Isoprenoids and phenylpropanoids are key components of the antioxidant defense system of plants facing severe excess light stress

Cecilia Brunetti[1,2], Lucia Guidi[3], Federico Sebastiani[4], Massimiliano Tattini[4*]

[1] The National Research Council of Italy, Department of Biology, Agriculture and Food Sciences, Trees and Timber Institute, Via Madonna del Piano 10, I-50019, Sesto Fiorentino, Florence (Italy)

[2] University of Florence, Department of Agri-Food Production and Environmental Sciences (DISPAA), Viale delle Idee 30, I-50019, Sesto Fiorentino, Florence (Italy)

[3] University of Pisa, Department of Agriculture, Food and Environment, Via del Borghetto 80, I-56124, Pisa (Italy)

[4] The National Research Council of Italy, Department of Biology, Agriculture and Food Sciences, Institute for Sustainable Plant Protection, Via Madonna del Piano 10, I-50019, Sesto Fiorentino, Florence (Italy)

* Corresponding author: Phone +39 0554574039; Fax + 39 055 4574017

Email address tattini@ipp.cnr.it

Summary

Plants face excess light stress on daily as well as on seasonal basis. The excess of excitation energy on cellular organelles prone to reactive oxygen species (ROS) generation is further enhanced when plants growing in full sun concurrently experience drought and heat stress. These are the very conditions that promote the biosynthesis of a wide range of secondary metabolites. Plants display a highly integrated arsenal of ROS-detoxifying agents to keep ROS concentration under control for efficient signalling, while avoiding cell death. There is evidence that primary antioxidants, i.e. antioxidant enzymes and low molecular-weight antioxidants, such as ascorbic acid and glutathione, are depleted under a severe excess of radiant energy. Here we discuss about how relevant secondary metabolites, namely isoprene, carotenoids, and flavonoids may complement the function of primary antioxidants to avoid irreversible oxidative damage, when plants experience intense, even transient stress events. We offer evidence of how plants orchestrate daily the antioxidant machinery, when challenged against multiple environmental stresses. It is indeed conceivable that daily variations in sunlight irradiance and air temperature may greatly alter the effectiveness of primary and secondary ROS-detoxifying agents. Finally, we discuss about the possible inter-relation between isoprenoid and flavonoid metabolism in plants facing high light coupled with drought stress, and hypothesize that abscisic acid might represent the missing link between these metabolic pathways.

Key words: antioxidant enzymes, flavonoids, isoprene, reactive oxygen species (ROS), singlet oxygen ($^1O_2$), zeaxanthin

1. Introduction

Plants routinely face a wide range of stress events, which fluctuate on daily as well as on seasonal basis. The inevitable consequence of living in an oxygen-rich environment when combined with environmental constraints is the accelerate production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\cdot-}$), and hydroxyl radical ($^{\cdot}OH$). Plants, as sessile organisms, have imperatively evolved a multiplicity of well-coordinated "defence" systems, aimed maintain sub-lethal levels of ROS while taking further advantage from their abilities to signal stressful conditions (Mittler et al., 2004; Foyer and Noctor, 2012, 2013). In plant cells, ROS are produced as by-products of plant metabolism in chloroplasts, mitochondria, and peroxisomes as well as in the apoplast by the action of NADPH-oxidase (Mittler et al. 2002, 2004; Apel and Hirt 2004; Maruta et al. 2012). There is clear evidence that ROS (as well as ROS-induced changes in the ratio of oxidized to reduced forms of low molecular weight antioxidants, i.e. redox couples, *sensu* Foyer and Noctor, 2015), constitute an important hub capable of fine tuning cell metabolism by 'transmitting' environment-induced perturbations, rather than representing dangerous by-products of aerobic metabolism (Foyer and Noctor, 2012).

For example, H_2O_2, due to its relatively long life-time and affinity for water channels (Bienert et al., 2006, 2007) is a perfect signal transducing molecule in plants growing under both 'optimal' and stress conditions (Pastori and Foyer, 2002). H_2O_2 may indeed mediate developmental processes because of its ability to activate Mitogen Activated Protein Kinases (MAPK)-induced signalling cascade (Kovtun et al. 2000; Foreman et al., 2003; Barba-Espín et al. 2011), and stress-induced H_2O_2 over production represents a local and systemic signal that allows plants to acclimate to different stress agents (Foyer et al., 1997; Rodriguez et al. 2002; Maruta et al. 2012). Even extremely reactive forms of oxygen, such as singlet oxygen ($^1O_2$) and $^1O_2$-generated oxylipins have also been involved in the retrograde signalling form chloroplast to the nucleus, thus tightly controlling cell metabolism (Wagner et al., 2004; Fisher et al., 2007; Kim et al., 2008). Changes in the concentration as well as in redox state of major low-molecular weight antioxidants, i.e. ascorbic acid and glutathione, represent systemic signals that profoundly alter cell metabolism conferring further resistance to over production of ROS (Schnaubelt et al., 2015). Nonetheless, the extent to which external perturbations enhance ROS generation and their successive diffusion from photosynthetic organs may result in severe cellular damage up to include programmed cell death (Van Breusegem and Dat, 2006; De Pinto et al., 2012). Therefore, plants have to make great efforts to finely tune ROS-derived signalling (preventing massive ROS generation and detoxify ROS once they are formed), when their capacity to use radiant energy to photosynthesis is severely constrained.

Main components of the antioxidant machinery of plants are low molecular-weight antioxidant metabolites and enzymes. These include ascorbate (ASA), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX), and constitute the first line of defence against oxidative stress that operates in cellular compartments in which photosynthesis and photorespiration take place (Apel and Hirt, 2004; Foyer and Shigeoka, 2011; Noctor et al., 2014). However, stress-induced enhancement in the first line of antioxidant defence is not a general rule. The activity of antioxidant enzymes increases in stress-tolerant species or genotypes, but may decrease steeply in stress-sensitive counterparts (Schwanz and Polle, 2001; Hernández et al., 2002, 2003). This simply means that primary antioxidants may be depleted depending on stress severity (Fini et al., 2011). Sensitivity to multifarious stressors is generally estimated in terms of a plant's ability to fix carbon and hence to promote new growth. It is therefore conceivable that the extent to which radiant energy reaching the photosynthetic apparatus exceeds the plant ability to use it to photosynthesis because of environmental stressors, may profoundly affect the effectiveness of primary ROS detoxifying agents (Fini et al., 2012, 2014). In other words, exposure to excess light stress may result into transient activation/inactivation of antioxidant enzyme activities (Polle, 2001; Mullineaux and Karpinski 2002; Mubarakshina et al., 2010; Fini et al. 2011; Fini et al. 2014).

Plants have evolved a variety of additional antioxidant systems, which are indeed activated in response to severe excess of sunlight irradiance (Agati et al., 2012, 2013; Esteban et al., 2014). Secondary metabolites are well suited to constitute a 'secondary' antioxidant system to transiently complement the action of primary antioxidants, as secondary metabolite biosynthesis is mostly activated in response to a severe excess of radiant energy (Agati et al., 2012, 2013). The cost, in terms of energy and carbon, for secondary metabolites biosynthesis is balanced by the multiplicity of functions that secondary metabolites may serve in plants suffering from severe excess light stress (Loreto and Schnitzler, 2010; Agati and Tattini, 2010; Ramel et al., 2012). Non-volatile isoprenoids, such as carotenoids, and flavonoids have the ability to avoid ROS generation as well as to counter ROS-induced damage (see next sections for details). In other words, they are potent antioxidants, following authoritative definitions given by Halliwell and Gutteridge (1989) and Halliwell (2009).

Here we explore the issue of how plants may orchestrate key components of the antioxidant machinery when severely stressed by an excess of radiant energy. In particular, our focus is on isoprenoids and flavonoids, and we discuss about the potential of this vast class of secondary metabolites to complement the functions of primary antioxidants in plants facing concurrently exposed to multiple stress agents. The matter has outstanding ecological significance for plants inhabiting most areas worldwide, particularly the arid and semi-arid regions, as the frequency of intense stress events,

such as scarcity of rainfall coupled with heat waves, is predicted to increase in the next future because of climate change (Mateasanz and Valladares, 2014; Tattini and Loreto, 2014).

2. Primary antioxidant defences decline under severe excess light stress

There is compelling evidence that enzymes aimed ROS detoxification decline in leaves, in which excitation energy to the chloroplast is in great excess (Casano et al., 1997; Streb et al., 1997; Polle, 2001; Mullineaux and Karpinski, 2002; Mubarakshina et a., 2010). This poses some concerns whether antioxidant enzymes constitute an efficient control system against stress induced ROS production (Peltzer and Polle, 2001; Peltzer et al., 2002; Apel and Hirt, 2004; Schutzendubel et al., 2001; Fini et al., 2012, 2014). APX and CAT are depleted in plants exposed to severe excess excitation energy (Polle, 2001; Mullineaux and Karpinsky, 2002; Hatier and Gould, 2008; Mubarakshina et al., 2010; Agati *et al.,* 2012) especially when other environmental constraints concurrently reduce the use of light energy for carbon fixation (Fini et al., 2011, 2012). There is evidence that APX activity is particularly sensitive to high temperature and sunlight irradiance (De la Haba et al., 2014) as revealed by both *in situ* and *in vitro* analysis of enzyme activity (Peltzer and Polle, 2001; Peltzer et al., 2002). Temperature dependent reduction of enzyme activities may be further enhanced under severe drought induced limitations of photosynthesis, which in turn contribute forming excess excitation energy (Fini et al*.,* 2011, 2012).

Similarly to antioxidant enzymes, also the concentration of ASA and GSH generally increases under mild to moderate stress, but may decrease when the stress become more severe, in concomitance with severe limitation of photosynthesis (Herbinger et al. 2002; Guo et al., 2006; Zechmann et al., 2011; Koffler et al., 2014). Notably, drought induced decrease in ascorbate and glutathione concentrations in *Arabidopsis* was mostly due to depletion in chloroplasts and peroxisomes, whereas the concentration of vacuolar ASA steeply increased (Koffler et al 2014). It was suggested that ASA may have a role as H_2O_2–detoxifying in the vacuole (Koffler et al 2014), possibly behaving as a 'secondary vacuolar antioxidant', as detailed below in section 4.

