethylacetate. The eluate was reduced to dryness and rinsed with 250 µL of extraction solvent. Finally 3 µL of sample solution were injected into the LC–ESI–MS/MS system consisting of an UPLC (Nexera UPLC Shimadzu Corporation) coupled with a MS/MS detector (TQ 8030) equipped with an ESI source (all from Shimadzu Corporation, Kyoto, Japan) operating in negative ion mode. Compounds were separated using a Poroshell C18 column (3.0 × 100 mm, 2.7 µm i.d., Agilent, USA). Gradient elution was performed with water acidified with 0.1% formic acid (solvent A) and acetonitrile/methanol (1/1) added with 0.1% of formic acid (solvent B) at a constant flow-rate of 300 µL min^{-1} ranging from 95% solvent A to 100% solvent B during a 30-min run. Quantification was conducted in multiple reaction mode (MRM) as reported in López-Carbonell *et al.* (2009).

Analysis of carotenoids and phenylpropanoids

Individual carotenoids were quantified using the protocol reported in Tattini *et al.* (2015). Briefly, fresh leaf tissue (300 mg) was extracted twice with 5 mL of acetone (added with 0.5 g L^{-1} of $CaCO_3$). 15 µL of the extracted solution were injected in a Perkin Elmer Flexar liquid chromatograph equipped with a quaternary 200Q/410 pump and a LC 200 diode array detector (DAD) (all from Perkin Elmer, Bradford, CT, USA). Photosynthetic pigments were separated in a Agilent Zorbax SB-18 (250 × 4.6 mm, 5 µm) termostated at 30 °C using a 18-min run and a linear gradient solvent system from 100% of solvent A (methanol/water 95/5) to 100% solvent B (methanol/ethylacetate 6.8/3.2) with a flow rate of 0.8 mL min^{-1}. Xanthophyll cycle pigments (violaxanthin, antheraxanthin and zeaxanthin, collectively named VAZ), neoxanthin, lutein, β-carotene and chlorophylls were identified using visible spectral characteristics and retention times. Individual carotenoids and chlorophylls were calibrated using authentic standards from Extrasynthese (Lyon-Nord, Genay, France) and from Sigma Aldrich (Milan, Italy).

Individual phenylpropanoids (mostly hydroxycinnamic acid, luteolin and apigenin derivatives) were identified and quantified using the protocol reported in Tattini *et al.* (2015). Fresh leaf material (300 mg) was extracted twice with 5 mL of ethanol/water (75/25) adjusted at pH 2.5 with formic acid and the supernatant partitioned with 3 × 5 mL of *n*-hexane. The ethanol fraction was reduced to dryness, and the residue was rinsed with 1 mL of methanol/water (90/10). Aliquots of 10 µL were injected into the Perkin Elmer liquid chromatography unit reported earlier. Phenylpropanoids were separated using a Agilent Zorbax SB-18 (250 × 4.6 mm, 5 µm), operating at 30 °C with a flow rate of 1 mL min^{-1} and eluted with a linear gradient solvent system from 100% solvent A (water adjusted to pH 2.5 with HCOOH/acetonitrile (90/10)) to 100% solvent B (acetonitrile/water adjusted to pH 2.5 with HCOOH (90/10)) over a 45-min run. Metabolites were identified using retention times and UV spectral characteristics of authentic standards (Extrasynthese, Lyon-Nord, Genay, France). Individual phenilpropanoids were calibrated using authentic standards from Extrasynthese.

Light microscopy

Leaf segments (4–5 mm^2) from the middle part of fully expanded leaves, taken from the 4th nodes, were fixed in 3% (m/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 12 h at 4 °C and used in microscopy studies. Hand-made transversal sections (at least 30 per species) were mounted on slides in glycerol. Observations were carried out and micrographs were taken using a light microscope and a camera (*Nikon Eclipse 50i*, Tokyo, Japan).

Transmission electron microscopy

Leaf segments (1–2 mm^2) were fixed in 3% (m/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and post-fixed in 1% (m/v) $KMnO_4$ in the same buffer for 2 h at room temperature. After dehydration by increasing concentrations of ethyl alcohol (from 25 to 100%), the samples were embedded in Durcupan (*Fluka*, Buchs, Switzerland) and cross-sectioned with a *Reichert-Jung* (Wien, Austria) ultramicrotome. Observations were performed with an electron microscope (JEOL 1200 EX, Tokyo, Japan). At least 20 photos for each species were analysed.

Leaf relative water content determination

Leaf sections chosen from at least five different plants were cut for RWC determination. The fresh weight of the leaf discs was immediately determined, followed by floatation on water for 24 h. The turgid weight was then recorded and the leaf tissue was subsequently oven dried at 80 °C for 48 h. RWC was calculated using the formula: RWC = (fresh weight – dry weight) / (turgid weight – dry weight).

Statistical analyses

All data represent the means ± SE from two independent experiments with at least three biological replicates of each plant species and treatments (control, drought-stress and rewatering). ANOVA followed by Tukey's test was used to separate plant species and treatments and significant differences at $P < 0.05$ are indicated by different letters.

RESULTS

Water relations, photosynthetic gas exchange and photosystem II performance

Leaf relative water content was similar in both plant species under control conditions (Table 1). Under drought stress, *A. donax* leaves lost more water than *H. macra* leaves. *A. donax* leaves completely recovered their turgor after rewatering, whereas leaves of *H. macra* did not recover their water status (RWC = 70.5%).

Photosynthesis (A_n) was different in the investigated species. *A. donax* leaves are characterized by significantly higher A_n than *H. macra* (Fig. 1a). Drought stress caused a stronger inhibition of A_n in *A. donax*, but after rewatering, A_n recovered to 73% of pre-stress level. Photosynthesis of *H. macra* was less affected by drought but did not recover after re-hydration. The WUE_i was significantly higher in drought-stressed *A. donax* plants compared with controls and rewatered plants. WUE_i was similar in *H. macra*, and *A. donax* leaves under control conditions. However, WUE_i in *H. macra* was significantly depressed under drought stress, whereas it reached pre-stress values after rewatering (Fig. 1b).

In control plants, stomatal conductance (g_s) was lower in *H. macra* than in *A. donax*. A significant decrease of g_s was

Table 1. Changes in leaf RWC (%), and photosynthetic parameters estimated from CO_2-response curves based on C_i (A_n/C_i) in control, drought stressed and recovered plants of *Arundo donax* and *Hakonechloa macra*

	A. donax			*H. macra*		
	Control	Drought	Recovery	Control	Drought	Recovery
RWC	95.0 ± 0.8 a	53.1 ± 2.6 c	97.1 ± 0.4 a	92.1 ± 1.2 a	62.6 ± 2.5 b	70.5 ± 2.5 b
V_{cmax}	177 ± 15 a	93 ± 8 c	151 ± 16 a	110 ± 7 b	47 ± 9 d	69 ± 9 c
J_{max}	155 ± 19 a	62 ± 5 c	146 ± 14 a	116 ± 7 b	52 ± 6 c	54 ± 6 c
TPU	11.2 ± 2.1 a	4.3 ± 0.1 b	11.0 ± 1.1 a	8.6 ± 0.6 a	4.6 ± 0.5 b	4.0 ± 0.5 b
A_{max}	33.6 ± 3.9 a	7.5 ± 2.4 c	30.3 ± 0.3 a	24.2 ± 1.4 b	10.1 ± 0.6 c	11.1 ± 1.1 c
V_{cmax}/g_s	0.24 ± 0.02 c	3.17 ± 0.66 a	0.47 ± 0.14 b	0.32 ± 0.02 c	0.27 ± 0.07 c	0.45 ± 0.08 b
$C_i - C_c$	52 ± 6 b	22 ± 3 c	48 ± 5 b	81 ± 8 a	73 ± 15 a,b	99 ± 18 a

Measured light intensity was 500 μmol m^{-2} s^{-1} PPFD and leaf temperature of 30 °C. Means are average of six replicates ± SE. Means were separated by Tukey's test and different letters indicate means statistically different with $P < 0.05$. V_{cmax} – maximum carboxylation rate (μmol m^{-2} s^{-1}); J_{max} – electron transport rate at saturating light and CO_2 (μmol m^{-2} s^{-1}); TPU – triose-phosphate use (μmol m^{-2} s^{-1}); A_{max} – net photosynthetic rate at saturating CO_2 concentration (μmol m^{-2} s^{-1}); V_{cmax}/g_s ratio (mmol mol^{-1}); C_i–C_c (μmol mol^{-1}). RWC, relative water content; TPU, triose phosphate utilisation.

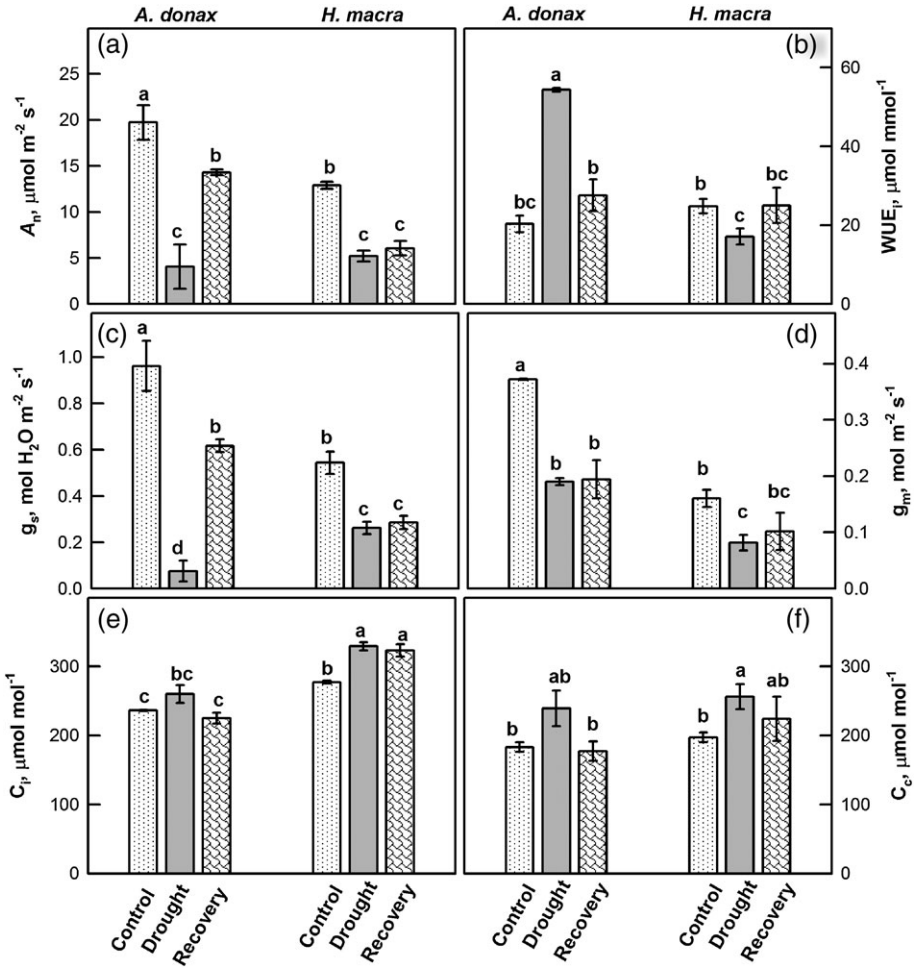

Figure 1. Photosynthesis (A_n) (a), intrinsic water-use efficiency (WUE_i) (b), stomatal conductance (g_s) (c), mesophyll conductance (g_m) (d), and internal (C_i) (e) and chloroplast (C_c) (f) concentration of CO_2 in the control, drought stressed and recovered plants of *Arundo donax* and *Hakonechloa macra*. Means ± SE ($n = 6$) were separated by Tukey's test and different letters indicate statistically different means with $P < 0.05$.

observed in both species after the drought treatment, but g_s dropped more severely in *A. donax* and only partly recovered after rewatering in both species (Fig. 1c). The mesophyll conductance (g_m) was also inherently lower in *H. macra* than in *A. donax*. The g_m was largely reduced in both *A. donax* and *H. macra* plants experiencing drought with respect to controls and did not recover after rewatering in either species (Fig. 1d). Considerable reductions of g_s and g_m in both drought-stressed species were accompanied by a significant increase in C_i and chloroplastic CO_2 concentration (C_c) only in *H. macra* plants, whereas C_i and C_c were unaffected in *A. donax* during the experimental period (Fig. 1e,f).

The initial slopes of A_n/C_i responses were different in control plants of the two species, indicating different Rubisco activities (Fig. 2). The CO_2-saturated rate of photosynthesis was also different between the two species. The higher A_n of *A. donax* compared with *H. macra* indicates higher Rubisco regeneration capacity sustained by higher electron transport rate. Estimates of V_{cmax} and J_{max} confirmed these species-specific differences being significantly higher in *A. donax* than in *H. macra* under optimal growth conditions, whereas TPU was similar in both species (Table 1). Drought stress reduced both the slope and the plateau of CO_2 response curves in leaves of the two plant species (Fig. 2) indicating adverse effects of the stress on both carboxilation efficiency and Rubisco regeneration rate. After rewatering, V_{cmax}, J_{max} and TPU were restored to control values in *A. donax*, while these parameters did not recover in *H. macra* (Fig. 2; Table 1).

The maximum yield of PSII photochemistry (F_v/F_m) did not change with drought in *A. donax*, whereas it significantly decreased in *H. macra* (Fig. 3a). The effective quantum yield of PSII (Φ_{PSII}) and the non-photochemical quenching of fluorescence (NPQ) were different in *A. donax* and *H. macra* under control conditions, being Φ_{PSII} higher in *A. donax* and NPQ higher in *H. macra* (Fig. 3b, c). A statistically significant reduction of Φ_{PSII} was caused by drought stress only in *H. macra* where Φ_{PSII} reached 56% of the control value did not recover after rewatering and was accompanied by significant increases of NPQ (Fig. 3b, c). The impact of drought was more attenuated on the photochemical parameters of *A. donax*.

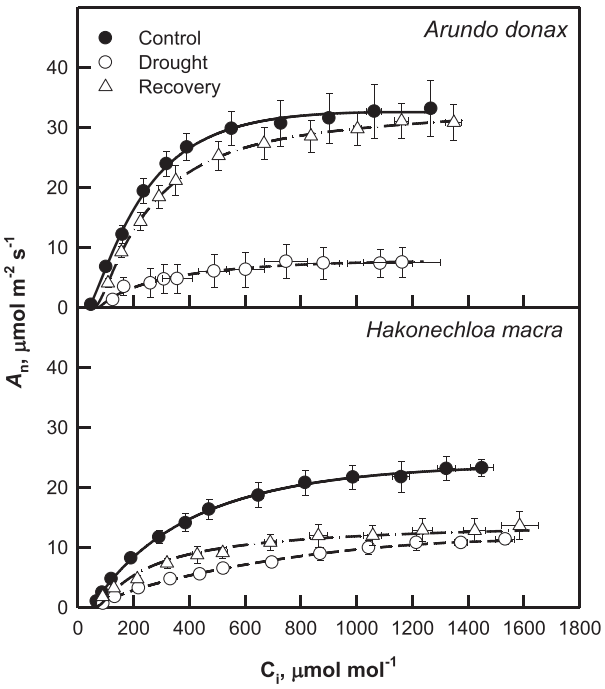

Figure 2. Photosynthesis (A_n) at different internal [CO_2] for *Arundo donax* (upper panel) and *Hakonechloa macra* (lower panel). Measured light intensity was 500 µmol m^{-2} s^{-1} PPFD. Closed circles – control, triangle – drought, open circle – recovery. Curves are averaged from six replicates at each time point of measurements obtained from six different plants of each species.

Isoprenoids

Xanthophyll pool

Changes in the pool of xanthophylls of the two plant species during the treatments are shown in Fig. 4a,b,c. Violaxanthin content decreased only in drought-stressed *H. macra* plants. Antheraxanthin levels increased in both drought-stressed species, although the differences were not statistically significant in *H. macra*. Finally, zeaxanthin was markedly higher in drought-stressed plants of both *A. donax* and *H. macra* but remained higher after rewatering only in *H. macra* leaves. The de-epoxidation status (DES) of xanthophylls and the ratio between xantophylls and chlorophylls (VAZ/Chl_{tot}) were significantly different in the two species already under control conditions, being DES lower and VAZ/Chl_{tot} higher in *A. donax* than in *H. macra* (Fig. 4d,e). DES increased significantly in both species under drought (Fig. 4d), whereas no significant changes in VAZ/Chl_{tot} were found in drought-stressed *H. macra* plants (Fig. 4e). After recovery, DES remained higher in *H. macra*, while in *A. donax*, it decreased to pre-stress level, mirroring zeaxanthin. The concentration of total carotenoids was significantly higher in *A. donax* compared with *H. macra*, and was not significantly affected by the treatment (Fig. 4f).

Isoprene emission

Isoprene emission was high in *A. donax* (Table 2) and undetectable in *H. macra* (data not shown, see also Ahrar *et al.* 2015).

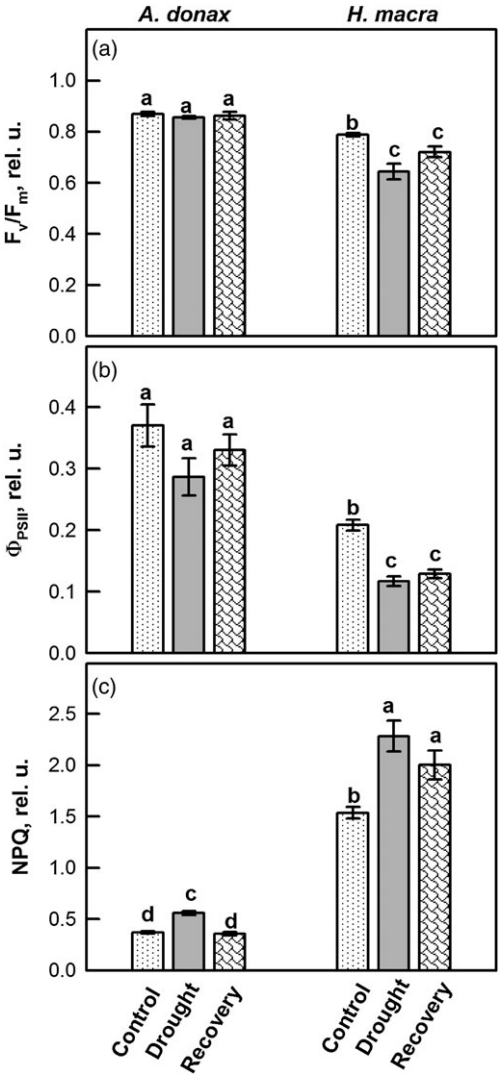

Figure 3. Maximum quantum yield of PSII (F_v/F_m) (a), quantum efficiency of PSII photochemistry (Φ_{PSII}) (b) and non-photochemical dissipation of absorbed light energy (NPQ) (c) in the control, drought stressed and recovered plants of *Arundo donax* and *Hakonechloa macra*. Means ± SE ($n = 6$) were separated by Tukey's test and different letters indicate statistically different means with $P < 0.05$.