The extent of stress-induced depletion of primary antioxidant defences determine cell and whole-organ fate. Indeed, ROS may represent an unsolvable dead threat for the cell or instead activate a network of defences (through ROS-signalling) conferring further stress tolerance (Suzuki et al., 2012; Barajas-Lopez et al., 2013; Van Breusegem and Dat, 2006) in a very narrow concentration range (Cheeseman, 2006). The matter needs further investigations, examining the responses of plants to multiple stressors on long-term basis.

3. Antioxidant functions of volatile and non-volatile isoprenoids in high light-stressed leaves

As mentioned above, the conditions that lead to depletion of primary antioxidants can activate the biosynthesis of relevant secondary metabolites. This is exactly the case of volatile (here the discussion is centred on isoprene, the functions of which have been deeply explored) and non-volatile isoprenoids (Esteban et al., 2014; Rasulov et al., 2014; Tattini et al., 2014a). These metabolites are therefore best suited to complement the function of primary chloroplast antioxidants, and equip the chloroplast with an extraordinarily versatile arsenal aimed at effectively countering the risk of irreversible photo-oxidative damage.

3.1 Isoprene

Isoprene biosynthesis is a feature of most hygrophylic and deciduous species (Loreto and Fineschi, 2015), and its emission is further stimulated when these plants experience intense, even transient stress events, e.g. water deficit coupled with heat waves (Bruggeman and Schnitzler, 2002; Sharkey et al., 2008; Harrison et al., 2013; Rasulov et al., 2014). Drought and heat stress-induced changes in physiological traits result into 'biochemical' conditions that stimulate isoprene biosynthesis. Photosynthesis mostly declines because of stomatal limitations early during drought stress without a concomitant decrease in electron transport rate. The generated excess of reducing power coupled with limited intercellular CO_2 partial pressure, are the very conditions that favour foliar emission of isoprene (Harrison et al., 2013; Morfopoulos et al., 2014). Severe reductions in stomatal conductance inevitably increase leaf temperature, thus approaching to the optimal temperature range for the activity of isoprene synthase (45-50 °C, Niinemets and Sun, 2015), which is, interestingly, much higher than optimal temperature for the activity of Rubisco and other cellular enzymes, including antioxidant enzymes (Peltzer and Polle, 2001; Cen and Sage, 2005). This may help explaining why in plants growing in full sunlight and concomitantly exposed to heat and drought stress, isoprene biosynthesis is stimulated, though photosynthesis is severely depressed (Behnke et al., 2007; Loreto and Schnitzler, 2010). In other words, isoprene biosynthesis is stimulated when plants potentially suffer from severe, though transient photo-oxidative stress.

The issue of why plants loose considerable amounts of fresh assimilated carbon (up to 10-20%) to isoprene emission, and continue to invest both fresh assimilated and organic carbon for isoprene biosynthesis even when carbon gain is severely constrained, is still an open question (Harrison et al., 2013). However, several empirical and mechanistic evidences supports the idea that isoprene, as also reported for other secondary metabolites, may serve a multiplicity of functions in plants suffering from severe excess light stress. Isoprene is long known to preserve leaves from photo-oxidative damage

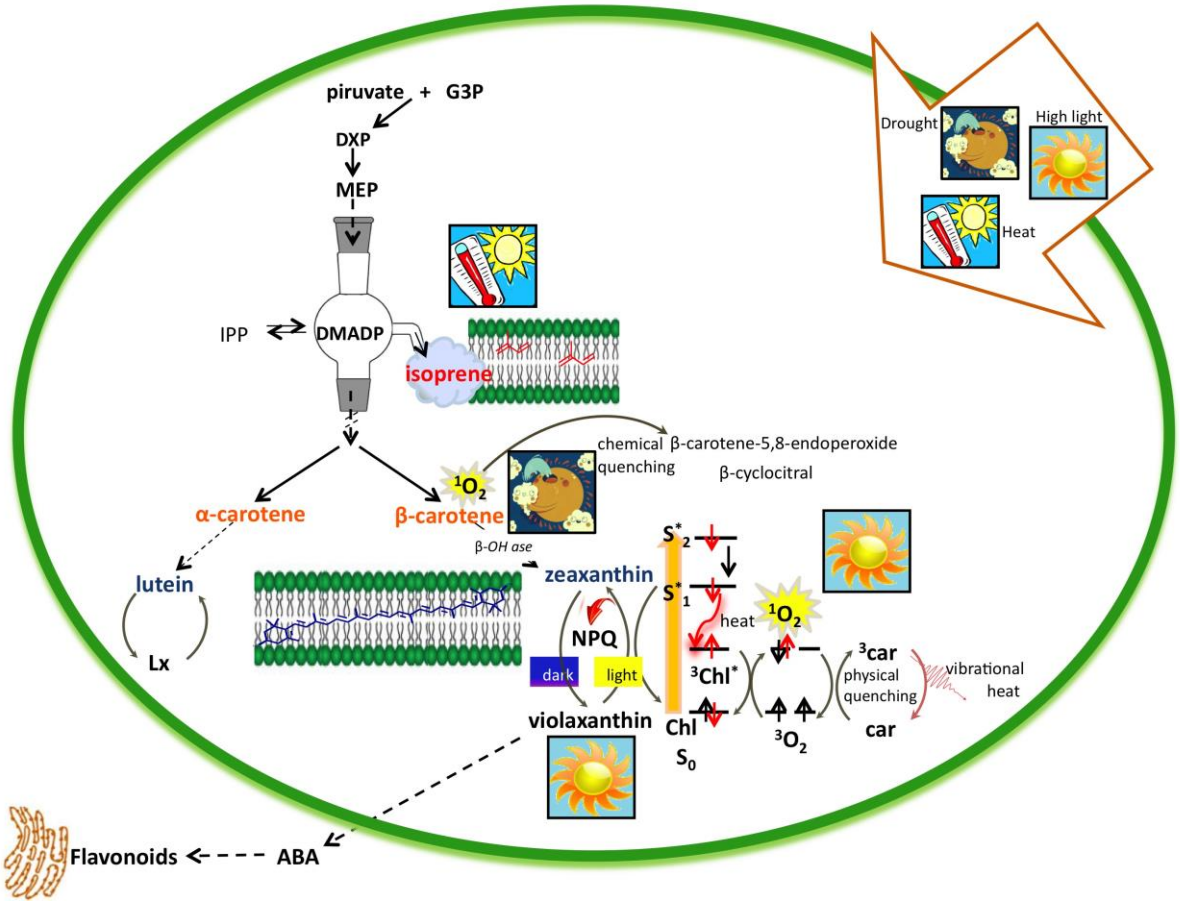
through its capacity to quench ROS/RNS and strength thylakoid membranes (Affek and Yakir, 2002; Velikova et al., 2004; Vickers et al., 2009). The idea that isoprene stabilizes chloroplast membranes was proposed two decades ago by Sharkey and Singsaas (1995). Recent studies have shown that isoprene may transiently enhance phospholipid layer packing in model membrane systems (Siwko et al., 2007). The high lipophilicity of isoprene is likely responsible for its capacity to stabilize pigment-protein complexes, and hence to preserve the functionality of membranes at elevated temperature, though isoprene can reside in thylakoid membrane only for short time (**Fig. 1**, Velikova et al., 2011). These findings may explain why isoprene-emitting plants usually display higher thermo-tolerance as compared to non-emitting counterparts (Singsaas et al., 1997; Sharkey et al., 2001; Velikova and Loreto 2005; Sasaki et al., 2007)

As mentioned above isoprene biosynthesis is stimulated when the ratio of electron transport to photosynthesis steeply increases (Morfopoulos et al., 2013). Isoprene emitting plants use a portion of this excess of reducing power (NADPH) to form DMADP (dimethylallyl diphosphate) (Harrison et al. 2013), in turn preventing ROS generation. The consumption of DMADP for isoprene biosynthesis prevents DMADP accumulation and its consequent feedback down-regulation of the whole MEP pathway (**Fig. 1**, Banerjee et al., 2013; Ghirardo et al., 2014). This regulatory effect of isoprene on the MEP pathway may have significance in plants concurrently facing severe drought and high light stress (**Fig. 1**, Rinnan et al., 2014; Tattini et al., 2014a). Isoprene biosynthesis may indeed channel more carbon to the synthesis of non-volatile MEP-pathway products **(Fig. 1)**, such as carotenoids (Tattini et al., 2014a), which play a role of outstanding significance in high light-stressed leaves (Esteban et al., 2014).

3.2. Carotenoids

Carotenoids are essential for the correct assembly and functioning of photosystems and protect the photosynthetic machinery from excessive light (Cazzonelli, 2011; Esteban et al., 2014; Havaux, 1998). They afford protection against photo-oxidative damage preventing and quenching ROS generated from triplet excited chlorophylls (^{3}Chl*; **Fig. 1**). Xanthophylls mediated non-photochemical quenching (NPQ) limits the formation of ^{3}Chl* from ^{1}Chl* (chlorophyll singlet excited state, Demmig-Adams, 1998). Briefly, cycles involved in the light-driven dynamic inter-conversion of xanthophylls are the VAZ cycle (VAZ, violaxanthin-antheraxanthin-zeaxanthin) and the Lx cycle (Lx, lutein epoxide), in which epoxided xanthophylls (violaxanthin and lutein epoxide) are converted to corresponding de-epoxided forms (antheraxanthin, zeaxanthin and lutein). Then changes in xanthophyll composition during dark-to-sun transition allow carotenoids to serve strikingly different functions from light harvesting to its dissipation

199 through NPQ (Ruban and Johnson, 2010), thus equipping leaves with flexible mechanisms to manage
200 properly radiant energy reaching PSII (Demmig-Adams and Adams, 2006).



201 **Fig. 1.** A proposed model for the integrated actions of isoprene, carotenoids, and flavonoids in leaves
202 suffering from a severe excess of radiant energy, following the possible depletion of chloroplastic antioxidant
203 defences (e.g. ascorbate peroxidase and ascorbic acid). Consumption of DMADP for isoprene biosynthesis channels
204 more carbon into the whole MEP pathway in full sunlight growing plants concomitantly challenged against heat
205 and drought stress. Isoprene improves the thermostability of thylakoids. Isoprene-induced enhancement of
206 carotenoids biosynthesis equips leaves with a versatile system that prevents the generation of ROS and quench
207 ROS once they are formed. De-epoxidation of violaxanthin to zeaxanthin, in addition to avoid the formation of
208 triplet chlorophyll (^{3}Chl*) from singlet chlorophyll (S^*_1, via NPQ), confers rigidity to thylakoid membranes, and
209 prevents lipid peroxidation. Carotenoids (car) also quench, physically and chemically singlet oxygen ($^1O_2$) through
210 dissipation of highly energetic molecular oxygen ($^1O_2$-$^3O_2$ transition) and direct $^1O_2$-oxidation of β-carotene (and
211 zeaxanthin). Isoprene-induced activation of the MEP pathway also promotes the biosynthesis of abscisic acid (ABA)
212 and, in turn the biosynthesis of flavonoids.

213

De-epoxidation of violaxanthin to zeaxanthin may have, however, a more subtle role in chloroplasts suffering from severe excess of radiant energy. There is compelling evidence that zeaxanthin may play antioxidant functions when the photosynthetic capacity of high light-grown leaves is severely constrained by concurrent stress agents, such as drought and salinity (Fini et al., 2014; Beckett et al., 2012). Indeed, these are the very conditions that steeply enhance the pool of violaxanthin-cycle pigments (VAZ) relative to the chlorophyll pool (Chl_{tot}). Zeaxanthin may therefore derive from 'free' - unbound to light-harvesting chlorophyll-protein complexes - violaxanthin, when the concentration of VAZ relative to Chl_{tot} concentration exceeds 50 mmol mol^{-1} (Havaux and Niyogy, 1999; Niinemets et al., 2003), as commonly observed in high light-grown leaves (Esteban et al., 2014). Zeaxanthin may therefore resides in other parts of the thylakoids thus conferring rigidity to the lipid bilayer membranes (**Fig. 1**), enhancing their thermo-stability during severe events of drought and heat stress, and in turn preventing membrane lipid peroxidation (Havaux, 1998; Havaux et al., 2007; Beckett et al., 2012; Tattini et al., 2014a). Beckett et al. (2012) observed that in *Xerophyta humilis* at very severe dehydration (RWC at 5%) chlorophyll levels became negligible whereas zeaxanthin accumulated to very high concentrations. Authors offered intriguing hypothesis that zeaxanthin tightly associated to thylakoid membranes preserved chloroplast from irreversible disruption, and allowed a prompt recovery of the photosynthetic apparatus when water was newly available to plants. Relevantly, zeaxanthin can also derive through direct hydroxylation of β-carotene (**Fig. 1**) when plants suffer from a wide range of stress agents, including high light, heat and drought stress (Davison et al., 2002; Du et al., 2010). Such conversion of β-carotene to zeaxanthin offers further stability to thylakoid membranes, as β-carotene is mostly involved in enhancing thylakoid membrane fluidity (consistent with its outstanding significance in cold acclimation, Havaux, 1998).

The magnitude of the reduction of ^{3}Chl* to its ground state by carotenoids depends, obviously, from the excess of radiant energy reaching the photosynthetic apparatus. Although ^{3}Chl* thermally decay to ground states in few μs, this time is long enough to generate the most dangerous chloroplast oxidant, i.e. singlet oxygen ($^1O_2$). Carotenoids have the peculiar capacity, among the wide array of chloroplast antioxidants, to quench $^1O_2$, both physically (Triantaphylidès and Havaux, 2009, Alboresi et al., 2011) and chemically (Ramel et al., 2012a), once it is formed. β-carotene, and to a lower degree zeaxanthin 'reduce' $^1O_2$ to its triplet state ($^3O_2$), producing a wide range of oxidation products, mostly endo-peroxides and volatile short-chain molecules (**Fig. 1** Ramel et al., 2012b, Havaux, 2014). Since β-carotene is exclusively located in the reaction centres, the major site of $^1O_2$ generation, β- carotene-5, 8-endoperoxide has been proposed as an 'early marker' of $^1O_2$-induced oxidative stress (Ramel et al., 2012b). β-carotene also produces volatile derivatives, mainly β-cyclocitral and dihydro-actinidiolide

(Ramel et al., 2012a,b; Shumbe et al., 2014). These carbonyl by-products are very reactive, thus supporting recent hypotheses that they may serve as secondary messengers in $^1O_2$ signalling, and significantly contribute to chloroplast-to-nucleus retrograde signalling under severe photooxidative stress (Laloi and Havaux, 2015; Ramel et al., 2012b; Shumbe et al., 2014).

3.3. Isoprene and carotenoids complement primary antioxidant defences in high light stressed leaves on daily basis

Fig. 2 shows daily changes in chloroplastic APX, isoprene and zeaxanthin in leaves of the isoprene emitting *Q. pubescens* growing in full sunlight, under either optimal irrigation or suffering from drought stress of increasing severity, during Mediterranean summer. As mentioned above, the activity of APX, which increased in response to mild to moderate drought, declined steeply at severe drought. In contrast, isoprene emission, and particularly zeaxanthin concentration increased in drought stressed leaves. All antioxidants had strikingly different daily variations in both well watered and drought stressed leaves. Interestingly, the activity of APX declined during the hottest hours of the day (in both well watered and drought stressed leaves), particularly in severely drought stressed leaves. On the contrary, isoprene biosynthesis was highest from 12:00 to 16:00 hrs, and zeaxanthin concentration was at its maximum at 12:00 h. Therefore, isoprene and zeaxanthin might play roles of increasing significance to preserve chloroplast from photo-damage, when leaves concurrently faced with multiple stressors (i.e. high solar irradiance, high air temperature and severe drought stress). This suggestion conforms to the notion that: (1) zeaxanthin biosynthesis is mostly activated in response to sunlight irradiance; (2) isoprene biosynthesis is stimulated by high air temperature; (3) a combination of high sunlight irradiance and high air temperature, broadly excess light stress, may depress the activity of APX.

There is increasing interest in understanding how plants cope with such a multiple stress condition, in view of future climate change (Flexas et al., 2014; Matesanz and Valladares, 2014; Tattini & Loreto 2014). This asks for future studies aimed at exploring how plants sense and respond to signals originated from multiple stress agents, at both physiological and biochemical levels, under field conditions. The ability of most species, which have not been evolved in harsh environments, to withstand intense, even transient stress events typical of future climate tightly depends on the so-called "metabolic plasticity" (Logemann et al., 2000). Our reasoning, suggests that 'secondary' metabolites, which are key components of the metabolic suite of plants, may serve functions of increasing significance for the survival of plants experiencing a severe excess of sunlight irradiance.

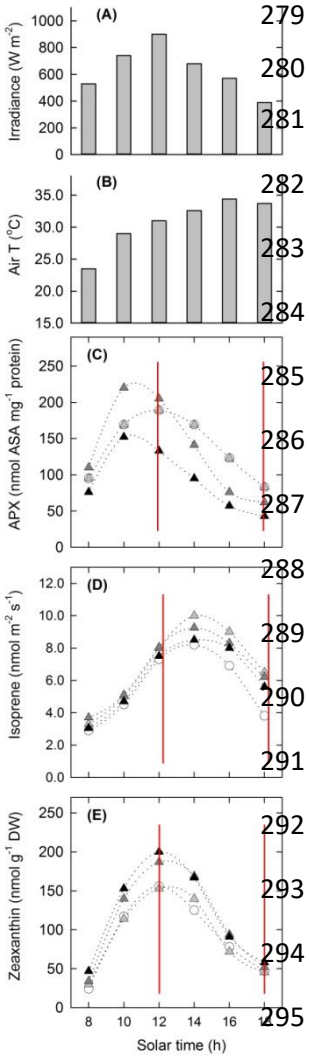


Fig. 2. Daily variation of: (A) sunlight irradiance, estimated over 200-3,000 nm spectral region and peaked approx. 900 Wm-2 at midday; (B) air temperature (min/max ranged from 19.7 ± 2.2 to 32.8 ± 2.7 °C) to which *Q. pubescens* plants were exposed during July 2014; the activity of ascorbate peroxidase (APX, C), the emission of isoprene (D) and the concentration of zeaxanthin (E) in leaves of plants growing under either well-watered (open circles) or drought stress conditions of increasing severity. Drought stress was imposed by withholding water for five (grey triangles), 10(dark grey).
triangles) or 15 days (closed triangles). Vertical red lines have been reported for illustrative purposes only.

4. The peculiar, still unappreciated antioxidant functions of flavonoids in high light-stressed plants

Flavonoids accumulate in a range of tissues and subcellular compartments, thus having the potential to reduce photo-oxidative damage in all order of plants (for a review see Agati et al., 2012). UV-absorbing flavonoids located in epidermal cells strongly reduce highly energetic solar wavelengths from reaching ROS-generating cells, and the consequential photo-oxidative stress and damage (Tattini et al., 2005). Nuclear located dihydroxy B-ring-substituted flavonols (e.g. quercetin, Agati et al., 2012) may effectively chelate Fe and Cu ions, thus avoiding the generation of the extraordinary reactive hydroxyl radical, when H_2O_2 may freely escape from the chloroplast under severe excess light stress conditions (Hernández et al., 2009). Nuclear-located flavonols in addition to preserve DNA from photo-oxidative

damage may also fine-tune the H_2O_2-induced signaling cascades that involve MAPKs. Flavonols have also the capacity to regulate the MAPK activity by directly competing with the ATP binding sites of the proteins (Pollastri and Tattini, 2011). The potential of nuclear flavonols in regulating key steps of cell growth and differentiation under photo-oxidative stress has not been deeply investigated, possibly because of technical difficulties on their visualization (Agati et al., 2012; Brunetti et al., 2013; Polster et al., 2006). However, the matter is intriguing, as key enzymes of flavonol biosynthesis have been also detected in the nucleus (Saslowsky et al. 2005; Kuhn et al., 2011).