Drought inhibited isoprene emission in *A. donax* leaves, and the emission was further depressed in plants recovering from the stress (Table 2). The concentration of isoprene inside leaves was 10 times higher in drought-stressed than in control plants of *A. donax*. The carbon lost as isoprene was also around eight times higher under drought than in control conditions (Table 2).

Abcisic acid and catabolites

The levels of free ABA under control conditions were different in the two species, being lower in *A. donax* compared with *H. macra* (Fig. 5a). Free ABA increased dramatically in both species in response to drought. These changes were associated with significant reductions of g_s (Fig. 5a insert). After rewatering, the ABA concentration decreased reaching pre-stress levels.

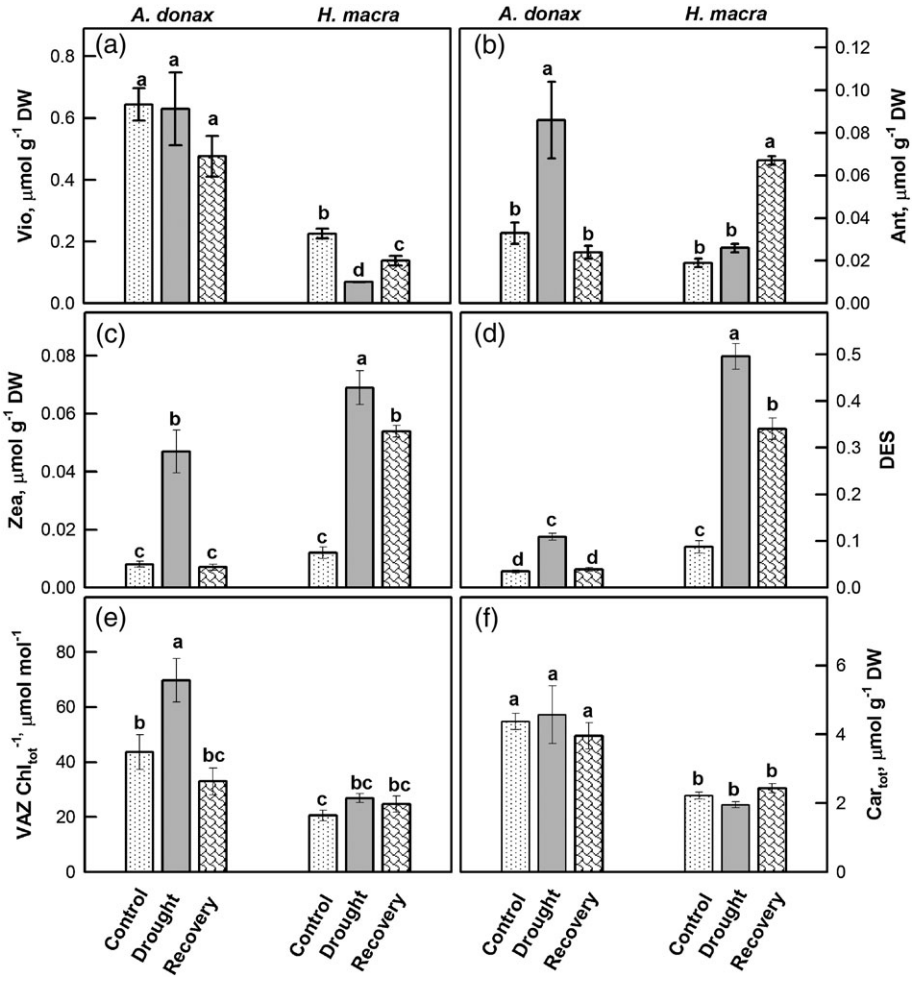

Figure 4. Violaxanthin (Vio) (a), anthneraxanthin (Ant) (b), zeaxanthin (Zea) (c) and de-epoxidation state (DES) (d), xanthophyll to chlorophyll ratio (VAZ Chl^{-1}) (e) and total concentration of carotenoids (Car_{tot}) (f) in the control, drought stressed and recovered plants of *Arundo donax* and *Hakonechloa macra*. Means ± SE ($n = 3$–6) were separated by Tukey's test and different letters indicate statistically different means with $P < 0.05$.

Phaseic and dihydrophaseic acid (PA + DPA) were significantly higher in *A. donax* than in *H. macra* under control conditions (Fig. 5b). In drought-stressed leaves, the concentration of PA + DPA increased by a factor of 2.5 in *A. donax*, whereas no significant changes were observed in *H. macra*. After rewatering, PA + DPA decreased to control levels in *A. donax* leaves, while increased further in *H. macra*.

Table 2. Effect of drought stress and consequent rewatering on isoprene emission rate (I_e, nmol m^{-2} s^{-1}), isoprene concentration inside the leaf (I_i, nmol mol^{-1}) and carbon lost as isoprene (C_{lost}, %) in *Arundo donax* leaves

	Control	Drought	Recovery
I_e	9.90 ± 1.77	6.19 ± 0.77	2.96 ± 0.43*
I_i	30.26 ± 2.09	371.51 ± 33.05*	12.78 ± 1.21*
C_{lost}	0.25 ± 0.02	1.95 ± 0.61*	0.12 ± 0.01*

Measurements were performed at 30 °C/500 μmol m^{-2} s^{-1} PPFD. Means ± SE ($n = 6$) are shown. Significant differences ($P < 0.05$) between control and treated plants are indicated by asterisk.

Phenylpropanoid compounds

Constitutive leaf contents of caffeic and ferulic acids (HCA) were significantly higher in *H. macra* than in *A. donax* (Fig. 6a). Under drought stress, HCA increased dramatically in *H. macra*, whereas no significant changes were found in *A. donax*. After rewatering, the levels of HCA returned similar to controls in the two species. The levels of flavonoids were similar in both species under control conditions (Fig. 6b,c). Drought stress caused a significant rise of flavonoid content (luteolin and apigenin) in both species, especially in *H. macra*. After rewatering, the level of luteolin remained high in both *A. donax* and *H. macra* compared with the corresponding controls, whereas apigenin dropped to pre-stress levels.

Leaf anatomy and chloroplast ultrastructure

As previously reported, control plants of the two studied species have clear differences of leaf anatomy and chloroplast structure (Ahrar *et al.* 2015). The main difference found in leaf anatomy is a higher number of bulliform cells in *A. donax* than

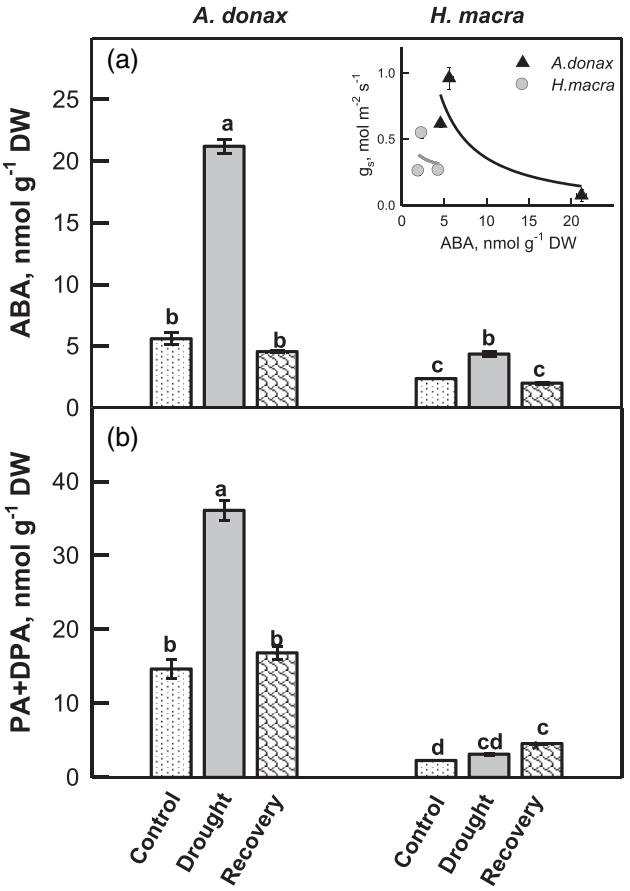

Figure 5. Levels of abcisic acid (ABA) (a) and sum of phaseic and dihydrophaseic acids (PA-DPA) (b) in the control, drought-stressed and recovered plants of *Arundo donax* and *Hakonechloa macra*. (insert) correlation between ABA content and stomatal conductance in *A. donax* ($r^2 = 0.659$) and *Hakonechloa macra* ($r^2 = 0.046$), symbols represent average value of 3 biological replicates. Means ± SE ($n = 3$) were separated by Tukey's test and different letters indicate statistically different means with $P < 0.05$.

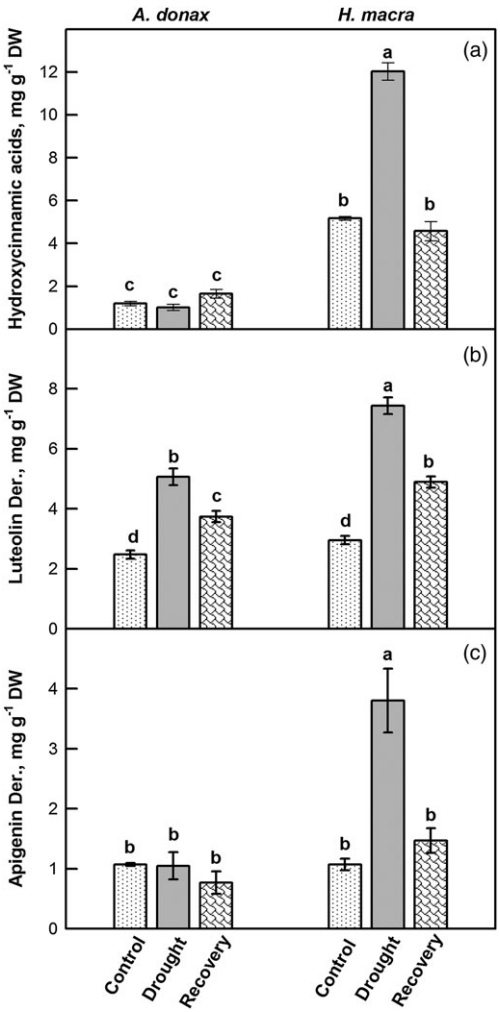

Figure 6. Hydroxycinamic acid (a), luteolin and (b), apigenin (c) derivatives in the control, drought stressed and recovered plants of *A. donax* and *H. macra*. Means ± SE ($n = 3$) were separated by Tukey's test and different letters indicate statistically different means with $P < 0.05$.

in *H. macra* (Fig. 7). Our further analyses in the present study showed no changes in leaf anatomy because of drought in both species (data not shown).

As also reported previously (Ahrar *et al.* 2015), *A. donax* chloroplasts are highly elongated with a well-structured inner membrane system (Fig. 8a), whereas in *H. macra* chloroplasts are more round and the membrane system consists of a relatively smaller number of grana thylakoids uniformly occupying the entire stroma space (Fig. 8d). Drought stress caused similar changes in the inner chloroplast membrane system of both species (Fig. 8b,e). The mesophyll chloroplasts of *A. donax* became more rounded (Fig. 8b). The internal membrane system consisted of grana that were still well-structured (from 10 to 30–35 thylakoids) but with various spatial orientation and fragmented stromal thylakoids. Fusion or total destruction in some of the granal and stromal thylakoids were observed in few occasions in drought-stressed *A. donax*, whereas these changes were more massive in *H. macra* chloroplasts (Fig. 8f). After rehydration, the chloroplasts of *A. donax* did not differ from the control ones both in shape and in the orientation of the internal membrane system (Fig. 8c). They acquired again an elongated shape with well-structured thylakoid membranes arranged along the longitudinal axis. In contrast, only a partial reconstruction of the membrane system was observed in rewatered *H. macra* chloroplasts (Fig. 8f). In particular, some grana with completely fused thylakoids were observed, whereas the stromal thylakoids were better reconstructed.

DISCUSSION

Our study provided functional and metabolic evidence 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 g_m and g_s, 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

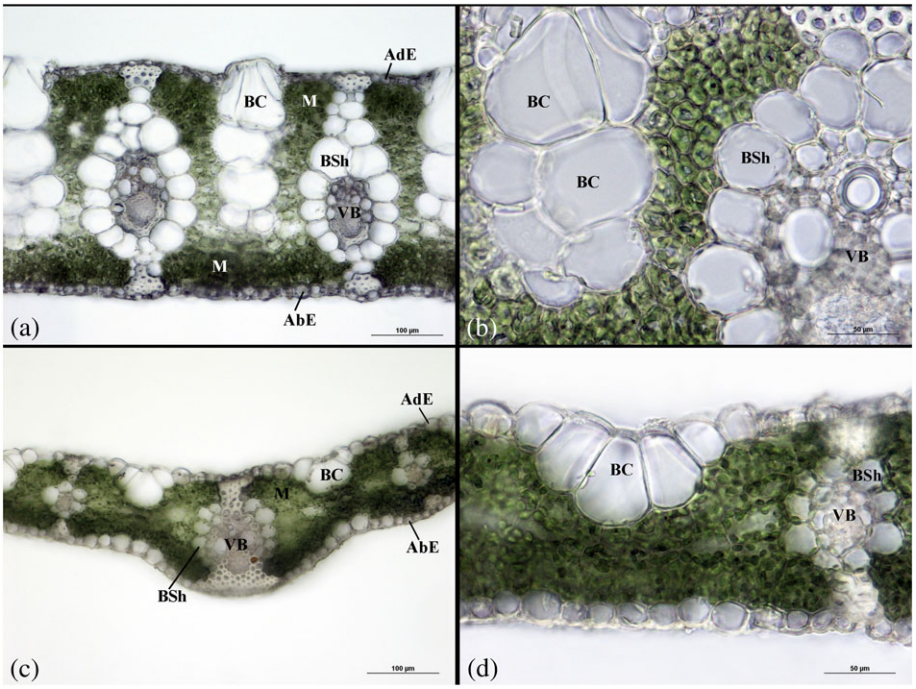

Figure 7. Light microscopy images of leaf cross section of *A. donax* (a, b) and *H. macra* control plants (c, d). AdE, adaxial epidermis; AbE, abaxial epidermis; BC, bulliform cells; M, mesophyll; VB, vascular bundle; BSh, bundle sheat.

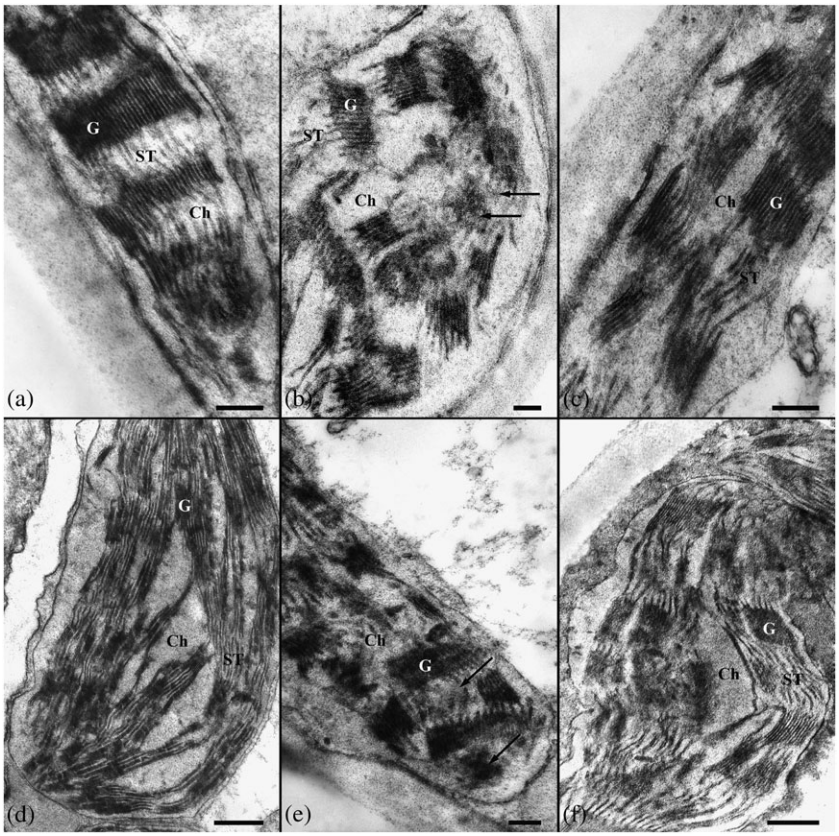

Figure 8. Electron micrographs of mesophyll chloroplasts in *Arundo donax* and *Hakonechloa macra* leaves. Chloroplasts in the leaves of the control plants of *A. donax* (a) and *H. macra* (d). Micrographs of chloroplasts illustrating thylakoid destruction (arrows) of the grana and the stroma thylakoids in drought stressed plants of *A. donax* (b) and *H. macra* (e). Chloroplasts in the leaves of recovered plants of *A. donax* (c) and *H. macra* (f). Bars = 200 nm. Ch, chloroplast; G, grana; ST, stroma thylakoid.

resilient after drought stress, achieving soon photosynthetic performances and structural features similar to unstressed leaves.

Functional and structural leaf traits determining Arundo donax and Hakonechloa macra responses to drought

Drought induced a very large reduction of g_s and g_m in *A. donax*. Stomatal closure was particularly heavy, allowing a dramatic reduction of transpiration and a very efficient use of water under drought stress conditions. Diffusive resistances generally also limit CO_2 entry and A_n under stress conditions (Evans & Loreto 2000; Medrano *et al.* 2002, 2009; Flexas & Medrano 2002; Flexas *et al.* 2008, 2016). However, this was not observed in *H. macra*, as the C_i and the C_c increased under stress, indicating availability of substrate for photosynthesis. Thus, A_n of *H. macra* was limited by biochemical and/or photochemical constraints under drought stress. Analysis of photosynthesis response to CO_2 (A_n/C_i) revealed a reduction of both Rubisco activity and RuBP regeneration rate. Rubisco activity inhibition (estimated by the slope of A_n/C_i response) might actually be lower than the estimate provided by A_n/C_i, because reduction of g_m (i.e. $C_c < C_i$) can make steeper the response of photosynthesis to CO_2 (Loreto *et al.* 1994). However, the limited drawdown between C_i and C_c under drought conditions indicates that this effect might be residual. Drought caused a decline in V_{cmax} in both species, but it was stronger in *H. macra* compared with *A. donax* suggesting again a predominant role of biochemical limitations in *H. macra*. V_{cmax} and J_{max} also drive the intrinsic WUE (for review, see Flexas *et al.* 2016). In our study WUE_i significantly increased only in A. *donax* when exposed to drought suggesting that *A. donax* leaves possess traits that allow better adaptation to drought than in *H. macra*. Decreased WUE_i in drought-stressed, *H. macra* could be due to metabolic impairment of the photosynthetic machinery. This is in accordance with the quantitative limitation analyses of photosynthesis performed on both species. Chlorophyll fluorescence parameters also indicate that the photochemistry of photosynthesis was more impaired in *H. macra* compared with *A. donax*. Overall, it seems that *A. donax* did not resist better than *H. macra* to drought stress, but that a larger plasticity of the photochemical apparatus allowed *A. donax* to swiftly and completely recover photosynthetic performances after the stress.