Agati et al. (2007), comparing shade- and full sun-adapted leaves of *P. latifolia*, gave compelling evidence that chloroplast located flavonols quenched $^1O_2$ generated in leaf cross sections exposed to severe excess of blue light. Light stressed shade and sun leaves did not differ for the actual efficiency of PSII photochemistry. Authors therefore hypothesized that flavonols were mostly involved in the $^1O_2$ quenching under these specific conditions. In-depth fluorescence-microscopy analyses have revealed that flavonols have probably a location in the chloroplast outer envelope membrane (Agati et al. 2007). Flavonols have the capacity to stabilize membranes, including membranes that contain non-bilayer lipids, such as monogalactosyl diacyl glycerol (MGDG) (Scheidt et al. 2004). Taking into account that the cytoplasmic side of OEM is poor in MGDG (Moellering and Benning, 2010), and that during dehydration there is highly specific decrease in MGDG at the OEM, flavonols may preserve the integrity of OEM, thus preventing the chloroplast from irreversible oxidative damage. The significance of flavonols as chloroplast antioxidants might increase in leaves experiencing severe excess light stress, when other ROS-detoxifying systems have been already compromised.

Nonetheless, the actual significance of flavonoids as antioxidants in high light-stressed leaves is a long-standing question, as these secondary metabolites are mostly confined in the vacuole, in which the risk of photo-oxidative stress is much less as compared to the chloroplast or the peroxisome. Whether vacuolar flavonoids may play a role in the whole-cell redox homeostasis has been a matter of intense conflicts during the last decade (Mittler et al., 2004; Hernández et al., 2009; Agati and Tattini, 2010, Ferreres et al., 2011; Agati et al., 2012). Recent experiments have shed new light on this complex matter, starting from clear evidence that H_2O_2 may cross the tonoplast membrane and enter the vacuole using aquaporins (Bienert et al., 2006, 2007, 2014). This further corroborates early hypothesis that flavonoids, particularly UV-responsive dihydroxy B-ring substituted flavonols may effectively quench H_2O_2 serving as substrates for vacuolar peroxidases (POX, Yamasaki et al., 1997; for a review see Takahama, 2004). Furthermore, there is compelling evidence that under severe conditions of excess light stress, declines in the activity of APX and CAT are paralleled by concomitant increases in the activity of POX and in the concentration of dihydroxy B-ring-substituted flavonols (Agati et al., 2011, Fini et al.,

2011, 2012, 2014). An in-depth analysis of subcellular compartmentation of ascorbate and glutathione add further insights on the antioxidant machinery that may operate in the vacuole in high light and drought stressed plants (Zechmann et al., 2011; Koffler et al., 2014). These authors have shown that the decrease in ASA and GSH in both experiments regards the chloroplast, while the concentration of ASA steeply increases in the vacuole. Since ASA has a much lower both ability to quench directly H_2O_2 and affinity for vacuolar POX than flavonols, it is supposed that H_2O_2 is scavenged by POX using flavonols as substrates, and then flavonoid radicals are recycled back to their reduced forms. This is the classical Takahama/Yamasaki (Yamasaki et al, 1997; Takahama et al., 2004) model, further corroborated more recently by studies conducted by Ferreres et al. (2011). Recent experiments conducted in sun-adapted *F. ornus* leaves reveal that coumarins accumulate as vacuolar inclusions in mesophyll cells, and that the antioxidant esculetin is preferentially distributed in the vacuolar portion proximal to the adaxial epidermis, whereas the poor-antioxidant, but effective UV-screener esculin was confined deeper in the vacuole (Tattini et al., 2014b). This finding supports, from one hand that vacuolar phenylpropanoids may serve as effective antioxidants in high light-stressed leaves, but from the other hand, highlights how many questions are still open concerning the transport mechanisms and the distribution of these vast class secondary metabolites in the vacuole.

5. Is ABA the missing link between isoprenoid and phenylpropanoid metabolism in plants suffering from severe excess light stress?

There is evidence that the isoprenoid and phenylpropanoid metabolism are inter-related, although both pathways are involved in the synthesis of specialized metabolites (Behnke et al., 2010; Zvi et al., 2012; Tattini et al., 2014a). Introduction of *PRODUCTION OD ANTHOCYANIN PIGMENT1 (PAP1)* in *Rosa hybrida* enhanced the production of volatile terpenoids (Zvi et al., 2012). A decline in phenylpropanoid biosynthesis was observed in transgenic poplar with suppressed isoprene biosynthesis (Behnke et al., 2010), and suggested as partially responsible for the greater oxidative damage suffered by transgenic than wild-type plants challenged against heat stress. Recently, in transgenic isoprene-emitting lines of tobacco the biosynthesis of phenylpropanoids (caffeic acid derivatives and flavonol glycosides) was significantly greater than in non-emitting lines in response to drought stress (Tattini et al., 2014a). Authors also observed that in isoprene-emitting lines the biosynthesis of ABA increased to a much greater degree than in non-emitting lines in response to drought stress, without a concomitant greater reduction in stomatal conductance and net assimilation rate. It was therefore hypothesized that isoprene biosynthesis under severe drought channels more carbon in the whole MEP pathway, thus stimulating ABA biosynthesis (**Fig. 1**). These findings conform to previous suggestions that isoprene may be a proxy of ABA (Barta and Loreto, 2006).

374 The stress-responsive phyto-hormone ABA plays multifarious roles in plant-environment
375 interaction, which go well beyond its control of stomata movements in response to changes in soil water
376 availability (Rook et al., 2006; Takezawa et al., 2011). Interestingly, sunlight, particularly UV-irradiance,
377 also promotes the ABA biosynthesis (Maruta et al., 2012; Lee et al., 2012; Tossi et al., 2012). The
378 biosynthesis of ABA in *Arabidopsis* during dark-to-high light transition originated through β-
379 deglucosylation of ABA-GE in the absence of hydraulic signal, which is long known to be responsible for
380 the activation of β-glucosidase (Lee et al., 2006; Jiang and Hartung, 2008). This conforms to previous
381 suggestions (Barta and Loreto, 2006; Christmann et al., 2007) that increase in foliar ABA concentration is
382 not only through the transport of ABA loaded in the root and leaf xylem, but directly through the
383 activation of the MEP pathway in the leaf. Therefore, ABA biosynthesis is stimulated in leaves suffering
384 from a severe excess of sunlight, the very conditions that also activate the biosynthesis of
385 phenylpropanoids.

386 It is long known that the ABA-signalling network indeed profoundly alters the metabolic
387 machinery of plants in response to a wide array of environmental stimuli. Dissecting individual
388 components of this network is out of the scope of this article, but for detailed reviews see Cutler et al.
389 (2010) and Hauser et al. (2011). The flavonoid biosynthesis is just one, though relevant, of the
390 extraordinary high number of metabolic pathways that are potentially regulated by the ABA signalling
391 network. Although ABA regulation of flavonoid biosynthesis has mostly regarded anthocyanin
392 biosynthesis (Wheeler et al., 2009; Medina-Puche et al., 2014), there is also compelling evidence that a
393 rise in foliar ABA is paralleled by an enhanced biosynthesis of UV-absorbing flavonoids, such as
394 kaempferol and quercetin derivatives (Castellarin et al., 2007; Berli et al., 2011; Perrone et al., 2012;
395 Tattini et al., 2014a).

396 It is therefore conceivable that the ABA-signaling network may constitute an important hub that
397 tightly regulates fundamental biochemical, not only physiological adjustments of plants that
398 concurrently face conditions that strongly limit the use of radiant energy to photosynthetic processes,
399 exactly plants facing severe excess light stress. In detail, we speculate that environmental-induced
400 enhancement in the isoprenoid, and hence in ABA biosynthesis, might consequentially result in the
401 biosynthesis of phenylpropanoids, particularly flavonoids (**Fig. 1**). The potential tight interrelation
402 between isoprenoid and phenylpropanoid metabolism deserves future experimentation, but open new
403 scenarios on the functional roles of secondary metabolites in plants severely stressed by an excess of
404 sunlight irradiance.

405

6. Primary vs. secondary antioxidant defences: Does it really matter?

We are aware our view on the relative significance of individual components of the antioxidant machinery of plants facing severe photo-oxidative stress has been from the secondary metabolites side. However, we are also aware that primary low molecular-weight antioxidants are closely related with secondary metabolite biosynthesis. ASA is a cofactor of violaxanthin de-epoxidase, which indeed assists zeaxanthin biosynthesis (Bratt et al., 1995; Forti et al., 1999). ASA is also a cofactor of 2-oxoacid-dependent dioxygenases involved in the synthesis of abscisic acid (Qin and Zeevaart, 1999) as well as of several enzymes involved in flavonoid biosynthesis (including anthocyanidin synthase, flavone 3-hydroxylase and flavonol synthase, Şahin and De Tullio, 2010). These findings also conform to early observations that enzymes involved in the phenylpropanoid metabolism have originated from those ancestrally regulating primary metabolism (Rausher, 2006). As reported above, ASA is also a key component of the H2O2-detoxifying system that operate in the vacuole (Ferreres et al., 2011). We also acknowledge relevant studies (e.g., from Dr. Foyer's lab, Foyer and Noctor, 2012, 2013), which highlight the relevance of redox couples (ASA/ASAH; GS/GSH) in stress-induced metabolic adjustments. Taylor and Grotewold (2005) offered the interesting hypothesis that the redox potential of the cell might tightly regulate the biosynthesis of flavonols, as R2R3MYBs transcription factors (well-known regulators of flavonoid biosynthesis, Dubos et al., 2010) are redox-controlled.

Here our main objective has been to pose the question of what may happen to the antioxidant machinery of high light-growing plants when concurrently challenged against stress agents that severely constrains their capacity to 'use' safely radiant energy. We have offered evidence that primary and secondary antioxidants are closely interconnected on daily basis in well-watered plants growing in full sunlight and high air temperature. The antioxidant functions of secondary metabolites might assume increasing significance when plants also face drought stress of increasing severity, particularly during the hottest hours of the day. Our critical review may open to new experimentation to unveil how plants may orchestrate individual components of the antioxidant machinery to cope with extreme, event transient stress events in the field. The survival of most plants not evolved in harsh environments when suffering from unpredictable environmental stressors greatly depends on their 'metabolic plasticity', and secondary metabolites might serve roles of primary significance, when severe limitations to the usage of radiant energy to photosynthesis lead to transient impairments of primary antioxidant defenses.