Our analyses reveal differences in the leaf structural characteristics of both species, which may play an important role in plant response to drought (Terashima *et al.* 2011; Peguero-Pina *et al.* 2012; Tomàs *et al.* 2013). *A. donax* leaves are characterized by larger number of bulliform cells than in *H. macra*. These cells are arranged in columns, which, in *A. donax* leaves, penetrate the mesophyll deeper than the middle half of the leaf lamina. The physiological significance of bulliform cells is still under debate. They may play a role in the unfolding of developing leaves and in the rolling and unrolling of mature leaves in response to drought and after adequate water is available (Moore *et al.* 1998). Bulliform cells are mainly water containing cells with little or no chlorophyll (Evert 2006). Fleurat-Lessard *et al.* (1997) indicated that these cells facilitate water fluxes across the vacuolar membrane and energization of the vacuole. Moreover, γ-TIP aquaporin and H^+-translocating ATPase (V-ATPase) are almost exclusively detected on aqueous vacuole found in mature bulliform cells (Fleurat-Lessard *et al.* 1997; Marty 1999; Sze *et al.* 1999).

Drought caused destructive changes of the chloroplast membrane compartment in both species, but these alterations were stronger in *H. macra* than in *A. donax*. After rewatering, mesophyll chloroplasts of *A. donax* were not different from control plants. It was recently demonstrated that isoprene plays an important role in thylakoid membrane organization through modification of the lipid environment and organization of the pigment-protein complexes in thylakoid membranes (Velikova *et al.* 2011, 2014, 2015). Moreover, we have estimated internal isoprene concentration 10 times higher in drought-stressed than in control *A. donax* leaves (Table 2). It is therefore speculated that maintenance of chloroplast integrity in drought-stressed *A. donax* be due to isoprene presence. The stronger negative effect of drought on chloroplast ultrastructure of the species that does not emit isoprene, *H. macra*, may also suggest a role for isoprene in keeping stable membranes under drought.

Involvement of isoprenoids in drought response of Arundo donax and Hakonechloa macra

Volatile and non-volatile isoprenoids in plants are synthesized via the plastidial methyl erythrol phosphate (MEP) pathway (Lichtenthaler 1999). Non-volatile isoprenoids, namely carotenoids (Lichtenthaler 1999) have a well-established biological role in stress tolerance (Demmig-Adams & Adams 1996). In our study, *A. donax* and *H. macra* displayed large differences in the concentration of non-volatile products. The concentration of carotenoids of *A. donax* leaves, expressed on a tissue mass basis, was 91.7% higher than in *H. macra*, irrespective of the water status of the plants. While the pool of xanthophyll cycle pigments was significantly higher in *A. donax* than in *H. macra* (expressed on both tissue mass and chlorophyll basis), the relative de-epoxidation of xanthophylls was dramatically higher in *H. macra*. This is consistent with the observation that mechanisms aimed at dissipating excess radiant energy operated much more in *H. macra* than in *A. donax*. Drought-induced enhancement in DES and NPQ has been widely reported, as a result of severe limitation to the use of radiant energy by photosynthesis (Lawlor & Tezara 2009; Fini *et al.* 2012). This occurred in either species, but at much greater degree in *H. macra*. Recovery in photosynthetic performance after rewatering in *A. donax* resulted in NPQ and DES values similar to pre-stress conditions. In contrast, in *H. macra* xanthophylls sustained dissipation of excess light in the chloroplast also after rewatering, as this species was unable to recover appreciable photosynthesis upon relief from drought stress.

Abcisic acid, which is also produced by the MEP pathway downstream of carotenoids, triggers stomatal closure (e.g. Wilkinson & Davies 2002). Regulation of g_s under severe

drought stress was likely caused by the large increase of ABA, particularly in *A. donax*. Indeed, significantly higher levels of free-ABA and its degradation products correlated with stronger reduction of g_s in *A. donax* than in *H. macra* (Seiler *et al.* 2011). However, we cannot rule out the hypothesis that large decreases in RWC may have contributed to regulation of stomatal closure, especially in *A. donax* (Christmann *et al.* 2005).

Up-regulation of the MEP pathway under drought stress may have a series of important consequences that are related to the observed tolerance to/resilience from stress of *A. donax*. In fact, isoprene and xanthophylls act as powerful antioxidants (Loreto & Velikova 2001; Vickers *et al.* 2009; Behnke *et al.* 2010a; Loreto & Schnitzler 2010) reducing the negative impact of ROS accumulation unavoidably occurring when photosynthesis is constrained while photochemical excitation pressure (the photons collected by photosystems) remains high. It is interesting to note that MEP stimulation leading to xanthophylls and ABA biosynthesis only largely occurred in the isoprene emitting species. Barta & Loreto (2006) showed that isoprene may proxy ABA biosynthesis, in turn allowing stomatal closure, as previously observed. Dani *et al.* (2016) speculated that isoprene and citokinins follow similar trends and that leaves enter senescence when the foliar content of the two metabolites drop. On the other hand, Ghirardo *et al.* (2014) demonstrated that more non-volatile isoprenoids are produced when isoprene synthesis is down-regulated or suppressed. Our results suggest that (1) isoprene emission occurs in species that have a large flux of carbon in the MEP pathway, and a better capacity to activate the overall pathway in response to stress and (2) species that do not emit isoprene and produce low MEP pathway metabolites, produce higher levels of de-epoxidized xanthophylls, which likely defend plants when photosynthesis is inhibited by the stress, but further decrease the substrate available for ABA synthesis (Lichtenthaler 2007).

Role of phenylpropanoids in drought response of Arundo doanx and Hakonechloa macra

Drought, as many other stresses, also promotes the biosynthesis of phenylpropanoids (Olsen *et al.* 2009; Tattini *et al.* 2004; Agati *et al.* 2012), likely as a consequence of the impairment of photosynthetic electron transport chain (Akhtar *et al.* 2010) or ROS homeostasis (Babu *et al.* 2003; Pollastri & Tattini 2011; Agati *et al.* 2012). Indeed, biosynthesis of phenolic compounds (HCA, luteolin and apigenin derivatives) was particularly high in drought-stressed *H. macra*, confirming that drought induced a more severe photochemical stress in *H. macra* than in *A. donax*, activating protection against ROS especially because of drought stress (Agati *et al.* 2012). The very high phenylpropanoid concentration detected in *H. macra* leaves, approximately $93\,\mu mol\,g^{-1}$ DW under drought, suggests that a large portion of these metabolites accumulate in the mesophyll rather than in the epidermal cells (Agati *et al.* 2009, 2012).

Indeed, isoprenoids and phenylpropanoids are synthesized and distributed in different foliar compartments, and protect leaves from stresses in different parts of the day, being isoprenoids more active in the morning and phenylpropanoids in the afternoon (Tattini *et al.* 2015). Supporting these functional synergies, there is evidence that isoprenoids and phenylpropanoids biosynthesis are tightly co-regulated, as plants with repressed isoprene biosynthesis produce less phenylpropanoids (Behnke *et al.* 2010b) and plant where isoprene synthesis is induced also produce more phenylpropanoids (Tattini *et al.* 2014; Harvey & Sharkey 2016). However, our experiment suggests that, under drought stresxs conditions, plants investing on isoprenoids activate a protection that allows better and faster resilience from transient stress, as also indicated by Loreto & Fineschi (2015) for isoprene emitters. Whereas, plants investing on phenylpropanoids are not able to avoid damage to the photosynthetic apparatus but are able to cope with prolonged exposure to oxidative species. Being our experiment limited to contrasting Arundinoideae species, this idea needs to be corroborated by additional experimentation on different plants.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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3.2. The role of isoprene in two Arundineae species exposed to progressive drought

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THE ROLE OF ISOPRENE IN TWO ARUNDINEAE SPECIES EXPOSED TO PROGRESSIVE DROUGHT

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Abstract

Isoprene is the most abundant biogenic volatile organic compound emitted from vegetation and has been suggested to have protective role in plants under different kinds of stress. In this study we compared the responses to drought stress of two Arundineae species which differ in their ability to emit isoprene- *Arundo donax*, a promising biofuel crop and isoprene emitter, and *Hakonechloa macra*, a non-emitting ornamental plant. Our results showed better recovery of photosynthesis in *A. donax* after rehydration in comparison to *H. macra*. *H. macra* had lower photosystem II (PSII) photochemical efficiency, increased non-photochemical quenching (NPQ) and high levels of leaf malondialdehyde (MDA) and proline content when exposed to drought stress. The isoprene emitting species *A. donax* showed enhanced drought tolerance and better recovery after rewatering than the non-emitting *H. macra*. Our results confirm the idea that in isoprene emitting species, the function of isoprene is closely associated with functioning of the photosynthetic apparatus.

Key words: isoprene, drought, *Arundo donax, Hakonechloa macra*

Introduction. Isoprene is the simplest plant isoprenoid. It is emitted from many plant species of different genera. As a result of the high volatility of isoprene once it is synthesized *in planta*, it is not stored but it is emitted through the stomata into the atmosphere. Biogenic isoprene emission represents 0.5-2% of carbon fixed by photosynthesis [1]. The question why plants invest in this non-trivial carbon loss is still under debate.

Although many authors have investigated the biological function of isoprene in plants, it still remains elusive. Evidence has been provided that isoprene can protect photosynthesis against damages caused by transient high-temperature stress [2, 3, 4] and oxidative stress [3] including drought [5]. Since isoprene is highly lipophilic, it may aggregate into membranes and can prevent the formation of water channels responsible for the membrane leakiness at high temperatures [2]. The protective effect of isoprene can also be related to the double bounds in the isoprene molecule that has been demonstrated in the experiment of [2]. A possible function of isoprene as an antioxidant which could quench reactive oxygen species (ROS) generated under oxidative stress is also hypothesized [3].

In terms of the constantly changing climate conditions and decreasing water availability on the Earth, the emission of isoprene may have an important role for plant sustainability and adaptation to the changing environment. Isoprene may serve as a short-term thermoprotective agent under drought stress, for example, in *Quercus spp.* [6].

The taxonomic distribution of isoprene emission is broad [7]. There are many studies on isoprene emission from dicots but not enough information about monocots is available. The grass family Poaceae (around 10000 species) has been poorly sampled.

In the present study we focused on two species from the Poaceae family, Arundinoideae subfamily – *A. donax* and *H. macra* which differ in their ability to emit isoprene [8]. *A. donax* (giant reed) is an isoprene emitting [8] fast-growing perennial crop that has received particular attention during the last decade as bioenergy crop [9]. *H. macra* is an ornamental, shade tolerant landscape grass that grows slowly [10]. Greater emphasis on the use of ornamental grasses in the landscape has significantly increased the interest in these perennials but there is a lack of scientific investigation into the physiology of ornamental grasses. In our recent research we have conducted a screening under control conditions covering six species from the Arundineae tribe. Our study showed that *H. macra* does not emit isoprene or other volatile isoprenoids at a detectable level [8]. We also found that isoprene emitting *A. donax* is characterized by much lower non-photochemical fluorescence quenching (NPQ) and higher intensity and up-shift of the main thermoluminescence B-band, compared to the non-emitting species *H. macra*. Differences in the chloroplast ultrastructure between the two species were also observed - the chloroplasts of *H. macra* had more disordered and less abundant grana stacking in the thylakoid membrane [8]. Based on these results we

suggest that the isoprene emitting species *A. donax* will be more stress tolerant compared to the non-emitter *H. macra*.

The aim of the current study was to investigate the significance of isoprene and other functional and biochemical factors involved in plant protection.

Material and methods. In the present study we carried out a comparative analysis based on some functional and biochemical parameters in *A. donax* and *H. macra* exposed to drought. The plants were grown from rhizomes, potted in 12 L and 2 L pots for *A. donax* and *H. macra*, respectively, filled with commercial soil mixture (Agroflora S.A, Greece) with addition of 5-10% sand. Plants were grown in a climatic chamber under controlled conditions (day/night temperature 27/25^{o}C, 12/12 h photoperiod, 300 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) and relative air humidity of 70-80%). Plants were regularly watered to full soil capacity.

During drought period, pots were wrapped in plastic film and weighted daily. Drought was imposed by withholding water. The level of drought stress was defined by fraction of transpirable soil water ($FTSW_n$, n is expressed in %) as explained in [11]. Then drought-stressed plants were rewatered to pot capacity and measurements were made after 7 d recovery period. Gas exchange and chlorophyll fluorescence measurements were carried out in dynamics – prior to stress ($FTSW_{100}$), under mild ($FTSW_{60}$ and $FTSW_{48}$), severe stress ($FTSW_{35}$), at the endpoint ($FTSW_{25}$), and after recovery. Determination of malondialdehyde (MDA) and proline accumulation was performed on frozen leaf samples collected prior to stress, at $FTSW_{25}$ and after recovery.

Measurements of photosynthetic parameters and chlorophyll fluorescence were done on fully expanded leaves (4th node from the apical meristem) of 5-6-weeks-old *A. donax* plants approximately 70-80 cm high and 8 weeks-old *H. macra* plants with 15-20 cm height at the beginning of the experiment. Leaf samples for biochemical analysis of MDA and proline were collected from the middle part of the same leaves after the gas exchange and chlorophyll fluorescence measurements, fixed in liquid nitrogen and stored at -80^{o}C.

Gas exchange parameters were measured with a portable infrared gas exchange system (LCpro+, ADC, BioScientific Ltd., UK). The middle part of a fully expanded leaf was clamped in 6.25 cm^2 gas exchange cuvette and exposed to atmospheric air with flow rate of 200 µmol s^{-1} and 380-390 ppm CO_2 concentration. During the measurements the cuvette temperature was maintained at about 26-27^{o}C and light intensity was about 500 µmol m^{-2} s^{-1} PPFD. The gas exchange parameters net CO_2 uptake (A_n) and stomatal conductance (g_s) were recorded after reaching the steady state of net photosynthesis. The intrinsic water use efficiency (WUEi) was calculated as A_n/g_s ratio.

Chlorophyll fluorescence was measured by Fluorescence Monitoring System (FMS, Hansathech Instruments, UK) immediately after the gas exchange measurements. Leaves were dark adapted for 20 minutes prior to the determination of minimum (F_0) and maximum (F_m) fluorescence. The maximum quantum yield of PSII photochemistry (F_v/F_m) was determined as $(F_m-F_0)/F_m$. After that leaves were adapted to the growth light intensity and saturating pulse of 0.8 s with over 6000 µmol m^{-2} s^{-1} was applied for determining the maximum (F_m') and the steady state (F_s) fluorescence during the actinic illumination. The quantum yield of PSII (Φ_{PSII}) was calculated according to [12]: $\Phi_{PSII} = (F_m' - F_s)/(F_m')$. NPQ due to dissipation of excess light energy was calculated as $NPQ = (F_m - F_m')/F_m'$ [13].

MDA was determined according to the method of [14]. Frozen leaf samples (0.3g) were homogenized in 3 ml 0.1% trichloroacetic acid. The homogenate was centrifuged and 0.5 ml from the supernatant was mixed with 1.5 ml 20% trichloroacetic acid, containing 0.5% thiobarbituric acid. The reaction mixture was boiled for 30 min and cooled on ice. The absorbance was measured spectrophotometrically (Specoll 11, Carl Zeiss, Jena, Germany) at 532 nm and 600 nm. The content of MDA was calculated as described in [14].

Proline content was determined following the method of [15]. About 0.5 g frozen leaf sample was homogenized with 8 ml 3% sulfosalicilic acid. The homogenate was centrifuged. The reaction mixture, which contained 2 ml supernatant with 2 ml glacial acetic acid and 2 ml ninhydrin reagent, was boiled for 60 min and than cooled on ice. The reaction mixture was extracted with 4 ml toluene and the absorbance at 520 nm was read by spectrophotometer (Specoll 11, Carl Zeiss, Jena, Germany).

The statistical analyses were carried out using one-way analyses of variance (ANOVA) tests. The mean differences were statistically tested with Tukey's test and considered to be significant at the 5% level.

Results and discussion. Under well watered conditions *A. donax* had about two times higher photosynthesis (A_n) and stomatal conductance (g_s) than *H. macra* (Fig. 1*A*, *B*). Drought caused an inhibition of photosynthesis in both species which was significant at $FTSW_{60}$ for *H. macra* and at $FTSW_{48}$ for *A. donax* respectively (Fig. 1*A*). A_n further decreased with increasing the drought stress and at FTSW end point

($FTSW_{25}$) it was highly suppressed in both species. After rewatering A_n of *A. donax* recovered almost completely. However, rehydration did not result in significant recovery of photosynthesis in *H. macra*. In recovered *H. macra* plants A_n was less than 50% of that in pre-stressed plants. The decline in photosynthesis during the drought period was associated with reduced g_s. Stomatal conductance decreased steeply in *A. donax* at $FTSW_{60}$ and decreased further at $FTSW_{35}$ and $FTSW_{25}$ (Fig. 1*B*). In *A. donax* g_s did not recover fully after rewatering but it was higher than that at $FTSW_{60}$. In *H. macra* g_s was less affected than photosynthesis and decreased slowly during dehydration. It was significantly affected under severe drought stress ($FTSW_{25}$). Rehydration did not result in recovery of g_s in *H. macra*.

The intrinsic water-use efficiency (WUEi) in pre-stressed plants was similar in both species (Fig. 1*C*). WUEi in *A. donax* was higher at $FTSW_{60}$ and $FTSW_{48}$ compare to control plants, and further increased at $FTSW_{35}$ and $FTSW_{25}$. At $FTSW_{25}$ WUEi was about 2.8 times higher than that in pre-stressed plants. After rehydration WUEi in *A. donax* recovered to control levels. Contrary, in *H. macra* drought resulted in decrease of WUEi. After rewatering WUEi increased and was similar to that in *A. donax* and to pre-stressed plants.

Thus, *A. donax* showed higher resistance of A_n to drought than *H. macra*. It has been shown that isoprene non-emitting tobacco plants suffer from stronger inhibition of photosynthesis caused by drought compared to transgenic isoprene-emitting tobacco [5]. A decline in g_s under mild stress may have protective effects against stress, by allowing plant water saving and improving plant WUE [16]. The early stomatal closure in *A. donax* suggests that it is better adapted to reduced water supply which is supported by almost full recovery of photosynthesis after rewatering.