Acknowledgments

Work in the authors' laboratory was funded by the PRIN Project TreeCity (MIUR, Rome, Italy).

References

439 Affek, H. P., Yakir D. 2002. Protection by isoprene against singlet oxygen in leaves. Plant Physiol. 129,
440 269–77.

441 Agati, G., Azzarello, E., Pollastri, S., Tattini, M. 2012. Flavonoids as antioxidants in plants: location and
442 functional significance. Plant Science 196, 67-76.

443 Agati, G., Brunetti, C., Di Ferdinando, M., Ferrini, F., Pollastri, S., Tattini, M. 2013. Functional roles of
444 flavonoids in photoprotection: new evidence, lessons from the past. Plant Physiol. Biochem.72, 35-45.

445 Agati, G., Cerovic, Z. C., Pinelli, P., Tattini, M. 2011. Light-induced accumulation of ortho-dihydroxylated
446 flavonoids as non-destructively monitored by chlorophyll fluorescence excitation techniques. Environ.
447 Exp. Bot., 73, 3–9.

448 Agati, G., Matteini, P., Goti, A., Tattini M. 2007. Chloroplast-located flavonoids can scavenge singlet
449 oxygen. New Phytol. 174, 77–89.

450 Agati, G., Tattini, M., 2010. Multiple functional roles of flavonoids in photoprotection. New Phytol. 186,
451 786–93.

452 Alboresi, A., Dall'Osto, L., Aprile, A., Carillo, P., Roncaglia, E., Cattivelli, L., Bassi, R. 2011. Reactive oxygen
453 species and transcript analysis upon excess light treatment in wild type Arabidopsis thaliana vs
454 photosensitive mutant lacking zeaxanthin and lutein. BMC Plant Biol. 11, 62-84.

455 Apel, K., Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction.
456 Annu. Rev. Plant Biol. 55, 373-399.

457 Banerjee, A., Wu Y., Banerjee, R., Li, Y., Yan, H., Sharkey, T. D., 2013. Feedback inhibition of deoxy-d-
458 xylulose-5-phosphate synthase regulates the methylerythritol 4-phosphate pathway." J. Biol. Chem. 288,
459 16926–36.

460 Barba-Espín, G., Diaz-Vivancos, P., Job, D., Belghazi, M., Job, C., Hernández, J.A. 2011. Understanding the
461 role of H2O2 during pea seed germination: a combined proteomic and hormone profiling approach.
462 Plant Cell Environ. 34, 1907-1919.

463 Barta, C., Loreto F. 2006. The relationship between the methyl-erythritol phosphate pathway leading to
464 emission of volatile isoprenoids and abscisic acid content in leaves. Plant Physiol. 141, 1676–83.

465 Beckett, M., Loreto, F., Velikova, V., Brunetti, C., Di Ferdinando, M., Tattini, M., Calfapietra, C., Farrant,
466 J.M., 2012. Photosynthetic limitations and volatile and non-volatile isoprenoids in the
467 poikilochlorophyllous resurrection plant Xerophyta humilis during dehydration and rehydration. Plant.
468 Cell Environ. 35, 2061–2074.

469 Behnke, K., Ehlting, B., Teuber, M., Bauerfeind, M., Louis, S., Hänsch, R., Polle, A., Bohlmann, J.,
470 Schnitzler, J.-P., 2007. Transgenic, non-isoprene emitting poplars don't like it hot. Plant J. 51, 485–499.

471 Behnke, K., Kaiser, A., Zimmer, I., Brüggemann, N., Janz, D., Polle, A., Hampp, R., Hänsch, R., Popko, J.,
472 Schmitt-Kopplin, P., Ehlting, B., Rennenberg, H., Barta, C., Loreto, F., Schnitzler, J.-P., 2010. RNAi-
473 mediated suppression of isoprene emission in poplar transiently impacts phenolic metabolism under

474 high temperature and high light intensities: a transcriptomic and metabolomic analysis. Plant Mol. Biol.
475 74, 61–75.

476 Berli, F.J., Fanzone, M., Piccoli, P., Bottini, R., 2011. Solar UV-B and ABA are involved in phenol
477 metabolism of Vitis vinifera L. increasing biosynthesis of berry skin polyphenols. J. Agric. Food Chem. 59,
478 4874–4884.

479 Bienert, G.P., Chaumont, F., 2014. Aquaporin-facilitated transmembrane diffusion of hydrogen peroxide.
480 Biochim. Biophys. Acta, 1840, 1596–1604.

481 Bienert, G.P., Møller, A.L.B., Kristiansen, K.A., Schulz, A., Møller, I.M., Schjoerring, J.K., Jahn, T.P., 2007.
482 Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. J. Biol. Chem. 282,
483 1183–1192.

484 Bienert, G.P., Schjoerring, J.K., Jahn, T.P., 2006. Membrane transport of hydrogen peroxide. Biochim.
485 Biophys. Acta 1758, 994–1003.

486 Bratt, C.E., Arvidsson, P.-O., Carlsson, M., Åkerlund, H.-E., 1995. Regulation of violaxanthin de-epoxidase
487 activity by pH and ascorbate concentration. Photosynth. Res. 45, 169–175.

488 Brüggemann, N., Schnitzler, J.-P., 2002. Relationship of isopentenyl diphosphate (IDP) isomerase activity
489 to isoprene emission of oak leaves. Tree Physiol. 22, 1011–1018.

490 Brunetti, C., Di Ferdinando, M., Fini, A., Pollastri, S., Tattini, M. 2013. Flavonoids as antioxidants and
491 developmental regulators: relative significance in plants and humans. Int. J. Mol. Sci., 14, 3540-3555.

492 Casano, L. M., Gomez, L. D., Lascano, H. R., Gonzalez, C. A., Trippi, V. S., 1997. Inactivation and
493 degradation of CuZn-SOD by active oxygen species in wheat chloroplasts exposed to photooxidative
494 stress. Plant Cell Physiol. 38, 433-440.

495 Castellarin, S.D., Gaspero, G. Di, 2007. Transcriptional control of anthocyanin biosynthetic genes in
496 extreme phenotypes for berry pigmentation of naturally occurring grapevines. BMC Plant Biol. 7, 46.

497 Cazzonelli, C.I., 2011. Carotenoids in nature: insights from plants and beyond. Funct. Plant Biol. 38, 833–
498 847.

499 Cen, Y.P., Sage, R.F., 2005. The regulation of rubisco activity in response to variation in temperature and
500 atmospheric CO2 partial pressure in sweet potato. Plant Physiol. 139, 979–990.

501 Cheeseman, J.M., 2006. Hydrogen peroxide concentrations in leaves under natural conditions. J. Exp.
502 Bot. 57, 2435-2444.

503 Christmann, A., Weiler, E.W., Steudle, E., Grill, E., 2007. A hydraulic signal in root-to-shoot signalling of
504 water shortage. Plant J. 52, 167–174.

505 Cutler, S.R., Rodriguez, P.L., Finkelstein, R.R., Abrams, S.R., 2010. Abscisic acid: emergence of a core
506 signaling network. Annu. Rev. Plant Biol. 61, 651–79.

507 Davison, P.A., Hunter, C.N., Horton, P., 2002. Overexpression of β-carotene hydroxylase enhances stress
508 tolerance in Arabidopsis. Nature 418, 203–206.

509 de Dios Barajas-López, J., Blanco, N. E., Strand, Å. 2013. Plastid-to-nucleus communication, signals
510 controlling the running of the plant cell.Biochimica et Biophysica Acta. 1833, 425-437.

511 De la Haba, P., De la Mata, L., Molina, E., Agüera, E., 2014. High temperature promotes early senescence
512 in primary leaves of sunflower (Helianthus annuus L.) plants. Can. J. Plant Sci. 94, 659-669.

513 De Pinto, M.C., Locato, V., De Gara, L., 2012. Redox regulation in plant programmed cell death. Plant Cell
514 Environ. 35, 234-244.

515 Demmig-Adams, B., 1998. Survey of thermal energy dissipation and pigment composition in sun and
516 shade leaves. Plant Cell Physiol. 39, 474–482.

517 Demmig-Adams, B., Adams, W. W., 2006. Photoprotection in an ecological context: the remarkable
518 complexity of thermal energy dissipation. New Phytol. 172, 11-21.

519 Du, H., Wang, N., Cui, F., Li, X., Xiao, J., Xiong, L., 2010. Characterization of the β-carotene hydroxylase
520 gene DSM2 conferring drought and oxidative stress resistance by increasing xanthophylls and abscisic
521 acid synthesis in rice. Plant Physiol. 154, 1304–1318.

522 Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C., Lepiniec, L., 2010. MYB transcription
523 factors in Arabidopsis. Trends Plant Sci. 15, 573–581.

524 Esteban, R., Fleta-Soriano, E., Buezo, J., Míguez, F., Becerril, J.M., García-Plazaola, J.I., 2014.
525 Enhancement of zeaxanthin in two-steps by environmental stress induction in rocket and spinach. Food
526 Res. Int. 65, Part B, 207–214.

527 Ferreres, F., Figueiredo, R., Bettencourt, S., Carqueijeiro, I., Oliveira, J., Gil-Izquierdo, A., Pereira, D.M.,
528 Valentão, P., Andrade, P.B., Duarte, P., Barceló, A.R., Sottomayor, M., 2011. Identification of phenolic
529 compounds in isolated vacuoles of the medicinal plant Catharanthus roseus and their interaction with
530 vacuolar class III peroxidase: an H2O2 affair? J. Exp. Bot. 62, 2841–2854.

531 Fini, A., Brunetti, C., Di Ferdinando, M., Ferrini, F., Tattini, M., 2011. Stress-induced flavonoid
532 biosynthesis and the antioxidant machinery of plants. Plant Signal. Behav. 6, 709–711.

533 Fini, A., Guidi, L., Ferrini, F., Brunetti, C., Di Ferdinando, M., Biricolti, S., Pollastri, S., Calamai, L., Tattini,
534 M., 2012. Drought stress has contrasting effects on antioxidant enzymes activity and phenylpropanoid
535 biosynthesis in Fraxinus ornus leaves: An excess light stress affair? J. Plant Physiol. 169, 929–939.