The photochemical efficiency of PSII under drought stress was estimated from the variation in chlorophyll fluorescence. Under well-watered conditions F_v/F_m in *A. donax* was higher than in *H. macra* but the difference was statistically insignificant. In *A. donax* F_v/F_m was not significantly affected by drought during the experiment (Fig. 2*A*). In. *H. macra* F_v/F_m decreased significantly only at $FTSW_{25}$ and recovered after rewatering. The fact that F_v/F_m was negatively affected only in *H. macra* which does not emit isoprene is consistent with responses observed in transgenic tobacco plants modified to emit isoprene [5] and supports the observation that isoprene provides protection to photosynthetic apparatus from oxidative damage [17]. The actual efficiency of PSII photochemistry (Φ_{PSII}) at pre-stress conditions was significantly higher in *A. donax* than in *H. macra* (Fig. 2*B*). Φ_{PSII} in *A. donax* was not significantly affected until $FTSW_{35}$ and recovered to 85% from the control values after rehydration. However, drought caused decrease in Φ_{PSII} in *H. macra* at $FTSW_{60}$ and decreased further at $FTSW_{35}$. Φ_{PSII} in *H. macra* did not recover after rewatering. At control conditions *A. donax* had statistically lower NPQ than *H. macra* and in the course of the drought stress the increase of NPQ was not statistically significant (Fig. 2*C*). In *H. macra* NPQ increased slowly during the drought period but the difference was significant only at $FTSW_{25}$. NPQ increased even further after rehydration, indicating biochemical damage affecting the linear transport associated with energy dissipation. Recent study has shown lower NPQ and higher PSII efficiency under control conditions and under stress in isoprene emitting plants compared to the non-emitting [4]. In another experiment researchers observed increased NPQ after chemically inhibited isoprene emission in poplar leaves [18]. The authors have demonstrated that isoprene emission makes PSII photochemistry more efficient and reduces the need for heat dissipation from chloroplast under normal and high temperatures [18]. This is consistent with our observation for lower NPQ and enhanced stress tolerance in *A. donax* which emits isoprene compared to the non-emitter *H. macra*.

Lipid peroxidation indicates the prevalence of free radical reactions in tissues. The level of lipid peroxidation is an indicator for cellular injury in plants as a result of oxidative stress. Under well watered conditions MDA levels in *A. donax* were lower compared to *H. macra* (Fig. 3*A*). Drought stress did not cause significant changes of MDA in *A. donax* leaves, and after rewatering MDA content was similar to control values. In contrast, MDA substantially increased in *H. macra* plants exposed to drought and it remained higher after rehydration. Increased MDA content has been observed under oxidative stress in *Phragmites australis* leaves whose isoprene emission was inhibited by fosmidomicin feeding [3]. In addition, increased lipid peroxidation under drought has been reported for isoprene non-emitting tobacco plants compared to isoprene emitting [19].

We analysed the changes in leaf proline content during drought stress because it is thought to play a crucial role as an osmoregulatory solute in plants subjected to hyperosmothic stresses, primarily drought and soil salinity. We did not observe any significant differences between *A. donax* and *H. macra* in proline concentration in pre-stressed plants (Fig. 3*B*). Drought stress and rewatering did not result in significant

changes in proline levels in *A. donax*. However, drought stress caused strong increase in proline content (about 10 times) in *H. macra* plants and after rehydration it remained about 7 times higher than that in pre-stressed plants. Proline is known to have a role in osmotic adjustment, but it is often also considered as a symptom of tissue damage by drought, probably resulting from an excessive protein breakdown during water deficit [20]. Similar proline accumulation under severe drought stress was found in cowpea cultivars [20]. The observed metabolic alterations in *H. macra* are most likely a consequence of stress rather than an adaptive response with beneficial effects on plant physiological functioning. The significantly greater accumulation of proline and MDA in *H. macra* demonstrated that it sustained a higher degree of drought-related injury than *A. donax*. We suggest that the changes in *H. macra* physiology are caused by an excessive ROS accumulation in result of the drought to which the plant cannot counteract. In contrast, in *A. donax* isoprene probably serves as an antioxidant and the plants accumulate less ROS ; therefore the changes in *A. donax* are reversible and it recovers after eliminating the stress factor.

In conclusion, the enhanced drought tolerance of *A. donax* could be due to its ability to emit isoprene. The higher sensitivity to drought of *H. macra* compared to *A. donax* could be explained by the absence of isoprene emission in *H. macra*. It has been shown that isoprene strengthens thylakoid membranes and scavenges stress-induced oxidative species [5]. The ability of *A. donax* to emit isoprene probably has a key role in its drought tolerance and helps the plants to deal with severe drought and to recover better than the non-emitting *H. macra*.

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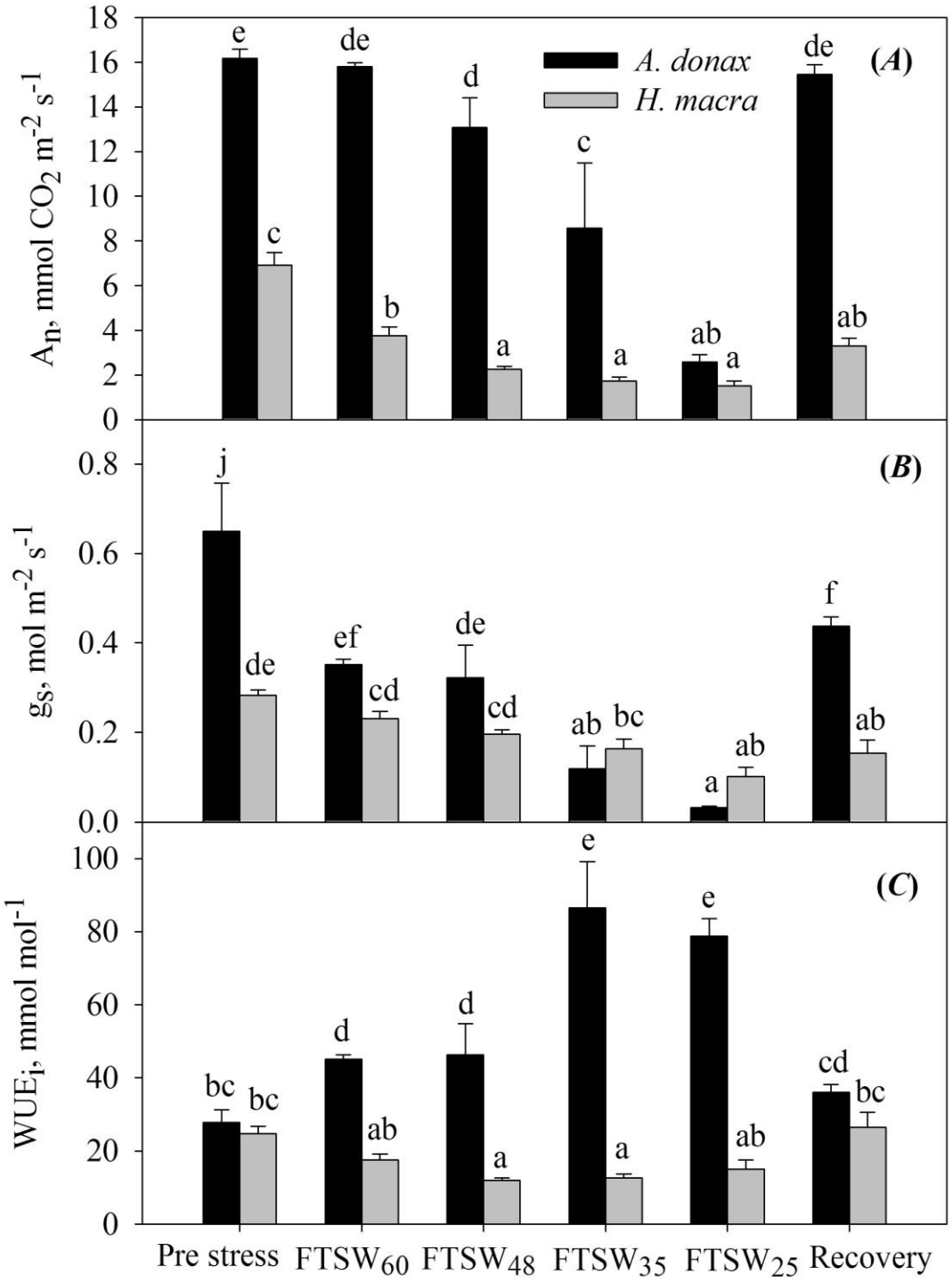

Fig. 1. Net photosynthetic rate (A_n) (*A*), stomatal conductance (g_s) (*B*) and water-use efficiency intrinsic (WUEi) (*C*) in *A. donax* and *H. macra* at pre-stress condition, during progressive drought stress (at different fraction of transpirable soil water (FTSW) points) and after 7 d recovery period. Measurements of gas exchange were performed at 500 µmol m^{-2} s^{-1} PPFD, 26-27°C air temperature and 380-390 ppm CO_2. Data are means ± SE (n=4-8). Different letters indicate statistically different means based on Tukey's test with $P < 0.05$.

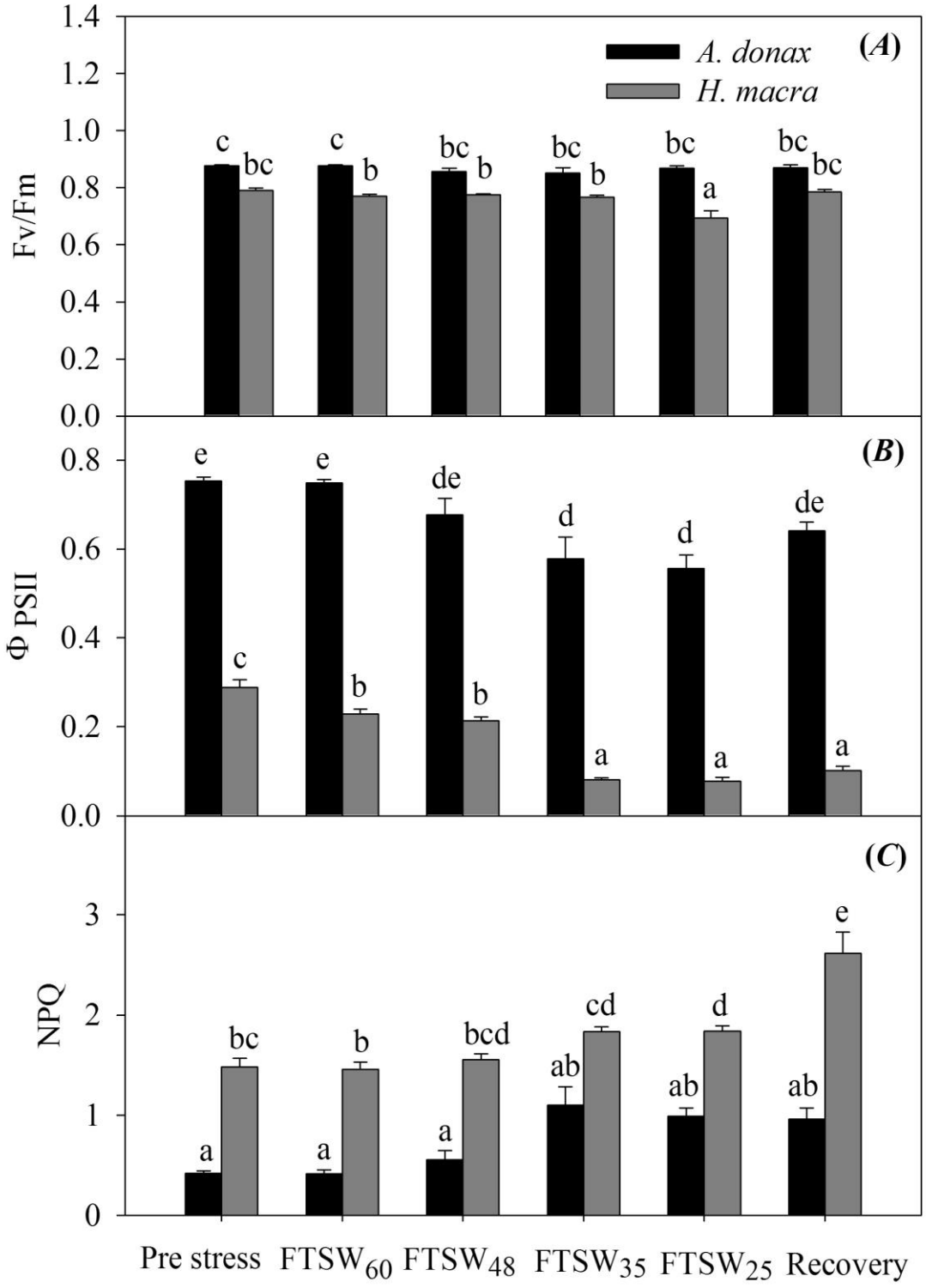

Fig. 2. Maximal (Fv/Fm) (*A*) and actual (Φ_{PSII}) (*B*) efficiency of PSII photochemistry and non-photochemical quenching (NPQ) (*C*) in *A. donax* and *H. macra* at pre-stress condition, during progressive drought stress (at different fraction of transpirable soil water (FTSW) points) and after 7 d recovery period. Data are means ± SE (n=4-8). Different letters indicate statistically different means based on Tukey's test with $P < 0.05$.

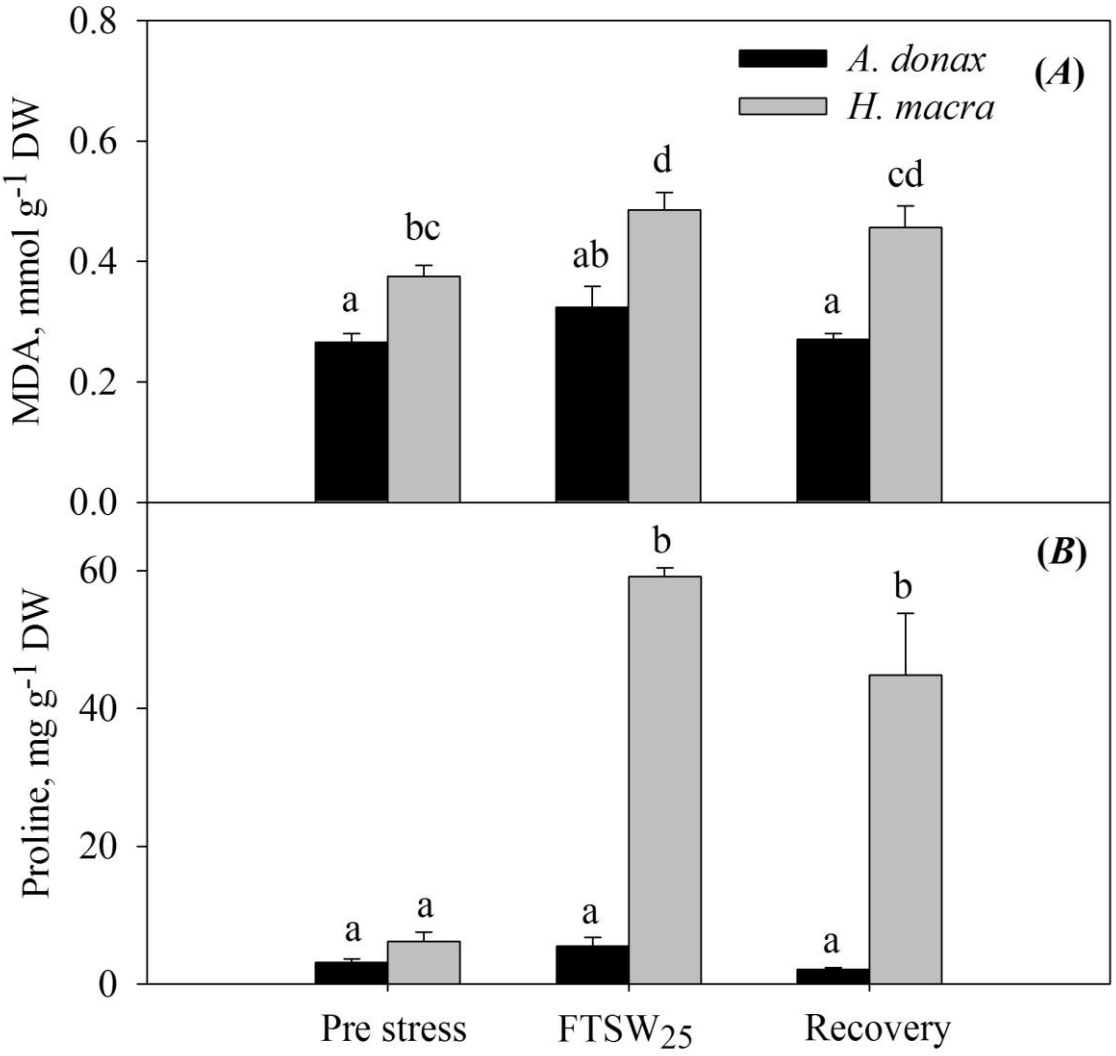

Fig. 3 MDA (*A*) and proline (*B*) content in *A. donax* and *H. macra* at pre-stress condition, at the end of progressive drought stress ($FTSW_{25}$) and after 7 d recovery period. Data are means ± SE (n=3-6). Different letters indicate statistically different means based on Tukey's test with $P < 0.05$.

Chapter 4

Isoprene emission enhances the photosynthetic performance of *Arundo donax* under drought stress

Manuscript in preparation

Isoprene emission enhances the photosynthetic performance of *Arundo donax* under drought stress

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Abstract

Isoprene biosynthesis is intimately connected to photosynthesis as it strongly relies on photosynthetic supply of carbon and energy. Moreover, isoprene has been proposed to prevent oxidation of the photosynthetic membranes under water deficit. *Arundo donax*, an important biofuel crop, is characterized by high isoprene emission rates like other fast-growing species. In this study we report on the isolation of Italian and Bulgarian ecotypes of *A. donax* and the characterization of their photosynthetic capacity under drought stress to test the hypothesis that isoprene emission is associated with an enhanced drought tolerance.

Under mild drought, the photosynthetic performance was mainly limited by the diffusional resistance to CO_2, while biochemical impairment ensued under more severe drought. Isoprene emission was less affected by water shortage than photosynthesis and it was stimulated by mild drought in the Bulgarian ecotype. We suggest that the synchronized function of volatile and non-volatile isoprenoids and higher induction of phenylpropanoids biosynthesis in the Bulgarian ecotype was related to its enhanced photosynthetic performance and better recovery of the Calvin cycle metabolism after re-watering. The data presented here support the signalling role of isoprene on the induction of the antioxidant response under stress and provide more evidence on the interrelation between isoprenoids and the phenylpropanoids pathway.

Key words: *Arundo donax*, Isoprene, Calvin cycle, Photosynthesis, Phenylpropanoids, Ecotype, Drought stress

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1. Introduction

Plants constitutively release considerable amounts of volatile organic compounds (VOC) and these biogenic emissions are receiving increasing attention because of their importance for the chemical/physical characteristics of the atmosphere and climate change. Isoprene constitutes the largest portion of biogenic VOC emissions from ecosystems to the atmosphere (Guenther et al. 2006). It is a highly reactive compound (Ashworth et al. 2013) and has a higher affinity than methane to oxidize hydroxyl radicals, which increases the lifetime of methane in the atmosphere (Guenther et al. 1995). Furthermore, isoprene significantly contributes to the formation of ozone (Folberth et al. 2006) and secondary organic aerosols (SOA), which have a detrimental impact on plants and human health (Claeys et al. 2004). Isoprene emission is exponentially increased by temperature (Guenther et al. 1993; Guenther et al. 2006; Niinemets et al. 2010). Moreover, water limitation can also stimulate isoprene emission (Brüggemann and Schnitzler 2002; Fortunati et al. 2008 ; Pegoraro et al. 2005).Therefore, climate change scenarios with a projected increase in temperature and drought events (IPCC 2013) could result in a dramatic increase of isoprene emission to the atmosphere (Pegoraro et al. 2004a; Heald et al. 2009; Pacifico et al. 2012), and hence its negative effect on air composition. This could be more significant in areas with large scale cultivation of strong isoprene emitting crops (Ashworth et al. 2012; Porter et al. 2012; Ashworth et al. 2013).

Drought stress is one of the major environmental factors constraining plant growth over most of the world's land surface. It is predicted that drought will significantly change agro-climatic conditions in the future (Trnka et al. 2011; Beringer et al. 2011). Generally, reduced water availability does not have remarkable effects on isoprene emission, since even under severe drought conditions largely impairing photosynthesis, isoprene emission is stable or shows only small decreases (Sharkey and Loreto 1993; Funk et al. 2004; Pegoraro et al. 2004b; Brilli et al. 2007). In some plant species, emission of isoprene is transiently induced under short (Brüggemann and Schnitzler 2002) or long periods of drought (Fortunati et al. 2008), which might be related to the supposed effect of isoprene on plant protection against oxidative stresses (Brüggemann and Schnitzler 2002; Brilli et al. 2007; Tattini et al. 2014). Contrary to the resistance of isoprene biosynthesis to drought, photosynthesis is one of the earliest physiological process that is impaired by water stress (Lawlor and Cornic 2002; Munns 2002; Signarbieux and Feller 2011). Photosynthetic suppression under drought could be due both to diffusive and metabolic limitations (Cornic 2000; Centritto et al. 2003; Chaves et al. 2009, 2011; Evans et al. 2009). Diffusion restrictions negatively affect photosynthesis by decreasing the

CO_2 concentration in chloroplasts. The reduction of stomatal conductance (g_s) is the main cause of photosynthetic suppression at early stages of drought. At more severe/later stages of drought, however, the metabolic impairment due to the limitation of phosphorylation and Calvin cycle metabolism becomes progressively more relevant among the factors suppressing photosynthesis (Long and Bernacchi 2003; Chaves et al. 2011). When the CO_2 carboxylation becomes limited under drought, the electron transport rate and the production of ATP and NADPH exceeds the demand of Calvin cycle. Under this circumstances the oversupply of excited electrons generates reactive oxygen species (ROS) that cause oxidative stress (Silva et al. 2010; Hernández et al. 2012). It has been suggested that isoprene could enhance the tolerance of plants to drought by regulating the ROS formation and improving the stability of thylakoid membrane functioning under oxidative stress (Loreto and Velikova 2001; Vickers et al. 2009a; Velikova et al. 2011, 2015; Ryan et al. 2014; Tattini et al. 2014).

Isoprene and non-volatile isoprenoids are formed through the same metabolic pathway (MEP) (for a review see Vranová et al. 2012). Drought-stressed plants continue to invest into isoprene biosynthesis even when carbon fixation is suppressed (Brilli et al. 2007; Tattini et al. 2014). The stimulation of the isoprene biosynthesis under stress might be associated to the excess of electrons/reducing power not used for assimilation and therefore are available for isoprene biosynthesis (Morfopoulos et al. 2013). It could be also due to the induction of the whole MEP pathway by stress, which consequently increase the production of other antioxidants (Tattini et al. 2014, 2015; Velikova et al. 2016). Non-volatile isoprenoids (e.g. carotenoids), indeed play a significant role in plants subjected to drought stress (Beckett et al 2012). There might be a co-regulation between isoprenoids production and another group of compounds with antioxidant function which are synthesized through the phenylpropanoid pathway (Agati et al. 2012; Brunetti et al. 2015). In contrast to carotenoids that are localized only in the chloroplast, phenylpropanoids are present in various organelles, which gives them the advantage to more readily transport within the plant cell and achieve complementary protective functions (Agati and Tattini 2010; Agati et al. 2012). It is also suggested that isoprene may have a signaling function under stress condition and could trigger the biosynthesis of other antioxidant metabolites such as carotenoids and phenylpropanoids (Tattini et al. 2014, 2015; Harvey and Sharkey 2016).

Arundo donax is a C_3 perennial grass originated from Asia and spread in the Mediterranean areas, USA, China, Australia and Southern Africa (Lewandowski et al. 2003). It can be found in a wide range of lands, from very humid to arid, (Mann et al. 2013) and produces high yield with low water and nutrients requirements. Therefore, *A.donax* is considered as a very promising crop for biofuel and bioethanol production, especially in Mediterranean regions (Lewandowski et al. 2003; Angelini

et al. 2005; Pilu et al. 2012, 2013). Hewitt et al. (1990) reported that *A. donax* leaves emit considerable amounts of isoprene.

In the present study we focused on the drought response of two *A. donax* ecotypes originated from areas with different climate conditions (temperature and water availability): Sesto Fiorentino, Tuscany, Italy (higher precipitation and lower temperature) and Srebarna, Silistra, Bulgaria (lower precipitation and higher temperature). We hypothesized that the Bulgarian ecotype originating from an area with lower water availability will exhibit greater drought tolerance than the Italian ecotype. Our aims were to (1) quantify the differences in isoprene emission, photosynthetic and stomatal responses to water deficit, (2) identify any traits confirming plant resistance to drought associated with ecotypic differences and (3) investigate the possible correlation between isoprene emission and biosynthesis of secondary metabolites (carotenoids and phenilpropanoids) under drought stress.

2. Materials and methods

2.1. Plant material and growth conditions

Arundo donax rhizomes were collected from natural habitats of this plant in Sesto Fiorentino, Florence, Italy (IT) (mean summer temperature of ~23°C and ~800 mm annual precipitation, (43°50´N11°12´E) and Srebarna, Silistra, Bulgaria (BG) (mean summer temperature of ~25°C and ~500 mm annual precipitation) (44°06´N27°04´E) (Fig. 1) during the spring of 2014. Plants were propagated vegetatively by rhizomes and each rhizome contained approximately 5-7 growth buds. Plants were grown in a climate chamber under controlled conditions (day/night temperature 27/25^{o}C, 12/12 h photoperiod, 300 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) and relative air humidity of 70-80%). The mixture of potting substrate consisted of commercial soil mixture (Agroflora S.A, Greece) with the addition of 5-10 % sand. After 30 days of regular irrigation to pot capacity, drought stress was initiated by stopping the watering and the pots were closed in plastic bags to avoid water loss by evaporation from the soil surface. In order to avoid anoxia the plastic bags were opened every day for 15 min. The water content of the pots, determined as the fraction of transpirable soil water (FTSW, %) as it is described in Brilli et al. 2007. Gas exchange and VOC emissions were measured at different stages during the experiment: (1) at control conditions before onset of drought stress (FTSW = 98%), during the drought exposure (2) at FTSW = 45% (mild stress), (3) at FTSW = 28% (severe stress) and (4) after re-watering (recovery to 98% of FTSW). The time lapse between the start of the drought treatment until its end (28% FTSW) was 4-6 weeks. The level of plant recovery was assessed after two weeks at FTSW = 98%, after the values of stomatal conductance remained stable for five days. Measurements were carried out on the fourth fully expanded leaf from the top. The relative leaf water content (RWC, %) was measured as (FW-DW)/ (TW-DW) × 100. Where the FW is the fresh weight, DW is the dry

weight after drying in the oven at 80 °C until constant weight is reached; TW was the turgor weight of the leaf after equilibrium in distilled water for 24 h.

2.2 Gas exchange and VOC measurements

Exchange of CO_2 and water vapor gas was measured with a portable leaf gas exchange system (LI-6400, Li-Cor, Lincoln, NE, USA). The middle part of the leaf was placed inside the 6 cm^2 gas exchange cuvette connected to the instrument. A constant flow (300 µmol s^{-1}) of synthetic air (21% O_2, 79% N_2, and 400 µmol mol^{-1} CO_2) was pumped over the leaf surface inside the cuvette. During all measurements, the temperature and light in the leaf chamber were maintained at 30°C and 1000 µmol m^{-2} s^{-1} PPFD. The intrinsic water use efficiency (WUEi) was calculated as the ratio between net photosynthetic rate (A_{net})/ stomatal conductance (g_s). To determine the photosynthetic efficiency related to carbon metabolism in the Calvin cycle A/C_i response curves were performed (Farquhar et al. 1980). A range of CO_2 concentrations from 50 to 1800 µmol mol^{-1} was generated, and photosynthesis together with other gas exchange parameters and isoprene emission rate were recorded after reaching the steady-state for each CO_2 concentration, typically 5-10 min after the change in ambient CO_2 concentration (C_a) (Centritto et al. 2003). The method of Sharkey et al. (2007) was used to calculate the V_{cmax} (µmol m^{-2} s^{-1}) - maximum carboxylation rate allowed by RuBisco (ribulose1,5 bisphosphate carboxylase/oxygenase), J_{max} (µmol m^{-2} s^{-1}) - rate of photosynthetic electron transport based on NADPH requirement for RuBP (ribulose 1,5 bisphosphate) regeneration, *TPU* (µmol m^{-2} s^{-1}) - triose phosphate utilization.

VOC emissions from *A. donax* leaves were analyzed by gas chromatography-mass spectrometry as detailed in Beckett et al. (2012). Briefly, part of the out flow from the leaf cuvette was directed into a silcosteel cartridge packed with 200 mg of Tenax (Markes International Limited, Llantrisant, UK). A volume of 2 L of air with a flow rate of 200 mL min^{-1} was passed through the trap. The cartridges were analysed with a Perkin Elmer Clarus 580 gas chromatograph and a Perkin Elmer Clarus 560S mass selective detector (Perkin Elmer Inc., Waltham, MA, USA). The gas chromatograph was coupled to a thermal desorber TurboMatrix 300 (Perkin Elmer, Inc.). Separation of the desorbed compounds was done by using a 30 m Elite-5 MS capillary column. Column temperature was first maintained at 40°C for 5 min, then increased with a 5°C min^{-1} ramp to 250°C, and kept at 250°C for 2 min. The NIST library was used for identification of the separated compounds.

Isoprene emission was also measured online by a high-sensitivity proton transfer reaction mass spectrometer (PTR-MS; Ionicon Analytik GmbH, Innsbruck, Austria). After reaching steady-state photosynthesis, the Li-Cor cuvette outflow was directed to the PTR-MS inlet by a T-connection. The VOCs were sampled from the headspace of the cuvette at the flow rate of 0.1 L min^{-1}. The operating parameters of PTR-MS were set at 80 °C drift tube temperature, 550V drift voltage and 2

mbar drift tube pressure, corresponding to an E/N ratio of about 140 Td (E being the electric field strength and N the gas number density; 1 Td = 10−17 Vcm^{-2}). The ion signal at m/z 69 corresponds to isoprene. Calibration of the instrument was performed by using a standard gas mixture (GCU; Ionicon Analytik GmbH, Innsbruck, Austria). The emission rate was calculated as described in Ahrar et al. (2015). Carbon lost as isoprene (%) was calculated as the ratio of isoprene emission to photosynthesis.

2.3. Analysis of carotenoids and phenylpropanoids leaf content

Carotenoids were identified and quantified as reported in Becket et al. (2012) and Tattini et al. (2014). Lyophilized leaf material was extracted with 2 x 2 mL acetone (added with 0.5 g L^{-1} $CaCO_3$) and 15 µL of aliquots were injected in a Perkin Elmer Flexar HPLC equipped with a quaternary 200Q/410 pump and a LC-200 photodiode array detector. Separation of photosynthetic pigments was performed with a 250 x 4.6 mm Waters Spherisorb ODS1 (5µm) column (Milano, Italy).The operating temperature of the column was set to 30°C and it was eluted with a linear gradient solvent system at a flow rate of 1.2 mL min^{-1}. Violaxanthin cycle pigments (violaxanthin, antheraxanthin and zeaxanthin) and β-carotene were detected by comparison of their retention times and UV spectra with those of authentic standards. Total chlorophyll was quantified as in Lichtenthaler and Bushmann (2001).

Individual phenylpropanoids were extracted and purified following the protocol of Tattini et al. (2004) and as described in Tattini et al. (2014). Briefly, 100 mg lyophilized leaf material was extracted with 3 x 2 mL of 75% C_2H_5OH/ H_2O (pH was adjusted to 2.5 with formic acid). The supernatant was portioned with 2 x 6 mL *n*-hexane, reduced to dryness and rinsed with 0.5 mL of CH_3OH/H_2O. Aliquotes of 10 µL were injected into the Perkin Elmer liquid chromatography unit reported earlier. The separation of phenylpropanoids was carried out by using a 250 x 4.6 mm Polaris C-18 Ether column (5µm) with operating temperature of 30°C. The eluent was H_2O (with addition of 0.1% triethylamine and pH adjusted to 2.5 with H_3PO_4) and CH_3CN. Phenylpropanoids were separated using a linear gradient solvent system as reported in Tattini et al. (2004) and identified using retention times and UV-spectral characteristics of authentic standards (Extrasynthese, Lyon-Nord, Genay, France), as well as by mass spectrometric data. HPLC-MS analysis was performed as described by Tattini et al. (2014).

2.4. Statistical analyses

Data points are reported as means of biological replicates, which consisted of leaves from different individual plants, as follow, for gas-exchange and VOC emission measurements 10 individuals, for parameters obtained from A/C_i curves 6-8 individuals, and for carotenoids and phenylpropanoids 3 individuals. The statistical analysis was carried out using a two-factorial ANOVA (ecotype, drought

levels and their interaction). The mean differences were statistically separated by Tukey's test and considered to be significant at the 5% level.

3. Results

3.1. Effect of drought on physiological characteristics of Arundo ecotypes

Leaf relative water content (RWC) decreased significantly and similarly in both ecotypes under severe drought conditions (~ 55%), compared to control (~ 90%). After re-watering the leaf water status of stressed plants reached control values.

Both *A. donax* ecotypes had similar isoprene emission rate under well watered conditions (Fig. 2A). Isoprene emission was stimulated by mild drought in BG ecotype, whereas it remained unchanged in IT being significantly lower than BG under mild drought. Severe drought stress caused significant inhibition of isoprene emission in both ecotypes being almost 60% less than control levels. After re-watering isoprene emission increased in BG and reached to 85% of the pre-stress values, whereas isoprene emission remained low in IT ecotype.

The BG ecotype had higher, but not significantly different, photosynthesis than IT under well-watered conditions (Fig. 2B). Both ecotypes responded to drought stress similarly. Photosynthesis was significantly suppressed under mild drought in both ecotypes being more pronounced in IT. Severe drought caused strong reduction of photosynthesis in both BG and IT (by about ~ 70- 80 %). After re-watering, both ecotypes recovered to a certain extent, but did not reach pre-stress values.

The changes in g_s followed those of photosynthesis, being significantly affected under mild drought with stronger effect in IT, and dramatically reduced in both ecotypes at severe stress (Fig. 2C). g_s did not recover significantly after re-watering in none of the ecotypes.

The interaction effect between ecotype and drought on WUEi was significant (P<0.01). The intrinsic WUE was similar in BG and IT ecotypes under control conditions (Fig. 2D). Drought stress significantly stimulated WUEi in both ecotypes. As the drought progressed, the WUEi in BG increased further and reached to its max value under severe drought. While in IT the WUEi under mild and severe drought did not change significantly. After re-watering the WUEi decreased again, however, it remained higher than pre-stress level in both ecotypes.

The interaction between drought and ecotype significantly affected (P<0.01) the intercellular CO_2 concentration (C_i) at ambient CO_2 (400 μmol mol^{-1} CO_2) (Fig. 2E).The two ecotypes had similar C_i under well-watered conditions. With the onset of drought C_i gradually decreased in both ecotypes. However, under mild drought C_i was not signifcanly different from control conditions in none of the ecotypes. By intensifying the drought C_i was significantly reduced in BG while in IT it showed

a slight but not significant increase compared with mild drought. After re-watering the C_i recovered in the BG ecotype and remained close to pre-stress levels in both ecotypes.

C lost as isoprene was significantly affected by the interaction between ecotype and drought (P<0.01) (Fig 2. F). Carbon lost as isoprene was similar in both ecotypes under control conditions and at mild drought, but dramatically increased in IT under severe drought stress being almost 6 times higher than pre-stress levels (Fig. 2F). Contrary, in BG carbon lost did not change and remained almost constant during the experiment.

In order to better understand the factors limiting photosynthesis in *A. donax* ecotypes exposed to drought the response of photosynthesis to changes in intercellular [CO_2] was measured. The parameters obtained after A/C_i fitting (maximum electron transport rate, J_{max}, maximum carboxylation rate, V_{cmax}, and the photosynthesis limitation by triose-phosphate use, TPU) at high [CO_2] when the CO_2 response shows a plateau or decrease (Sharkey 1985) are listed in Table 1. Under well-watered conditions the differences between ecotypes were not statistically significant. Both ecotypes exhibited a decline in all photosynthetic parameters with the onset of drought, being stronger under severe stress with a significant reduction in J_{max}, V_{cmax} and TPU. After re-watering the photosynthetic parameters recovered better in BG compared with IT ecotype and they attained closer to the pre-stress values.

3.2. Effect of drought on carotenoids and phenylpropanoids content in A. donax ecotypes

The content of β-carotene was significantly affected by the interaction between drought and ecotype (*P<0.01*; Fig. 3A). At pre-stress level IT had significantly lower concentration of β-carotene than BG. Drought stress resulted in a reduction of β-carotene level in BG while in IT the values were similar to pre-stress level. After rehydration the β-carotene content did not change in none of the ecotypes and the recovered IT and BG plants had similar values.