536 Fini, A., Guidi, L., Giordano, C., Baratto, M.C., Ferrini, F., Brunetti, C., Calamai, L., Tattini, M., 2014.
537 Salinity stress constrains photosynthesis in Fraxinus ornus more when growing in partial shading than in
538 full sunlight: consequences for the antioxidant defence system. Ann. Bot. 114, 525–538.

539 Fischer, B.B., Krieger-Liszkay, A., Hideg, É., Šnyrychová, I., Wiesendanger, M., Eggen, RI.L., 2007. Role of
540 singlet oxygen in chloroplast to nucleus retrograde signaling in Chlamydomonas reinhardtii. FEBS Letters
541 581, 5555-5560.

542 Flexas, J., Diaz-Espejo, A., Gago, J., Gallé, A., Galmés, J., Gulías, J., Medrano, H., 2014. Photosynthetic
543 limitations in Mediterranean plants: A review. Environ. Exp. Bot. 103, 12–23.

544 Foreman, J., Demidchik, V., Bothwell, J. H., Mylona, P., Miedema, H., Torres, M. A., Linstead, P., Costa, S.,
545 Brownlee, C., Jones D.G. J., Davies, M. J., Dolan, L., 2003. Reactive oxygen species produced by NADPH
546 oxidase regulate plant cell growth. Nature 422, 442-446.

547 Forti, G., Barbagallo, R.P., Inversini, B., 1999. The role of ascorbate in the protection of thylakoids
548 against photoinactivation. Photosynth. Res. 59, 215–222.

549 Foyer, C. H., Lopez-Delgado, H., Dat, J. F., Scott, I. M., 1997. Hydrogen peroxide-and
550 glutathione-associated mechanisms of acclimatory stress tolerance and signalling. Physiol. Plantarum
551 100, 241-254.

552 Foyer, C. H., Noctor, G., 2012. Managing the cellular redox hub in photosynthetic organisms. Plant Cell
553 Environ.35, 199-201.

554 Foyer, C. H., Noctor, G., 2013. Redox signaling in plants. Antioxid Redox Sign 18, 2087-2090.

555 Foyer, C., Noctor, G., 2015. Defining robust redox signalling within the context of the plant cell. Plant
556 Cell Environ 38, 239.

557 Foyer, C.H., Shigeoka, S., 2011. Understanding oxidative stress and antioxidant functions to enhance
558 photosynthesis. Plant Physiol. 155, 93-100.

559 Ghirardo, A., Wright, L.P., Bi, Z., Rosenkranz, M., Pulido, P., Rodríguez-Concepción, M., Niinemets, Ü.,
560 Brüggemann, N., Gershenzon, J., Schnitzler, J.P., 2014. Metabolic flux analysis of plastidic isoprenoid
561 biosynthesis in poplar leaves emitting and nonemitting isoprene. Plant Physiol. 165, 37–51.

562 Guo, Z., Ou, W., Lu, S., & Zhong, Q., 2006. Differential responses of antioxidative system to chilling and
563 drought in four rice cultivars differing in sensitivity. Plant Physiol. Biochem. 44, 828-836.

564 Halliwell, B, Gutteridge, J.M.C., 1989. Protection against oxidants in biological systems: The super oxide
565 theory of oxygen toxicity. In: HalliwellB, GutteridgeJMC (eds) Free Radicals in Biology and Medicine.
566 Clarendon Press, Oxford, pp 86–123.

567 Halliwell, B., 2009. The wanderings of a free radical. Free Radical Bio. Med. 46, 531-542.

568 Harrison, S.P., Morfopoulos, C., Dani, K.G.S., Prentice, I.C., Arneth, A., Atwell, B.J., Barkley, M.P.,
569 Leishman, M.R., Loreto, F., Medlyn, B.E., Niinemets, Ü., Possell, M., Peñuelas, J., Wright, I.J., 2013.
570 Volatile isoprenoid emissions from plastid to planet. New Phytol. 197, 49-57.

571 Hatier, J. H. B., Gould, K. S., 2008. Foliar anthocyanins as modulators of stress signals. J. Theor. Biol. 253,
572 625-627.

573 Hauser, F., Waadt, R., Schroeder, J.I., 2011. Evolution of abscisic acid synthesis and signaling
574 mechanisms. Curr. Biol. 21, R346–R355.

575 Havaux, M. 2014. Carotenoid oxidation products as stress signals in plants. Plant J. 79, 597-606.

576 Havaux, M., 1998. Carotenoids as membrane stabilizers in chloroplasts. Trends Plant Sci. 3, 147–151.

577 Havaux, M., Dall'Osto, L., Bassi, R., 2007. Zeaxanthin has enhanced antioxidant capacity with respect to
578 all other xanthophylls in Arabidopsis leaves and functions independent of binding to PSII aantennae.
579 Plant Physiol. 145, 1506–1520.

580 Havaux, M., Niyogi, K.K., 1999. The violaxanthin cycle protects plants from photooxidative damage by
581 more than one mechanism. Proc. Natl. Acad. Sci. 96, 8762–8767.

582 Herbinger, K., Tausz, M., Wonisch, A., Soja, G., Sorger, A., Grill, D., 2002. Complex interactive effects of
583 drought and ozone stress on the antioxidant defence systems of two wheat cultivars. Plant Physiol.
584 Biochem. 40, 691-696.

585 Hernández, I., Alegre, L., Van Breusegem, F., Munné-Bosch, S., 2009. How relevant are flavonoids as
586 antioxidants in plants? Trends Plant Sci. 14, 125–132.

587 Hernández, J.A., Aguilar, A., Portillo, B., López-Gómez, E., Mataix Beneyto, J., García-Legaz, M.F., 2003.
588 The effect of calcium on the antioxidant enzymes from salt-treated loquat and anger plants. Funct. Plant
589 Biol. 30, 1127-1137.

590 Hernández, J.A., Almansa, M.S. 2002. Short-term effects of salt stress on antioxidant systems and leaf
591 water relations of pea leaves. Physiol. Plantarum 115, 251-257.

592 Jiang, F., Hartung, W., 2008. Long-distance signalling of abscisic acid (ABA): the factors regulating the
593 intensity of the ABA signal. J. Exp. Bot. 59, 37–43.

594 Kim, C., Meskauskiene, R., Apel, K., Laloi, C., 2008. No single way to understand singlet oxygen signalling
595 in plants. EMBO reports 9, 435-439.

596 Koffler, B.E., Luschin-Ebengreuth, N., Stabentheiner, E., Müller, M., Zechmann, B., 2014. Compartment
597 specific response of antioxidants to drought stress in Arabidopsis. Plant Sci. 227, 133–144.

598 Kovtun, Y., Chiu, W. L., Tena, G., Sheen, J., 2000. Functional analysis of oxidative stress-activated
599 mitogen-activated protein kinase cascade in plants. Proc. Natl. Acad. Sci. 97, 2940-2945.

600 Kuhn, B. M., Geisler, M., Bigler, L., Ringli, C., 2011. Flavonols accumulate asymmetrically and affect auxin
601 transport in Arabidopsis. Plant Physiol., 156, 585-595.

602 Laloi, C., Havaux, M., 2015. Key players of singlet oxygen-induced cell death in plants. Plant Physiol. 6,
603 39.

604 Lee, K. H., Piao, H. L., Kim, H. Y., Choi, S. M., Jiang, F., Hartung, W., Hwang, I., Kwak, J.M., Lee, I.J.,
605 Hwang, I. 2006. Activation of glucosidase via stress-induced polymerization rapidly increases active
606 pools of abscisic acid. Cell, 126, 1109-1120.

607 Lee, S.C., Luan, S., 2012. ABA signal transduction at the crossroad of biotic and abiotic stress responses.
608 Plant. Cell Environ. 35, 53–60.

609 Logemann, E., Tavernaro, A., Schulz, W., Somssich, I.E., Hahlbrock, K., 2000. UV light selectively
610 coinduces supply pathways from primary metabolism and flavonoid secondary product formation in
611 parsley. Proc. Natl. Acad. Sci. 97, 1903–1907.

612 Loreto, F., Fineschi, S., 2015. Reconciling functions and evolution of isoprene emission in higher plants.
613 New Phytol. n/a, doi:10.1111/nph.13242

614 Loreto, F., Schnitzler, J.P., 2010. Abiotic stresses and induced BVOCs. Trends Plant Sci. 15, 154–166.

615 Maruta, T., Noshi, M., Tanouchi, A., Tamoi, M., Yabuta, Y., Yoshimura, K., Ishikawa, T., Shigeoka, S.,
616 2012. H2O2-triggered retrograde signaling from chloroplasts to nucleus plays specific role in response to
617 stress. J. Biol. Chem. 287, 11717–11729.

618 Matesanz, S., Valladares, F., 2014. Ecological and evolutionary responses of Mediterranean plants to
619 global change. Environ. Exp. Bot. 103, 53–67.

620 Medina-Puche, L., Cumplido-Laso, G., Amil-Ruiz, F., Hoffmann, T., Ring, L., Rodríguez-Franco, A., ... &
621 Blanco-Portales, R. 2014. MYB10 plays a major role in the regulation of flavonoid/phenylpropanoid
622 metabolism during ripening of Fragaria× ananassa fruits. J. Exp. Bot. 65, 401-417.

623 Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7, 405-410.

624 Mittler, R., Vanderauwera, S., Gollery, M., Van Breusegem, F., 2004. Reactive oxygen gene network of
625 plants. Trends Plant Sci. 9, 490–498.

626 Moellering, E.R., Benning, C., 2010. Galactoglycerolipid metabolism under stress: a time for remodeling.
627 Trends in Plant Sci. 16, 1360-1385.

628 Morfopoulos, C., Prentice, I.C., Keenan, T.F., Friedlingstein, P., Medlyn, B.E., Peñuelas, J., Possell, M.,
629 2013. A unifying conceptual model for the environmental responses of isoprene emissions from plants.
630 Ann. Bot. 112, 1223–1238.

631 Morfopoulos, C., Sperlich, D., Peñuelas, J., Filella, I., Llusià, J., Medlyn, B.E., Niinemets, Ü., Possell, M.,
632 Sun, Z., Prentice, I.C., 2014. A model of plant isoprene emission based on available reducing power
633 captures responses to atmospheric CO2. New Phytol. 203, 125–139.

634 Mubarakshina, M.M., Ivanov, B.N., Naydov, I.A., Hillier, W., Badger, M.R., Krieger-Liszkay, A., 2010.
635 Production and diffusion of chloroplastic H2O2 and its implication to signalling. J. Exp. Bot. 61, 3577-
636 3587.

637 Mullineaux, P., Karpinski, S., 2002. Signal transduction in response to excess light: getting out of the
638 chloroplast. Curr. Opin. Plant Biol. 5, 43-48.

639 Niinemets, Ü., Kollist, H., García-Plazaola, J.I., Hernández, A., Becerril, J.M., 2003. Do the capacity and
640 kinetics for modification of xanthophyll cycle pool size depend on growth irradiance in temperate trees?
641 Plant. Cell Environ. 26, 1787–1801.

642 Niinemets, Ü., Sun, Z., 2015. How light, temperature, and measurement and growth [CO2] interactively
643 control isoprene emission in hybrid aspen. J. Exp. Bot. 66, 841–851.

644 Noctor, G., Mhamdi, A., Foyer, C. H., 2014. The roles of reactive oxygen metabolism in drought: not so
645 cut and dried. Plant Physiol. 164, 1636-1648.

646 Page, M., Sultana, N., Paszkiewicz, K., Florance, H., Smirnoff, N., 2012. The influence of ascorbate on
647 anthocyanin accumulation during high light acclimation in Arabidopsis thaliana: further evidence for
648 redox control of anthocyanin synthesis. Plant Cell Environ. 35, 388-404.

649 Pastori, G. M., Foyer, C. H., 2002. Common components, networks, and pathways of cross-tolerance to
650 stress. The central role of "redox" and abscisic acid-mediated controls. Plant Physiol. 129, 460-468.

651 Peltzer, D., Dreyer, E., Polle, A., 2002. Differential temperature dependencies of antioxidative enzymes
652 in two contrasting species: Fagus sylvatica and Coleus blumei. Plant Physiol. Biochem. 40, 141-150.

653 Peltzer, D., Polle, A., 2001. Diurnal fluctuations of antioxidative systems in leaves of field-grown beech
654 trees (Fagus sylvatica): Responses to light and temperature. Physiol. Plant. 111, 158–164.

655 Perrone, I., Pagliarani, C., Lovisolo, C., Chitarra, W., Roman, F., Schubert, A. 2012. Recovery from water
656 stress affects grape leaf petiole transcriptome. Planta, 235, 1383-1396.

657 Pollastri, S., Tattini, M. 2011. Flavonols: old compounds for old roles. Ann. Bot. 108, 1225-1233

658 Polster, J., Dithmar, H., Burgemeister, R., Friedemann, G., Feucht, W., 2006. Flavonoids in plant nuclei:
659 Detection by laser microdissection and pressure catapulting (LMPC), in vivo staining, and uv–visible
660 spectroscopic titration. Physiol. Plant., 128, 163-174.

661 Qin, X., Zeevaart, J.A.D., 1999. The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of
662 abscisic acid biosynthesis in water-stressed bean. Proc. Natl. Acad. Sci. 96, 15354–15361.

663 Ramel, F., Birtic, S., Cuiné, S., Triantaphylidès, C., Ravanat, J. L., Havaux, M. 2012a. Chemical quenching
664 of singlet oxygen by carotenoids in plants. Plant Physiol. 158, 1267-1278.

665 Ramel, F., Birtic, S., Ginies, C., Soubigou-Taconnat, L., Triantaphylidès, C., Havaux, M., 2012b. Carotenoid
666 oxidation products are stress signals that mediate gene responses to singlet oxygen in plants. Proc. Natl.
667 Acad. Sci. 109, 5535–5540.

668 Rasulov, B., Bichele, I., Hüve, K., Vislap, V., Niinemets, Ü. 2014. Acclimation of isoprene emission and
669 photosynthesis to growth temperature in hybrid aspen: resolving structural and physiological controls.
670 Plant Cell Environ. n/a DOI: 10.1111/pce.12435

671 Rausher, M.D., 2006. The evolution of flavonoids and their genes, in: Grotewold, E. (Ed.), The Science of
672 Flavonoids. Springer New York, pp. 175–211.

673 Rinnan, R., Steinke, M., Mcgenity, T., Loreto, F., 2014. Plant volatiles in extreme terrestrial and marine
674 environments. Plant. Cell Environ. 37, 1776–1789.

675 Rodríguez, A. A., Grunberg, K. A., Taleisnik, E. L., 2002. Reactive oxygen species in the elongation zone
676 of maize leaves are necessary for leaf extension. Plant Physiol. 129, 1627-1632.

677 Rook, F., Hadingham, S.A., Li, Y., Bevan, M.W., 2006. Sugar and ABA response pathways and the control
678 of gene expression. Plant. Cell Environ. 29, 426–434.

679 Ruban, A. V., Johnson, M. P. 2010. Xanthophylls as modulators of membrane protein function. Arch.
680 Biochem. Biophys., 504, 78-85.

681 Şahin, G., Tullio, M.C. De, 2010. A winning two pair: role of the redox pairs AsA/DHA and GSH/GSSG in
682 signal transduction, in: Anjum, N.A., Chan, M.-T., Umar, S. (Eds.), ascorbate-glutathione pathway and
683 stress tolerance in plants. Springer Netherlands, pp. 251–263.

684 Sasaki, K., Saito, T., Lämsä, M., Oksman-Caldentey, K.-M., Suzuki, M., Ohyama, K., Muranaka, T., Ohara,
685 K., Yazaki, K., 2007. Plants utilize isoprene emission as a thermotolerance mechanism. Plant Cell Physiol.
686 48, 1254–1262.

687 Saslowsky, D. E., Warek, U., Winkel, B. S., 2005. Nuclear localization of flavonoid enzymes in Arabidopsis.
688 J. Biol. Chem. 280, 23735-23740.

689 Scheidt, H. A., Pampel, A., Nissler, L., Gebhardt, R., & Huster, D. 2004. Investigation of the membrane
690 localization and distribution of flavonoids by high-resolution magic angle spinning NMR spectroscopy.
691 Biochimica et Biophysica Acta, 1663, 97-107.

692 Schnaubelt, D., Queval, G., Dong, Y., Diaz-Vivancos, P., Makgopa, M.E., Howell, G., De Simone, A., Bai, J.,
693 Hannah, M.A., Foyer, C.H., 2015. Low glutathione regulates gene expression and the redox potentials of
694 the nucleus and cytosol in Arabidopsis thaliana. Plant Cell Environ. 38, 266-279.

695 Schützendübel, A., Schwanz, P., Teichmann, T., Gross, K., Langenfeld-Heyser, R., Godbold, D. L., Polle,
696 A., 2001. Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and
697 differentiation in Scots pine roots. Plant Physiol. 127, 887-898.

698 Schwanz, P., Polle, A., 2001. Differential stress responses of antioxidative systems to drought in
699 pendunculate oak (Quercus robur) and maritime pine (Pinus pinaster) grown under high CO2
700 concentrations. J. Exp. Bot. 52, 133-143.

701 Sharkey, T. D., Wiberley, A. E., Donohue, A. R. 2008. Isoprene emission from plants: why and how. Ann.
702 Bot. 101, 5-18.

703 Sharkey, T.D., Chen, X., Yeh, S., 2001. Isoprene increases thermotolerance of fosmidomycin-fed leaves.
704 Plant Physiol. 125, 2001–2006.

705 Sharkey, T.D., Singsaas, E.L., 1995. Why plants emit isoprene. Nature 374, 769.

706 Shumbe, L., Bott, R., Havaux, M., 2014. Dihydroactinidiolide, a high light-induced β-carotene derivative
707 that can regulate gene expression and photoacclimation in Arabidopsis. Mol. Plant 7, 1248–1251.

708 Singsaas, E.L., Lerdau, M., Winter, K., Sharkey, T.D., 1997. isoprene increases thermotolerance of
709 isoprene-emitting species. Plant Physiol. 115, 1413–1420.

710 Siwko, M.E., Marrink, S.J., de Vries, A.H., Kozubek, A., Schoot Uiterkamp, A.J.M., Mark, A.E., 2007. Does
711 isoprene protect plant membranes from thermal shock? A molecular dynamics study. Biochim. Biophys.
712 Acta, 1768, 198–206.

713 Streb, P., Tel-Or, E., Feierabend, J.,1997. Light stress effects and antioxidative protection in two desert
714 plants. Funct. Ecol. 11, 416-424.

715 Suzuki, N., Mittler, R., 2012. Reactive oxygen species-dependent wound responses in animals and plants.
716 Free Radical Biol. Med. 53, 2269-2276.

717 Takahama, U., 2004. Oxidation of vacuolar and apoplastic phenolic substrates by peroxidase:
718 Physiological significance of the oxidation reactions. Phytochem. Rev. 3, 207–219.

719 Takezawa, D., Komatsu, K., Sakata, Y., 2011. ABA in bryophytes: how a universal growth regulator in life
720 became a plant hormone? J. Plant Res. 124, 437–453.

721 Tattini, M., Di Ferdinando, M., Brunetti, C., Goti, A., Pollastri, S., Bellasio, C., Giordano, C., Fini, A., Agati,
722 G. 2014b. Esculetin and esculin (esculetin 6-O-glucoside) occur as inclusions and are differentially
723 distributed in the vacuole of palisade cells in Fraxinus ornus leaves: A fluorescence microscopy analysis.
724 J. Photoch. Photobio. B, 140, 28-35.

725 Tattini, M., Guidi, L., Morassi-Bonzi, L., Pinelli, P., Remorini, D., Degl'Innocenti, E., Giordano, C., Massai,
726 R., Agati, G., 2005. On the role of flavonoids in the integrated mechanisms of response of Ligustrum
727 vulgare and Phillyrea latifolia to high solar radiation. New Phytol. 167, 457–470.

728 Tattini, M., Velikova, V., Vickers, C., Brunetti, C., Di Ferdinando, M., Trivellini, A., Fineschi, S., Agati, G.,
729 Ferrini, F., Loreto, F., 2014a. Isoprene production in transgenic tobacco alters isoprenoid, non-structural
730 carbohydrate and phenylpropanoid metabolism, and protects photosynthesis from drought stress.
731 Plant. Cell Environ. 37, 1950–1964.