At pre-stressed level the ratio between xantophylls and chlorophylls (VAZ/Chl tot^{-1}) was equal in both ecotypes (Fig. 3B). As the drought stress progressed, the xanthophyll cycle pigments relative to total chlorophyll increased significantly in BG, while in IT the ratio did not change significantly. After re-watering the values decreased to pre stressed level in both ecotypes.

The interaction between drought and ecotype had a significant effect on the de-epoxidation state of xanthophylls (DES= (antheraxanthin + zeaxanthin)/(antheraxanthin + zeaxanthin + violaxanthin)) (P<0.01). The two ecotypes showed similar DES at the pre-stress level (Fig. 3C). Drought stress caused an increase of DES in IT and BG but it was more pronounced in IT plants. After rehydration DES decreased to pre-stressed values in both ecotypes.

The flavonoids content was similar in both ecotypes before stress. In drought stressed leaves flavonoids content increased in both ecotypes, being more pronounced in BG (Fig. 4A). After rehydration the leaf concentration of flavonoids decreased to pre-stress levels in both ecotypes.

The interaction effect of drought and ecotype significantly affected the HCA content. At pre-stressed level IT had higher concentration of HCA than BG. Drought induced significant increase of HCA concentration in BG, while in IT the values did not change compared to pre-stress level. (Fig. 4B). After re-watering, the HCA content decreased to the pre-stress level in BG, but in the IT ecotype it increased significantly in comparison with control and drought levels.

4. Discussion

In the present study we tested the hypothesis that two ecotypes of *A. donax* adapted to different environmental conditions could exhibit different isoprene emission rates, photosynthetic traits and level of secondary metabolites with antioxidant function, which in turn would determine the drought tolerance of the plants.

4.1. Isoprene emission and photosynthetic response of A.donax ecotypes under drought stress

In both ecotypes of *A. donax*, IT and BG, photosynthesis decreased earlier than isoprene emission during the progression of the drought. The same observation has been reported in other isoprene emitting plants (Sharkey and Loreto 1993; Brüggemann and Schnitzler 2002; Fortunati et al. 2008). The higher isoprene emission of BG compared with IT ecotype under mild drought could be partially the result of higher photosynthesis of this ecotype which leads to higher carbon fixation and carbon supply to isoprene pathway (Fig. 2A, B). Furthermore, after re-watering the BG ecotype showed better recovery in isoprene emission than IT, which could be associated to higher Calvin cycle metabolism in this ecotype and therefore a larger carbon pool for isoprene production. However, recovery of photosynthesis in BG was limited by diffusive resistance to CO_2 after re-watering, which possibly prevented significant difference between the ecotypes. In addition, higher J_{max} in BG after recovery could represent a higher rate of linear electron transport in this ecotype and larger availability of electrons for isoprene biosynthesis (Dani et al. 2014). However, incomplete recovery of *ISPS* mRNA transcript and protein level after re-watering, as it is reported in earlier studies, could also limit the isoprene emission of IT ecotype under recovery (Brilli et al. 2007; Fortunati et al. 2008).

Under severe drought, isoprene emission dropped significantly, similar to photosynthesis. The reduction of isoprene emission under drought is mainly the result of limitation in carbon and energy supply through photosynthesis for isoprene biosynthesis (Brilli et al. 2007; Fortunati et al. 2008; Tattini et al. 2014). However, alternative carbon sources such as xylem-transported glucose (Kreuzwieser et al 2002; Schnitzler et al. 2004), chloroplastic starch (Karl et al. 2002) or re-fixation

of CO_2 generated by light respiration (Anderson et al. 1998; Loreto et al. 2004) can contribute to provide the carbon demand of isoprene biosynthesis when photosynthesis is constrained. In the case of *Arundo* ectypes examined here, the photosynthesis was not completely supressed under drought. Thus, it probably could still provide carbon for isoprene biosynthesis. However, labelling experiments must be done to determine the contribution of assimilated carbon and alternative carbon sources in isoprene biosynthesis under drought (Brilli et al. 2007).

Comparison between different levels of drought revealed that the lower photosynthesis observed during mild drought in both ecotypes was likely due to a limitation in g_s compared to pre stress level and consequently less CO_2 availability (Fig. 2C). However BG maintained slightly higher g_s and C_i which resulted in higher photosynthesis of this ecotype compared with IT under mild drought. The reduction of g_s and consequently of photosynthesis was intensified under severe drought in both ecotypes. A strong linear correlation between g_s and photosynthesis over the period of drought has been reported for *Arundo* ecotypes previously (Sánchez et al. 2015). ABA-mediated stomatal closure, which is the first physiological response to drought, is known to limit the photosynthetic capacity of plants due to the reduced availability of CO_2 at the carboxylation centers (Flexas et al. 2004; Grassi and Magnani 2005; Erismann et al. 2008; Chaves et al. 2009). According to our results both ecotypes had almost similar stomatal response under drought. This observation is in accordance with the results of Haworth et al. (2016) on two Italian ecotypes of *A. donax*, which had identical stomatal response despite differences found in ABA concentration (both free and glycosylated form) in the early stage of drought. However, as drought progressed both ecotypes showed similar increases in ABA concentration (Haworth et al. 2016).

The intrinsic WUE (WUEi) representing the ratio of photosynthesis to stomatal conductance, is an important ecophysiological trait under drought conditions, which can provide a reliable assessment of stomatal limitation and its effect on photosynthesis (Medrano et al. 2009). The two ecotypes showed the same WUEi at pre-stress level (Fig. 2D). Compared to the recent report of Webster et al. (2016) on a natural plantation of *Arundo* in southern Portugal, the ecotypes tested here had lower WUEi at control conditions due to their high g_s (Webster et al. 2016). However, WUEi increased in both ecotypes with the onset of drought, which indicates a more conservative control of stomatal conductance (Ma et al. 2014; Velikova et al. 2016). The values observed here under drought were higher than in other herbaceous species (25-70 µmol mol^{-1}) (Medrano et al. 2009), especially in the case of BG which could highlight the potential of *A.donax* to cope with soil water deficits. The significant difference of ecotypes under severe drought could be partially the result of a better control on stomatal conductance under drought in BG. In addition, maintaining the photosynthetic rate slightly higher in BG than IT under severe drought could contribute to this effect. This

characteristic of BG suggests better adaptation of this ecotype to reduced water supply (Ares et al. 2000; Chaves et al. 2009).

Following the trend of change in intercellular CO_2 concentration (C_i) represented the steady decrease of C_i under mild drought compared with pre-stress level, which indicates the stronger reduction of g_s than A_{net} (Fig. 2E). This shows that the photosynthesis was limited by CO_2 transport during the early stage of drought (Cornic 2000). The two ecotypes showed a difference in C_i under severe drought being higher in IT than BG, while there was no difference in stomatal conductance between ecotype. This could suggest stronger limitation of CO_2 fixation in IT than BG due to the severe damage to photosynthetic carbon metabolism in water stressed leaves of this ecotype and therefore higher concentration of CO_2 in intercellular space (Lawlor and Cornic 2002; Centritto et al. 2011).

The carbon lost as isoprene represents the amount of assimilated carbon released as isoprene. It was shown that in isoprene emitting species the proportion of fixed carbon allocated to isoprene production increases under stress, which is associated to the protective role of isoprene under stress conditions (Centritto et al. 2011; Ryan et al. 2014). Therefore, we suggest that in the IT ecotype the significant increase of invested carbon in isoprene biosynthesis under severe drought might be the result of higher oxidative stress perceived by this ecotype (Fig. 2F).

Metabolic impairment is known to become progressively more relevant in photosynthetic limitation with the increase of drought stress (Ashraf et al. 2004; Ashraf and Harris 2013). Slowing down of the Calvin cycle metabolism under drought is mostly the consequence of the decrease in Rubisco carboxylation (Parry et al. 2002) and regeneration of RUBP (Medrano et al. 2002). The change of photosynthetic parameters (Table 1) represented the metabolic impairment of photosynthesis under severe drought in both ecotypes. As we observed here during drought, V_{cmax} decreased in both ecotypes, which indicates a reduction in Rubisco activity (Centritto et al. 2011). Overall, the BG ecotype had higher V_{cmax} than IT during the experiment, which might be related to a higher amount of activated Rubisco in this ecotype (Cheng and Fuchigami 2000; Perez et al. 2011). RUBP regeneration is another energy-dependent rate limiting step in the Calvin cycle. The saturation of RUBP or low supply of NADPH can decelerate this phase and result in lower J_{max}. We observed higher J_{max} in BG ecotype than IT during the experiment. It has been shown that the presence of isoprene enhances the fluidity of thylakoid membranes and flow of electrons along photosystems (PS) I and II, especially under stress (Velikova et al. 2011). Therefore, we suggest that the observed differences between ecotypes could be due to a more efficient electron transport rate in BG ecotype with higher isoprene emission under mild drought and recovery (Lawlor and Cornic 2002; Wentworth et al. 2006; Ashraf and Harris 2013). According to the observation of Haworth et al. 2016, the quantum yield of PSII in *A. donax* ecotypes under severe drought was significantly better

conserved in the ecotype with slight increase of isoprene emission under early stages of drought compared with the other ecotype with no stimulated isoprene emission under drought (Haworth et al 2016). The last phase of A/Ci curves is determined by the TPU (triose phosphate utilization). The Calvin cycle becomes limited also by the accumulation of triose phosphate in the chloroplast, which in turn decreases the transport of inorganic phosphate to the chloroplast, which is required in several steps of the Calvin cycle metabolism (Foyer and Noctor 2000; Drozdova et al. 2004). Any growth reduction under drought decreases the export of triose phosphate from photosynthetic leaf cells to other organs acting as a sink of assimilates. In addition, it has been suggested that prolonged drought triggers the conversion of starch to sucrose and elevates the triose phosphate concentration (Maroco et al. 2002; Drozdova et al. 2004; Diaz-Espejo et al. 2006). BG had higher TPU compared with IT showing higher utilization rate of assimilates which indicates a more active metabolism of this ecotype. Taken together these results confirm that assimilation was mainly affected by diffusional limitations to CO_2 at the early stages of drought. As the drought stress intensified, the metabolic limitation became dominant as demonstrated by a dramatic decrease in V_{cmax}, J_{max} and TPU in both ecotypes (Cornic 2000; Centritto et al. 2003; Centritto et al. 2011). The metabolic impairment of photosynthesis was better adjusted in BG than IT after re-watering. The photosynthetic parameters got closer to the pre-stress values in BG than IT (Table 1). We conclude that, analogously to the early phases of drought and during the recovery, the main limitation for photosynthesis in BG ecotype was the diffusional resistance to CO_2 transport and its availability for carboxylation (Galmes et al. 2007; Centritto et al. 2011). However, also the biochemical limitation contributed to the impairment of photosynthesis in IT during recovery, causing a more prolonged damage to the photosynthetic machinery in IT compared to BG (Flexas and Medrano 2002; Flexas et al. 2004).

Considering our results, we suggest that the stimulation of isoprene emission under mild drought in the BG ecotype could be involved in protecting the photosynthesis from oxidative stress under drought conditions. Our results are consistent with previous studies in transgenic non-isoprene emitting plants (Behnke et al. 2007; Ryan et al. 2014; Tattini et al. 2014) and support the antioxidant role of isoprene biosynthesis under abiotic stress (Vickers et al. 2009b; Velikova et al. 2011; Selmar and Kleinwachter 2013).

4.2. Interaction between isoprene emission capacity and biosynthesis of carotenoids and phenylpropanoids

At pre-stress levels the BG ecotype showed higher amounts of β-carotenoids than IT, which might indicate a higher activity of the MEP pathway in this ecotype (Cazzonelli and Pogson 2010) (Fig. 3A). While by intensifying the drought the two ecotypes showed the same β-carotene level. It is likely that the decline in β-carotene was due to its expenditure as an antioxidant to protect the plant

from oxidative damage during drought (Telfer 2002). β-carotene reduction may also occur if similar sources of carbon that produce isoprenoids is temporarily used for biosynthesis of other protective compounds, which are more effective (Beckett et al. 2012). This explanation could be relevant for BG which was a stronger isoprene emitter under mild drought, however as drought progressed the two ecotypes showed similar emission. Furthermore, BG represented a higher induction of VAZ/Chl_{tot} under severe drought that could be linked to its enhanced adaptation to cope with the excess light energy (Havaux and Niyogi 1999; Niinemets et al 2003; Fini et al 2014) (Fig. 3B). The photo-protective role of non-volatile isoprenoids (e.g. carotenoids) as a ROS scavenger under stress condition such as drought has been reported before (Munné-Bosch and Alegre 2000; Havaux et al. 2007; Du et al. 2010). Under severe and long-term drought the non-volatile isoprenoids can substitute isoprene to provide better protection against prolonged oxidative stress (Tattini et al. 2014; Loreto and Fineschi 2015; Velikova et al. 2016). According to our results we could suggest that the antioxidant isoprenoids are functioning more actively in BG than IT, represented by higher β-carotene, isoprene and VAZ/Chl_{tot} respectively at pre stress, under mild and severe drought. In particular, the higher stimulation of VAZ/Chl_{tot} under severe drought when isoprene was decreased could indicate the cooperation between volatile and non-volatile isoprenoids under drought, which can be regulated according to the severity of the water deficit in order to protect the photosynthetic machinery (Beckett et al. 2012; Tattini et al. 2014, 2015; Velikova et al. 2016). This is also supported by our observation on less photosynthetic impairment in BG than IT. The de-epoxidation state (DES) of xanthophyll cycle pigments is a known indicator of oxidative status of the plants. This mechanism dissipates the excess energy under stress conditions and has a crucial role in non-photochemical quenching (NPQ) (Cazzonelli 2011; Jahns and Holzwarth 2012). As we observed here, the IT ecotype characterized with higher DES under severe drought which could indicate the higher oxidative stress perceived by this ecotype (Munné-Bosch and Peñuelas 2004) (Fig. 3C). This coincides with our observation of larger carbon losses as isoprene in the IT ecotype under severe drought, which could trigger the isoprene production as a protective mechanism for immediate response to oxidative signals (Ramel et al. 2012). Tattini et al. (2014) have concluded that drought–induced isoprene biosynthesis may up-regulate the MEP-derived isoprenoid metabolism such as carotenoids biosynthesis (Tattini et al. 2014). According to recent research of Ghirardo et al. (2014), the DMADP accumulation in non-isoprene emitting poplar might cause feedback inhibition of 1-deoxy-D-xyluloso-5-phosphate synthase (DXS) activity, the first enzyme of the MEP pathway, which in turn results in suppression of the entire MEP pathway. Therefore, it is suggested that in isoprene emitting plants the continued uptake of DMAPP for isoprene production reduces the DMADP pool size of the chloroplast and consequently might stimulate the downstream pathway of

isoprenoids biosynthesis (Tattini et al. 2014). This mechanism could partially explain the higher level and stronger stimulation of non-volatile isoprenoids in BG with higher isoprene emission.

Flavonoids increased in both ecotypes under severe drought, while BG represented a more remarkable induction than IT (Fig. 4A). The phenylpropanoids, in particular flavonoids, play a fundamental role in photoinhibition of stressed plants (Schiedt et al. 2004; Agatti et al. 2007; Moellering et al. 2010; Agati and Tattini 2010; Agarti et al. 2012; Brunetti et al. 2015). Increase of flavonoids in water deficit leaves of *A. donax* ecotypes could indicate the flavonoids function to protect oxidative damage (Hernández et al. 2004; Tattini et al. 2004). Hydroxycinnamates (HCA) are another group of phenylpropanoids some of which are strong UV-B absorbents and therefore, could imply significant protection against UV damage and better adaptation to grow under excess light energy (Landry et al. 1995; Kolb et al. 2001; Tattini et al. 2004). The effect of UV radiation on plants can be influenced by drought (Alexieva et al. 2001; Basahi et al. 2014). Therefore, we propose that the significant induction of HCA in BG under severe drought could be a protective mechanism to cope with the harmful impact of excess light in water deficit leaves. While in the IT ecotype, although the HCA concentration was higher than BG at pre-stress level, no increase was observed under drought. The induction of HCA after re watering in IT could represent a higher susceptibility to UV-B light, due to the a more permanent damage to the photosynthetic apparatus (Kolb et al. 2001). There is evidence to support the interrelation between isoprenoids and the phenypropanoid pathway in plants experiencing excess light energy, e.g drought stress conditions (Brunetti et al. 2015). It is suggested that in drought-stressed plants the stimulation of the isoprenoid pathway affects the ABA content and indirectly regulates the phenylpropanoids production (Tattini et al. 2014). In *A. donax* ecotypes exposed to drought the ecotype with slightly higher isoprene emission under mild stress showed higher ABA content (Haworth et al 2016). Based on this evidence and according to our observations it is likely that the stimulated isoprenoids biosynthesis in BG could contribute to a more significant increase of phenylpropanoids (flavonoids and HCA) in this ecotype under severe drought. In addition, the sugar signalling also could induce phenylpropanoid production (Tattini et al. 2014; Morkunas and Ratajczak 2014). The reduction of TPU accompanied by induction of flavonoids under severe drought could indicate the activity of this mechanism in the *A. donax* ecotypes examined here.

5. Conclusion

In conclusion, the data presented here show that the photosynthetic capacity of both *A. donax* ecotypes was severely inhibited by prolonged drought. The two ecotypes represented remarkable differences in secondary metabolites biosynthesis in response to drought. We suggest the stimulated biosynthesis of isoprenoids and phenylpropanpids (flavonoids and HCA) in BG to be associated with its enhanced photosynthetic performance under drought and better recovery of the Calvin cycle

metabolism after re-watering (Tattini et al. 2014, 2015; Brunetti et al. 2015). Therefore, we conclude that the components of the antioxidant machinery were cooperating more efficiently in the BG ecotype to prevent the detrimental effect of oxidative stress under long-term drought. Considering the global limitation in water resources and interest to cultivate *A. donax* in marginal lands (Pilu et al. 2013), finding drought resistant cultivars is of large practical and economical value. Due to the sterility of *Arundo*, conventional breeding through sexual hybridization cannot be performed. On the other hand, vegetative propagation of *A. donax* results in low genetic variability in the current population and decreases the chances of finding new genotypes or varieties (Ahmad et al. 2008; Mariani et al. 2010; Pilu et al. 2012, 2013). Therefore, the differences in drought tolerance of the ecotypes examined in this study are of practical relevance for the cultivation of *A. donax* in regions with limited water resources.

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Fig. 1. Original sampling locations for the *Arundo donax* clonal populations used in this study. Italian ecotype was collected from Sesto Fiorentino, Florence, Italy (43°50´N11°12´E) and Bulgarian ecotype was collected from Srebarna, Silistra, Bulgaria (44°06´N27°04´E).