732 Taylor, L.P., Grotewold, E., 2005. Flavonoids as developmental regulators. Curr. Opin. Plant Biol. 8, 317–
733 323.

734 Tossi, V., Cassia, R., Bruzzone, S., Zocchi, E., Lamattina, L., 2012. ABA says NO to UV-B: a universal
735 response? Trends Plant Sci. 17, 510–517.

736 Triantaphylidès, C., Havaux, M., 2009. Singlet oxygen in plants: production, detoxification and signaling.
737 Trends Plant Sci. 14, 219–228.

738 Van Breusegem, F., Dat, J.F., 2006. Reactive oxygen species in plant cell death. Plant Physiol. 141, 384-
739 390.

740 Velikova, V., Edreva, A., Loreto, F., 2004. Endogenous isoprene protects Phragmites australis leaves
741 against singlet oxygen. Physiol. Plant. 122, 219–225.

742 Velikova, V., Loreto, F., 2005. On the relationship between isoprene emission and thermotolerance in
743 Phragmites australis leaves exposed to high temperatures and during the recovery from a heat stress.
744 Plant. Cell Environ. 28, 318–327.

745 Velikova, V., Várkonyi, Z., Szabó, M., Maslenkova, L., Nogues, I., Kovács, L., Peeva, V., Busheva, M.,
746 Garab, G., Sharkey, T.D., Loreto, F., 2011. Increased thermostability of thylakoid membranes in isoprene-
747 emitting leaves probed with three biophysical techniques. Plant Physiol. 157, 905–916.

748 Vickers, C.E., Gershenzon, J., Lerdau, M.T., Loreto, F., 2009. A unified mechanism of action for volatile
749 isoprenoids in plant abiotic stress. Nat. Chem. Biol. 5, 283–91.

750 Wagner, D., Przybyla, D., op den Camp, R., Kim, C., Landgraf, F., Lee, K. P., Würsch, M., Laloi, C., Nater,
751 M., Hideg, E., Apel, K., 2004. The genetic basis of singlet oxygen–induced stress responses of
752 Arabidopsis thaliana. Science 306, 1183-1185.

753 Wheeler, S., Loveys, B., Ford, C., Davies, C., 2009. The relationship between the expression of abscisic
754 acid biosynthesis genes, accumulation of abscisic acid and the promotion of Vitis vinifera L. berry
755 ripening by abscisic acid. Aust. J. Grape Wine Res. 15, 195–204.

756 Yamasaki, H., Sakihama, Y., Ikehara, N., 1997. Flavonoid-peroxidase reaction as a detoxification
757 mechanism of plant cells against H2O2. Plant Physiol. 115, 1405–1412.

758 Zechmann, B., Stumpe, M., Mauch, F. 2011. Immunocytochemical determination of the subcellular
759 distribution of ascorbate in plants. Planta, 233, 1-12.

760 Zvi, M.M. Ben, Shklarman, E., Masci, T., Kalev, H., Debener, T., Shafir, S., Ovadis, M., Vainstein, A., 2012.
761 PAP1 transcription factor enhances production of phenylpropanoid and terpenoid scent compounds in
762 rose flowers. New Phytol. 195, 335–345.

763

Captions

Fig. 1. A proposed model for the integrated actions of isoprene, carotenoids, and flavonoids in leaves suffering from a severe excess of radiant energy, following the possible depletion of chloroplastic antioxidant defences (e.g. ascorbate peroxidase and ascorbic acid). Consumption of DMADP for isoprene biosynthesis channels more carbon into the whole MEP pathway in full sunlight growing plants concomitantly challenged against heat and drought stress. Isoprene improves the thermostability of thylakoids. Isoprene-induced enhancement of carotenoids biosynthesis equips leaves with a versatile system that prevents the generation of ROS and quench ROS once they are formed. De-epoxidation of violaxanthin to zeaxanthin, in addition to avoid the formation of triplet chlorophyll (^{3}Chl*) from singlet chlorophyll (S^*_1, via NPQ), confers rigidity to thylakoid membranes, and prevents lipid peroxidation. Carotenoids (car) also quench, physically and chemically singlet oxygen ($^1O_2$) through dissipation of highly energetic molecular oxygen ($^1O_2$-$^3O_2$ transition) and direct $^1O_2$-oxidation of β-carotene (and zeaxanthin). Isoprene-induced activation of the MEP pathway also promotes the biosynthesis of abscisic acid (ABA) and, in turn the biosynthesis of flavonoids.

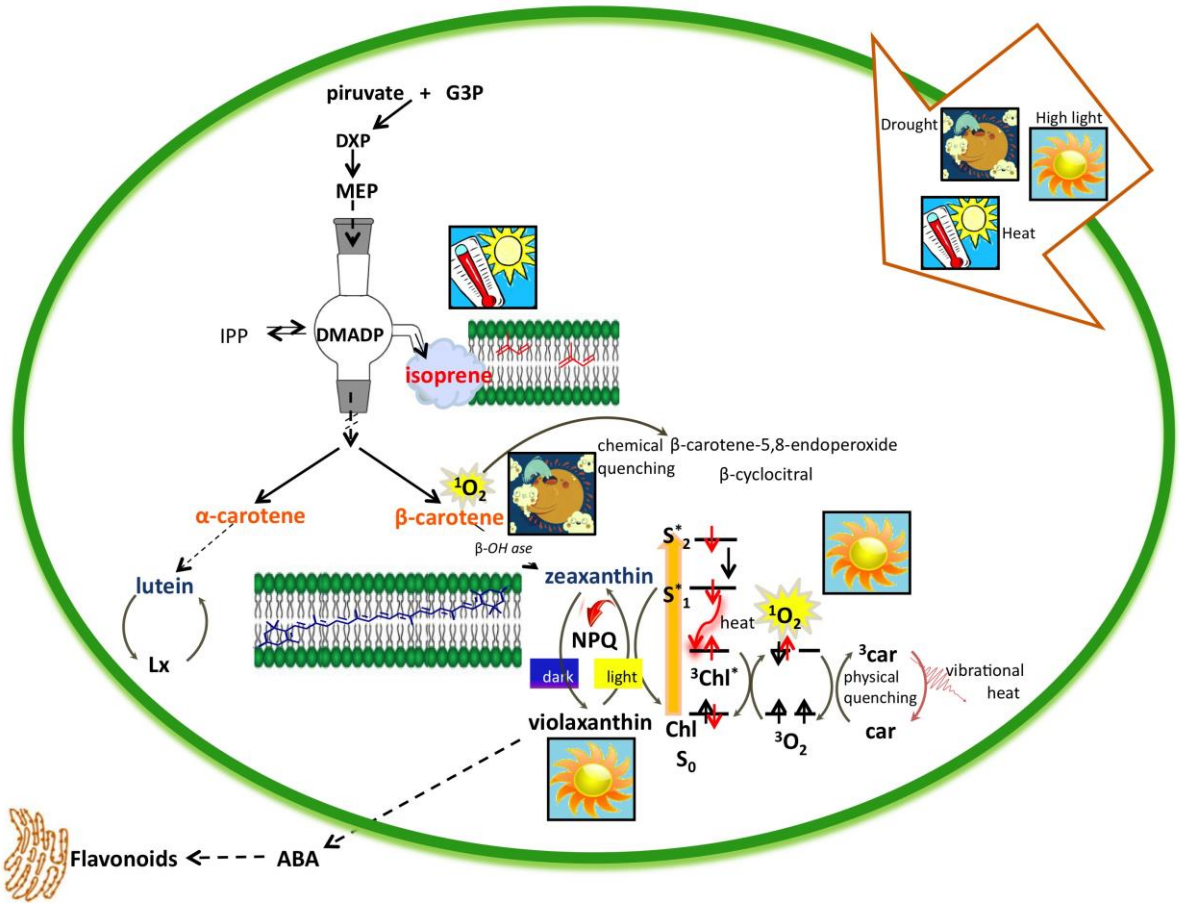
Fig. 2. Daily variation of: (A) sunlight irradiance, estimated over 200-3,000 nm spectral region and peaked approx. 900 Wm-2 at midday; (B) air temperature (min/max ranged from 19.7 ± 2.2 to 32.8 ± 2.7 °C) to which Q. pubescens plants were exposed during July 2014; the activity of ascorbate peroxidase (APX, C), the emission of isoprene (D) and the concentration of zeaxanthin (E) in leaves of plants growing under either well-watered (open circles) or drought stress conditions of increasing severity. Drought stress was imposed by withholding water for five (grey triangles), 10(dark grey).

785 **Figure 1**

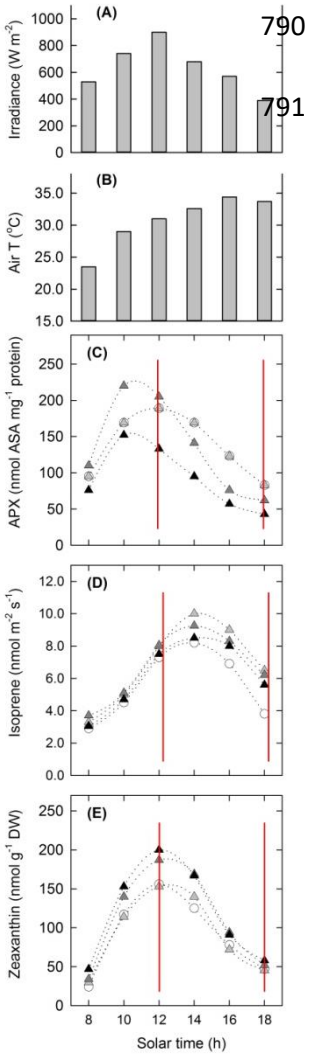
786

787

788



789 **Figure 2**


790
791
(A)
Irradiance (W m-2)
1000
800
600
400
200
0
(B)
Air T (°C)
35.0
30.0
25.0
20.0
15.0
(C)
APX (nmol ASA mg-1 protein)
250
200
150
100
50
0
(D)
Isoprene (nmol m-2 s-1)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
(E)
Zeaxanthin (nmol g-1 DW)
250
200
150
100
50
0
8
10
12
14
16
18
Solar time (h)