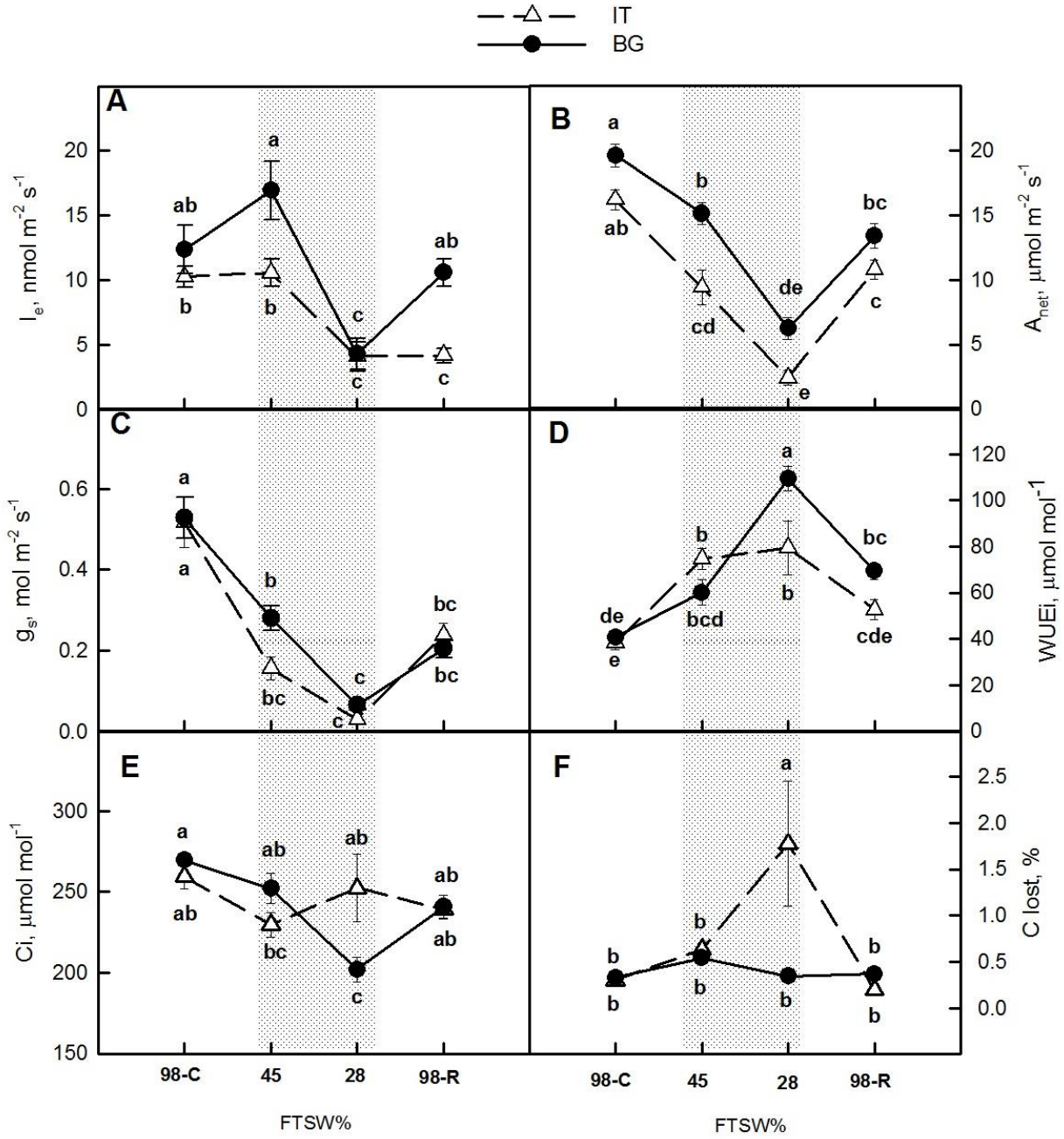

Fig. 2. Changes in isoprene emission rate (I_e) (A), net photosynthetic rate (A_{net}) (B), stomatal conductance (g_s) (C) and intrinsic water use efficiency (WUE_i) (D), intercellular CO_2 concentration (C_i) (E), percentage of carbon lost as isoprene (C lost %) (F) In two *Arundo donax* ecotypes, IT (dash line, triangle) and BG (solid line, circle) subjected to mild (45% FTSW) and severe (28% FTSW) drought. Measurements were performed at 1000 µmol m^{-2} s^{-1} PPFD, 30°C leaf temperature and 400 µmol mol^{-1} [CO_2]. Data are means ± SE (n=10). Different letters indicate statistically different means based on Tukey's test with $P < 0.05$.

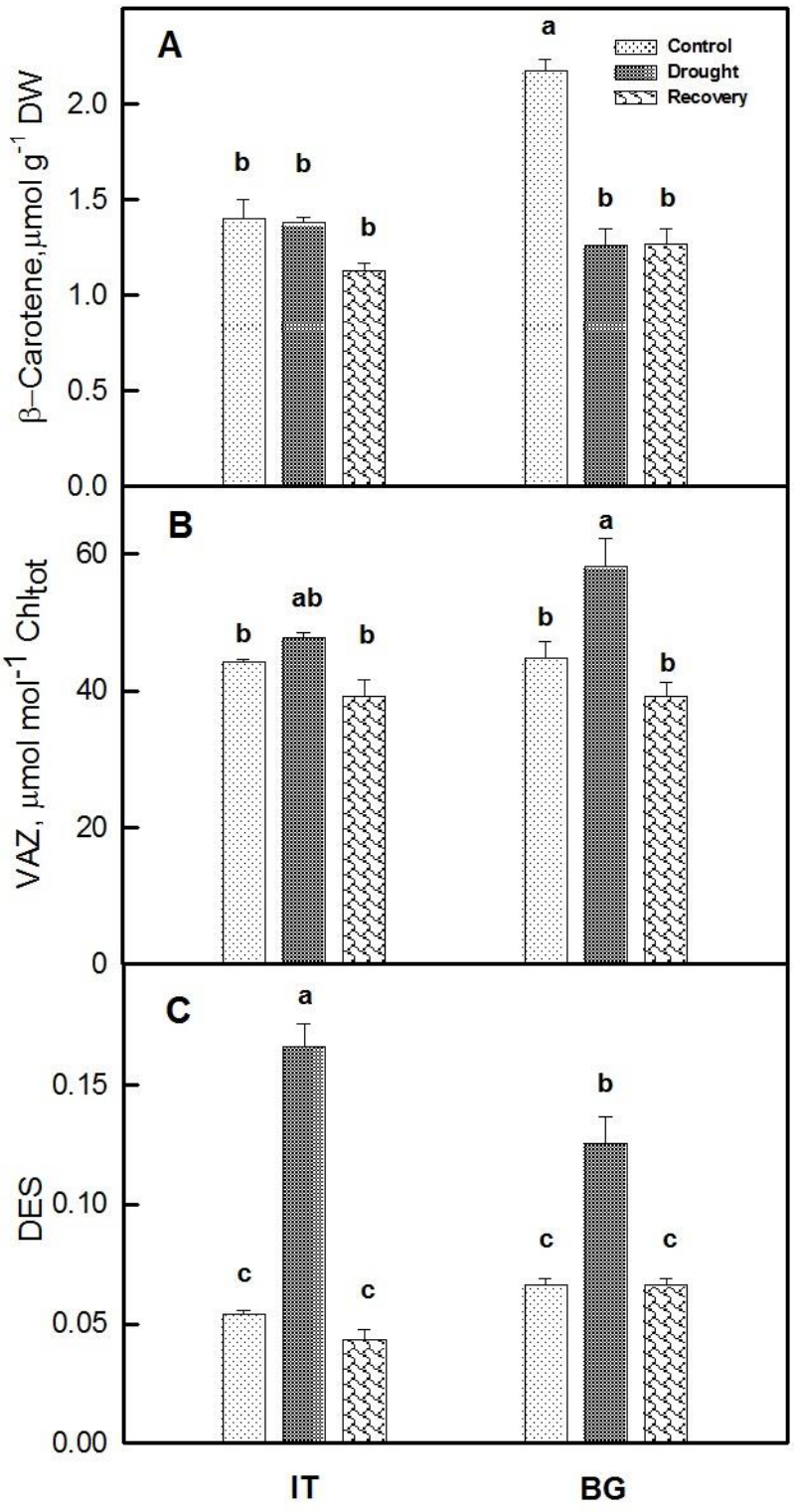

Fig. 3. Concentration of β-carotene (A), total concentration of violaxanthin, antheraxanthin, zeaxanthin (V+A+Z) on a chlorophyll basis (B), de-epoxidation state of violaxanthin cycle pigments [DES=(A+Z) $(V+A+Z)^{-1}$] (C) in leaves of two ecotypes *A. donax* (IT, BG) at pre-stress level , severe drought (28% FTSW) and recovery. Data are means ± SE (n=3). Different letters indicate statistically different means based on Tukey's test with $P<0.05$.

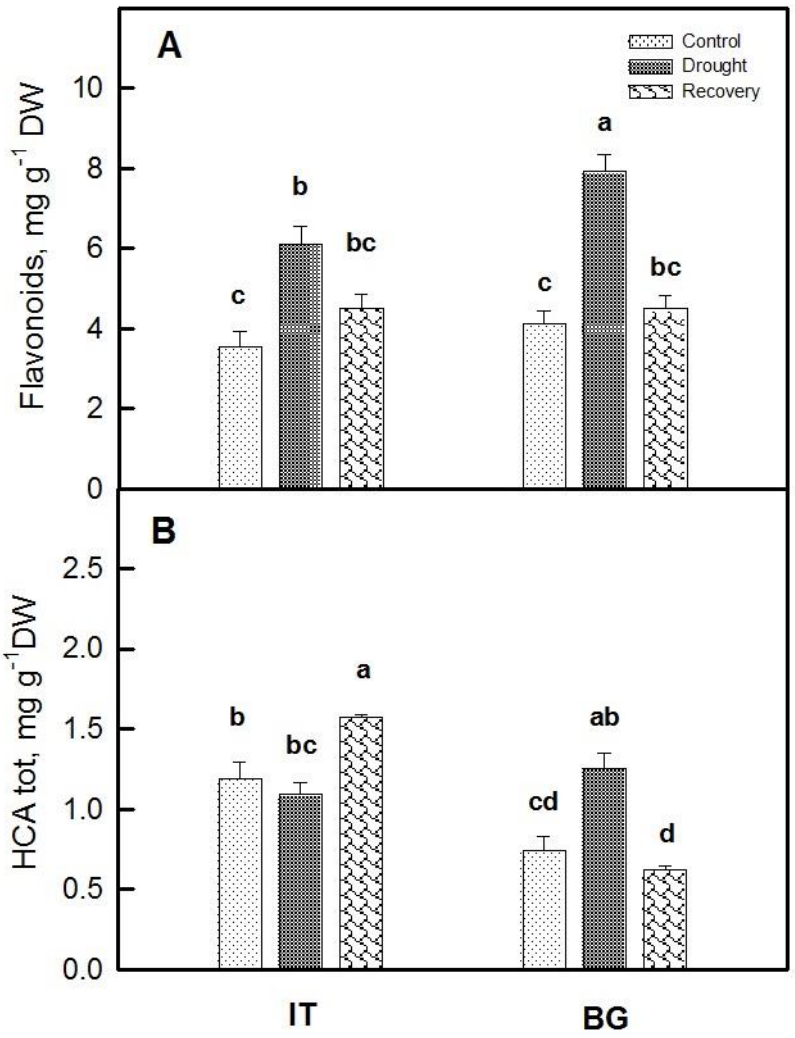

Fig. 4. Concentration of total flavonoids (A) and total hydroxycinamic acids (HCA) (B) in leaves of two ecotypes *A. donax* (IT, BG) at pre-stress level, severe drought (28% FTSW) and recovery. Data are means ± SE (n=3). Different letters indicate statistically different means based on Tukey's test with $P<0.05$.

Table 1. The photosynthetic parameters obtained from fitting the A-Ci responses curves (V_{cmax} - maximum carboxylation rate ,µmol m^{-2} s^{-1}, J_{max} – electron transport rate at saturating light and CO_2, µmol m^{-2} s^{-1}, *TPU* - triose-phosphate use, µmol m^{-2} s^{-1}),measured at pre stress , mild (45% FTSW) and severe (28% FTSW) drought and recovery . Results are means ±SD of 6-8 biological replicates. Different letters indicate statistically different means based on Tukey's test with $P<0.05$.

	Control		Mild drought		Severe drought		Recovery	
	IT	BG	IT	BG	IT	BG	IT	BG
V_{cmax}	196.2 ± 15. 9ab	210.0 ± 17.8ab	161.5 ± 15.6b	171.2 ± 28.7ab	58.6 ± 2.4c	75.6 ± 19.2c	167.4 ± 9.6b	253.1 ± 18.3a
J_{max}	145.6 ± 5.7abc	166.5 ± 7.6a	121.1 ± 10.9bc	137.3 ±18.2abc	45.1 ± 1.7d	57.9 ± 14.0d	112.1 ± 5.9c	163.7 ± 8.9ab
TPU	9.8 ± 0.3abc	11.8 ± 0.6a	8.0 ± 0.8 bc	9.1 ± 1.3abc	2.5 ± 0.2d	3.6 ± 0.8d	7.1 ± 0.4c	10.5 ± 0.9ab

Chapter 5

Discussion & Outlook

Isoprene is a highly reactive biogenic volatile organic compound (VOC) affecting the oxidative capacity of atmosphere (Guenther et al., 1995; Ashworth et al., 2013) and it has been suggested to have protective role in plants against environmental stresses (Loreto and Velikova, 2001; Vickers et al., 2009; Velikova et al., 2015, 2011; Ryan et al., 2014; Tattini et al., 2014). *Arundo donax*, a member of the Arundineae tribe, is an important biofuel crop thanks to its high productivity, low input requirement and adoptability to adverse environmental condition such as drought (Angelini et al., 2005; Lewandowski et al., 2003; Pilu et al., 2013, 2012). Although, *A.donax* and *P.australis*, also a member of Arundineae tribe, are known to emit considerable amount of isoprene, no information were available about other species of this tribe. Therefore, in the present work we used Arundineae tribe as a case study to investigate the interaction between isoprene emission capacity, physiological and biochemical performance of plants under control and drought stress conditions (Fig 3). Firstly, we studied the isoprene emission in relation to the photosynthetic capacity of six different species of the Arundineae tribe. This comparative study was the first to cover the isoprene emission of a monocot tribe.

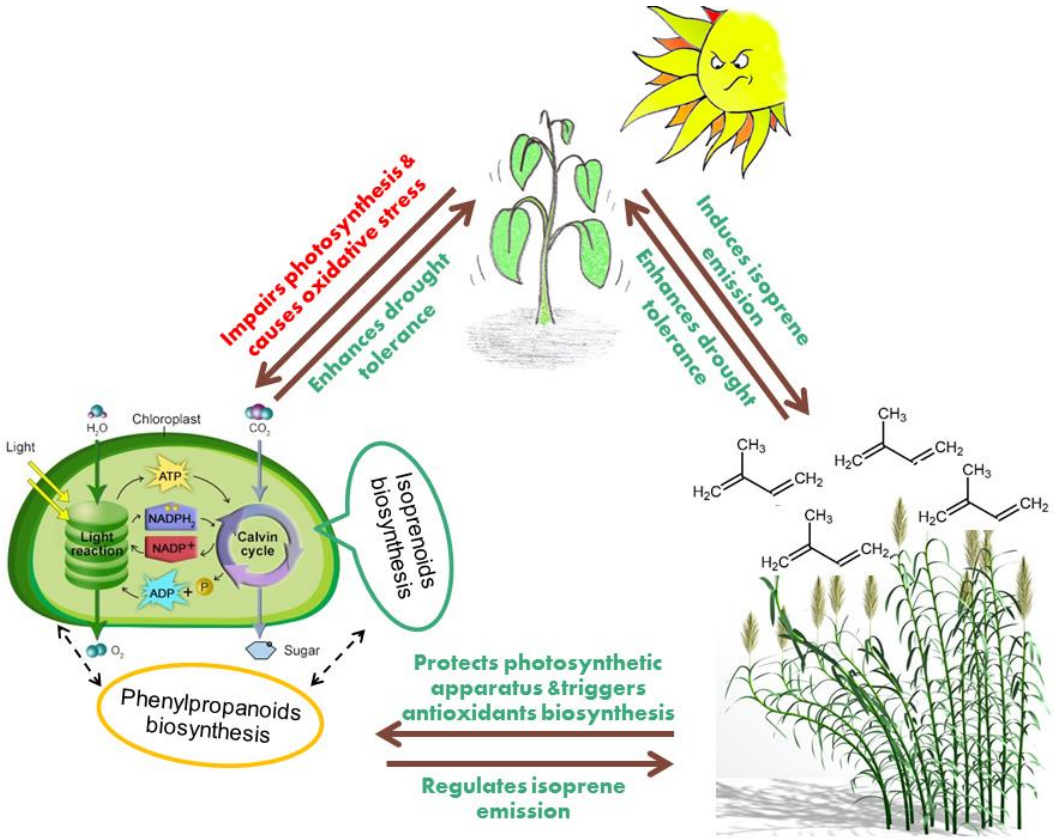

Fig 3: Schematic figure showing the complex interactions between isoprene emission, environmental stress (drought) and plant physiological and biochemical performance (photosynthesis and secondary metabolites). Individual cartoons obtained from : http://www.buzzle.com/images/diagrams/chloroplast-function-simplified.jpg; http://www.onyxtree.com/index.html; http://www.lancaster.ac.uk/staff/robertmr/abiotic.html.

Subsequently, we studied the effect of isoprene emission on the function and structure of the photosynthetic apparatus in two isoprene emitting (*A. collina, A. donax*) and one non-isoprene emitting (*H. macra*) species (Chapter 2). Secondly, we studied the effect of drought on the

photosynthetic performance and secondary metabolism (isoprenoids and phenylpropanoids) of the isoprene emitting (*A.donax*) and non-isoprene emitting species (*H. macra*) (Chapter 3). Eventually, to further investigate the role of isoprene in the drought tolerance of *A. donax*, we characterized the physiological response of two *A. donax* ecotypes from Italy and Bulgaria associated with a more humid and a more arid habitat, respectively (Chapter 4). According to our results, all the studied members of the Arundineae tribe except *H. macra* are isoprene emitters. Isoprene emission capacity enhanced the quantum yield and efficiency of PSII and chloroplast ultrastructure of the species under control conditions. Drought hampered the photosynthetic performance in both isoprene emitting and non-isoprene emitting species. However, the damage was more severe in the absence of isoprene emission. The isoprene emitting species represented an enhanced ability to recover structural and functional integrity of the photosynthetic apparatus after re-watering. The better ability of isoprene emitting species (*A. donax*) to recover from drought stress was associated with increased production of non-volatile isoprenoids (carotenoids), while non-emitting *H. macra* invests more on phenylpropanoids that are less efficient in preserving photosynthesis. We found physiological ecotypic differences between *A.donax* ecotypes (IT, BG) adapted to varied environmental condition. Overall, the conserved control over stomatal opening under drought, stimulated metabolism of isoprenoids and phenylpropanoids in response to drought and the high plasticity of the photosynthetic apparatus were identified as the main reasons underlying the drought tolerance of *A. donax*.

Among the 6 Arundineae species examined in this study *(A. donax, A. plinii, A. collina, P. australis, H. macra and Molinia caerulea)* only *H.macra* did not show detectable levels of isoprene emission, while the rest of the species were characterized by variable levels of emission (Chapter 2). Recent phylogenetic analysis supports the monophyletic grouping of *H. macra* with *P. australis* and *M. caerulea* (Christin et al., 2013; Mathews et al., 2000). This indicates a post-speciation loss of the isoprene biosynthetic capacity. Similar cases of multiple gains and losses of the ability to produce isoprene over the course of evolution have been suggested even among closely related species (Monson et al., 2013; Sharkey, 2005). Moreover, the lower isoprene emission in *M. caerulea* could indicate an ongoing sub-functionalization of the trait within this clade. It is thus possible that the evolution of the isoprene biosynthesis trait in Arundineae is driven by similar selective forces as it has been reported before in dicots (Harley et al., 1999). Finally, all the Arundineae species studied here are riparian, except *H. macra*. This could indicate a possible association between hydrophily and isoprene emission capacity, as was previously suggested (Loreto and Fineschi, 2015). The developmental control over isoprene emission differed remarkably between Arundineae (monocots) and dicots (Chapter 2). In sharp contrast with dicots, *A. donax* leaves are able to emit isoprene during earlier stages of leaf development (Sharkey et al., 2008).

Moreover, contrary to dicots, we did not detect monoterpene emission in young *A. donax* leaves. This evidence suggests differential transcriptional regulations of the isoprenoids pathway (e.g. *IspS* and monoterpene synthase genes) between dicots and monocots.

We found a direct correlation between isoprene emission and photosynthesis within the Arundineae tribe (Chapter 2), which is supporting the same observation on dicot plants (Harley et al., 1994; Litvak et al., 1996). Under physiological conditions, photosynthesis is the major source of carbon and energy for isoprene biosynthesis, therefore this interaction was expected. Recently, Morfopoulos et al. (2013, 2014) developed a mechanistic model based on the energetic status of the leaf showing that the isoprene emission rate is regulated by the balance between reducing power produced by light reactions of photosynthesis and the demand of the Calvin cycle. In our study, *A. donax* represented a higher efficiency of PSII, a parameter related to linear electron transport, while it had lower photosynthesis and isoprene emission compared with *A. collina* (Chapter 2). Therefore, we suggest the presence of another electron sink e.g. photorespiration in *A. donax*, which competes with isoprene biosynthesis for the residual reducing power not used for carbon fixation, as it was demonstrated before in eucalyptus plants (Dani et al., 2014). We also confirm that photosynthesis decreased earlier than isoprene emission during the progression of the drought (Chapter 4) (Brüggemann and Schnitzler, 2002; Fortunati et al., 2008; Sharkey and Loreto, 1993). The well-watered plants of *A. donax* ecotypes had similar isoprene emission. However, they responded differently under drought (Chapter 4). The BG ecotype showed an induction of isoprene emission under mild drought, whereas IT had the same emission with pre-stress level (Chapter 4). This effect could be the result of higher photosynthesis and consequently increased carbon supply to the MEP pathway in BG compared with IT. By intensifying the drought the isoprene emission decreased similarly in both ecotypes of *A. donax* (Chapter 4). This effect could be the consequence of the limited carbon and energy supply through photosynthesis in drought stressed plants. In addition, the alternative carbon sources could contribute to isoprene biosynthesis when photosynthesis is suppressed, but the possibility of this mechanism in *A. donax* needs to be explored further by labeling studies (Brilli et al., 2007). After re-watering, isoprene emission did not recover in IT, while it showed a high percentage of recovery in BG (Chapter 4). This could be explained by the higher Calvin cycle metabolism in BG during recovery, which could increase the carbon source for isoprene biosynthesis. Moreover, higher J_{max} in BG than IT after re-watering could reflect the high electron transport rate and therefore a larger availability of energy/electrons which can be directed to the isoprene biosynthesis (Dani et al., 2014) (Chapter 4). However, we cannot exclude the possibility of incomplete recovery of *ISPS* mRNA transcript and protein level in recovered IT plants, which could limit isoprene emission. This effect was observed in other isoprene emitting plants (Brilli et al., 2007; Fortunati et al., 2008). Isoprene emitting species (*A. donax* and *A. collina*)

had higher quantum yield and efficiency of PSII (F_v/F_m, Φ_{PSII}) under control conditions (Chapter 2). Comparing the isoprene emitting (*A. donax*) and non-emitting (*H. macra*) species under different stages of drought revealed an adverse impact on Φ_{PSII} during earlier stages of drought in *H. macra*, which did not recover after re-watering (Chapter 3). Moreover, we found higher J_{max} in the *A. donax* ecotype with higher isoprene emission at early stages of drought (BG) compared with other ecotype (IT), which could indicate a higher electron transport rate and quantum efficiency of PSII in BG (Chapter 4). These results support the role of isoprene to enhance the fluidity of thylakoid membranes and to improve the electron flow through photosynthetic membranes, as it has been suggested in previous studies (Velikova et al., 2015, 2011). In addition, *A. donax* and *A. collina* showed lower NPQ than *H. macra* under control conditions (Chapter 2). These results were consistent also under drought conditions (Chapter 3), when *H. macra* had higher NPQ than *A. donax* at different stages of drought, as well as after re-watering. NPQ is an important alternative mechanism to protect the photosynthetic machinery from the oxidative damage caused by excess light energy (Ashraf and Harris, 2013; Müller et al., 2001). The lower NPQ of *A. donax* could indicate the positive role of isoprene on the efficiency of photochemical quenching of light energy, which consequently decreases the need to dissipate the excess energy through an alternative mechanism such as NPQ. This result is in line with the previous reports on isoprene emitting plants (Behnke et al., 2007; Pollastri et al., 2014). *A. donax* showed higher carboxylation rate of Rubisco (V_{cmax}), more electron/energy demand in the Calvin cycle (J_{max}) and higher usage of assimilated sugar (TPU) compared with *H. macra*. This indicates more active photosynthetic carbon metabolism in isoprene emitting species (Chapter 3). The positive relationship between isoprene emission and J_{max}, V_{cmax} and TPU was also observed between *A. donax* ecotypes having different isoprene emission capacities. The biochemical limitation of photosynthesis under severe drought was persistent regardless of the isoprene emission capacity of the studied plants (Chapters 3, 4). However, we propose that the isoprene emission enhanced the recovery of the Calvin cycle metabolism and prevented the permanent damage to the photochemistry of photosynthesis (Chapters 3, 4). According to our results, isoprene emission was correlated to the photosynthetic performance of Arundineae species (Chapter 2) by enhancing the photosynthetic efficiency of PSII and carbon fixation in the Calvin cycle (Chapters 3, 4). These results provide more evidences for the protective role of isoprene on the photosynthetic apparatus under drought stress consistent with previous reports (Behnke et al., 2007; Ryan et al., 2014; Tattini et al., 2014).

Investigations of the chloroplast ultrastructure showed differences in chloroplast shape and its internal thylakoid membrane system between Arundineae species (Chapter 2). Isoprene emitting species (*A. donax* and *A. collina*) had more elongated chloroplasts and a higher density of thylakoid grana compared with non-isoprene emitting species (*H. macra*) under control conditions (Chapter

2). Moreover, a comparison of *A. donax* and *H. macra* under drought showed the destructive drought effect on chloroplast shape and thylakoid structure with more severe impact on *H. macra* (Chapter 3). Complete recovery of chloroplast structure after re-watering was observed in *A. donax*, while in *H. macra* the recovery was only partial (Chapter 3). The isoprene emission capacity is associated with enhanced chloroplast membrane integrity and functionality under stress condition (Velikova et al., 2014, 2011). In transgenic non-isoprene emitting poplar the level of chloroplastic proteins involved in photosynthesis were reduced (Velikova et al., 2014) and the lipid composition of the thylakoid membranes was modified, which resulted in less fluidity of this membrane (Velikova et al., 2015). Based on this evidence and according to our observations, we speculate that our results indicate the positive role of isoprene emission on the development of the chloroplast membrane and its protection from irreversible damage under drought. The higher photosynthetic efficiency of PSII was observed in species with well-structured chloroplasts and particularly abundant grana thylakoids (Chapter 2). In addition, the photosynthetic capacity recovered more readily in the species with complete reconstruction of chloroplastic membranes during recovery (Chapter 3). These findings revealed the direct relation between photosynthetic capacity and chloroplast structure in Arundineae species. This is consistent with observations on species from other plant families (Chen et al., 2008; Shao et al., 2014; Staehelin, 2003; Velikova et al., 2015) . The main difference of leaf structure between Arundineae species examined here was in the number of bulliform cells which were higher in *A. donax* and *A. collina* than *H. macra*. This might increase the capacity of these species (*A. donax* and *A. collina)* to capture light energy and therefore contribute to their higher photosynthesis (Lambers et al., 2008; Vogelmann and Gorton, 2014). However, the physiological function of bulliform cells and their possible role in drought tolerance need to be elucidated further.

The analysis of the water use efficiency (WUE) among Arundineae species did not reveal any direct relation between isoprene emission capacity and instantaneous WUE (WUE_i) in non-stressed plants (Chapter 2). Isoprene emitting (*A. donax*) and non-emitting species (*H. macra*) exhibited similar WUE (both instantaneous and intrinsic) under control conditions (Chapters 2, 3). Therefore, we suggest that this trait is independent from isoprene emission capacity in unstressed plants. However, following exposure to drought, *A. donax* and *H. macra* showed different trends of changes in WUE_i (Chapter 3). In *A. donax*, the WUE_i increased during the progression of the drought (Chapter 3) and the response was similar in both ecotypes (Chapter 4). In contrast, *H. macra*, showed a reduction of WUEi under drought conditions (Chapter 3). This difference could be partially due to the more conservative control of stomatal opening in drought-stressed leaves of *A. donax* compared with *H. macra*. This effect could be attributed to the higher capacity of the isoprenoids pathway in *A. donax*, which elevates the supply of substrates for ABA biosynthesis and consequently increases the foliar

ABA concentration and stomatal closure (Barta and Loreto, 2006; Tattini et al., 2014). In addition, the metabolic limitation of photosynthesis in *H. macra* could also contribute to its reduced WUEi under drought (Chapter 3). In the case of *A. donax* ecotypes, the higher WUEi under severe drought in BG than IT was mostly the result of the capability of BG to maintain the photosynthesis at slightly higher levels under extreme water deficit, rather than differences between ecotypes in their stomatal responses to drought (Chapter 4).

We found a significant interaction between isoprene emission capacity and concentration of non-volatile isoprenoids (Chapters 3, 4). Isoprene emitting species (i.e. *A. donax*) was characterized by higher carotenoids levels and xanthophyll cycle pigments compared with non-isoprene emitting species (*H. macra*) (Chapter 3). Also in the case of *A. donax* ecotypes, the ecotype (BG) with higher isoprene emission at early stages of drought had higher carotenoids content at pre-stress level and a stronger induction of VAZ/Chl in drought-stressed plants (Chapter 4). As it was shown before, isoprene emission could enhance the turnover of the MEP pathway as a consequence of less feedback inhibition due to a lower accumulation of DMAPP. Therefore, it was suggested that isoprene can trigger the biosynthesis of other isoprenoids such as carotenoids and xanthophylls (Ghirardo et al., 2014; Tattini et al., 2014). Our results support this hypothesis. The important role of non-volatile isoprenoids for plant protection under oxidative stress has been reported for different plant species (Beckett et al., 2012; Brunetti et al., 2015). We suggest that the combined effect of isoprene and non-volatile isoprenoids (carotenoids) could imply effective protection against oxidative damage in desiccated leaves (Chapters 3, 4). This is supported by our observation on more conserved structure and function of chloroplast and also lower de-epoxidation status of xanthophyll (DES) under drought in plants with higher isoprenoids metabolism (Chapters 3, 4). In addition, the absence of significant induction in malondialdehyde (MDA) and proline content under extreme drought in *A. donax* shows the smaller lipid peroxidation and osmotic stress perceived by this species.

Phenylpropanoids are another group of secondary metabolites with antioxidant function (Brunetti et al., 2015; Tattini et al., 2015, 2014, 2004). Comparison between an isoprene emitting (*A. donax*) and a non-emitting (*H. macra*) species revealed a different response in phenylpropanoids production induced by drought stress. *H. macra* showed a higher content of flavonoids and hydrocinnamic acid (HCA) compared with *A. donax*, likely due to the impairment of the electron transport chain (Akhtar et al., 2010) or ROS homeostasis (Agati et al., 2012; Babu et al., 2003; Pollastri and Tattini, 2011). Furthermore, when we compared two different ecotypes of *A. donax* originating from different habitats we found out that the ecotype with better adaptation to drought and less photosynthetic impairment had higher induction of phenylpropanoids. Taken together, these results suggest the important role of phenylpropanoids in drought response. In *H. macra*,

despite the increased metabolism of phenylpropanoids, the photosynthetic apparatus was not efficiently protected from oxidative damage under drought. Therefore, we suggest that the high production of phenylpropanoids in *H. macra* may confer more ability to cope with prolonged oxidative stress. However, in *A. donax* the induction of phenylpropanoids under drought conditions provide an additional antioxidant defence supplementing the protective role of isoprenoids and confer additional protection to the photosynthetic machinery. Based on our results, we propose that the protective role of phenylpropanoids and/or the strength of the protection under drought could vary between different plant species. Interestingly, phenylpropanoids induction in *A. donax* was correlated with a higher isoprenoids production. This is supporting previous reports on co-regulation of isoprenoids and phenylpropanoids (Brunetti et al., 2015a; Harvey and Sharkey, 2016; Tattini et al., 2014).

Among the Arundineae species tested here, *A. donax* presented the highest biomass production. However, we did not find any direct relation between biomass and other physiological characteristics of species, like, e.g: WUE, isoprene emission and photosynthesis (Chapter 2). The high productivity of *A. donax* could be the outcome of the combined effect of morphological and physiological characteristics of this crop, which need to be further explored.

In conclusion, the comparison of *A. donax* and *H. macra* under drought stress highlighted some of the strategies to cope with unfavourable environment conditions in *A. donax* (Chapter 3). The rapid adjustment of stomatal opening with the onset of drought played an important role in reducing the water loss of *A. donax* and resulted in an efficient water use of this species (Chapters 3, 4) as it was reported in former studies (Cosentino et al., 2016; Webster et al., 2016). In addition, the high photosynthetic efficiency of photosystem II and Calvin cycle metabolism could sustain the photosynthesis for extended periods during the progression of the drought (Chapters 3, 4). This result is in accordance with previous reports on the high photosynthetic capacity of *A. donax* (Haworth et al., 2016a; Webster et al., 2016) and delayed photosynthetic decline under drought (Cosentino et al., 2016; Haworth et al., 2016b). Considering the suggested antioxidant role of isoprenoids and phenylpropanoids in plant protection under drought (Beckett et al., 2012; Brunetti et al., 2015; Tattini et al., 2015, 2014), the higher content of isoprenoids in *A. donax* could be an important reason for its enhanced drought resistance (Chapters 3, 4). Moreover, the induced phenylpropanoids production co-regulated by isoprenoids metabolism could also strengthen this effect (Chapter 4). Another characteristic of *A.donax* that could contribute to its drought adaptation is the remarkable capacity of its chloroplast to recover (structurally and functionally) after passing the drought period (Chapters 3, 4). The clonal reproduction of *A.donax* results in low genetic diversity in the natural population of this plant (Ahmad et al., 2008; Mariani et al., 2010; Pilu et al.,

2013, 2012). However, our results indicate the possibility of variation among *A.donax* ecotypes as it was reported in former studies (Haddadchi et al., 2013; Haworth et al., 2016a). Therefore, studying *A.donax* ecotypes originated from arid areas might lead to find the drought tolerant ecotypes which can be cultivated in water limited regions.

Compared with common approaches which are used for studying the isoprene biological role under stress e.g. genetic modification (Behnke et al., 2007; Loivamäki et al., 2007; Sasaki et al., 2007) or chemically inhibition of isoprene biosynthesis (Velikova and Loreto, 2005; Velikova et al., 2006), using closely related species which are naturally emitting different amounts of isoprene, such as Arundineae species, has several potential advantages, like the absence of adverse impacts of transformation through *in vitro* regeneration or chemical inhibition of the MEP pathway which could result in an over/under estimation of the role of isoprene emission (Velikova, 2008; Vickers et al., 2009). Therefore, this work introduces a comparative approach to study the physiological role of biogenic isoprene which can be tested in plant species of other families. In particular, it would be important to compare the physiological and biochemical responses of species with different isoprene emission capacity under combined environmental stresses including high temperature, drought and elevated CO_2 concentration. This information could provide a better understanding of the complex plant-environment interactions under future climate scenarios (IPCC, 2013). Although this thesis focused on secondary metabolites with antioxidant activity (isoprenoids and phenylpropanoids), the primary antioxidants, e.g. ascorbate (ASA), glutathione (GSH), superoxide dismutase (SOD), etc., also play an important role in plant defence against oxidative stress (Apel and Hirt, 2004; Brunetti et al., 2015; Foyer and Shigeoka, 2011; Noctor et al., 2014). Investigating the primary antioxidants metabolism and function in relation to isoprene emission capacity of plant species under stress can unveil more aspects on the interrelation between primary and secondary antioxidants in response to stressful environmental conditions. Studies at the molecular level could determine the genes and transcription factors regulating the physiological and biochemical performance (Akhtar et al., 2010; Chaves et al., 2009; Fanciullino et al., 2014; Harb et al., 2010). Therefore, transcriptional and post-transcriptional studies of MEP and phenylpropanoids pathways, as well as photosynthesis could further improve the molecular mechanisms underlying the observed differences among the species studied here. Considering that isoprene biosynthesis is highly dependent on photosynthesis, including the interaction between these two mechanisms in isoprene emission models could lead to more accurate simulations (Martin et al., 2000; Morfopoulos et al., 2014, 2013; Niinemets et al., 1999; Zimmer et al., 2000). Therefore, gathering more parallel data on isoprene emission and photosynthetic parameters under varied environmental conditions, especially similar to future climate scenarios, can improve the mechanistic models of isoprene emission and

further contribute to more accurate estimation of isoprene emissions at regional and global scale and its effects on atmospheric quality.

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