UNIVERSITAT DE BARCELONA

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Dissertation submitted by Paula Muñoz Roldán to have the right to obtain the degree of Doctor by the University of Barcelona.

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The present dissertation has been conducted at the Department of Evolutionary Biology, Ecology and Environmental Sciences from the School of Biology of the University of Barcelona under the supervision of Prof. Sergi Munné-Bosch.

PhD student: Thesis Director and Tutor:

Paula Muñoz Roldán Prof. Sergi Munné-Bosch

I stand
On the sacrifices
Of a million women before me
Thinking
What can I do
To make this mountain taller
So that women after me
Can see farther
- Rupi Kaur

A la meva família,
per ser la base de tot.

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ABSTRACT

Environmental constraints and developmental processes in plants can lead to light energy excess in chloroplasts by lowering the threshold at which light becomes saturating by a lack of electron acceptors in the light-processing structures. Inactivation of photosystems due to high energy quanta is generally known as photoinhibition, a process that can compromise growth and organ development in plants due to an imbalance of the cellular redox state that leads to enhanced oxidative stress. Hence, it is essential to determine environmental factors and stages of organ development that influence the extent of photoinhibition and mechanisms triggered to avoid photoinhibition and cope with photo-oxidative stress, so that plant survival is not put in jeopardy. For this reason, leaves from an ecologically important Mediterranean plant, flowers from a relevant commercial crop and fruits from a highly appreciated agronomic tree, were selected to analyse events leading to photoinhibition and strategies to allow plant acclimation and correct organ development. With this regard, mastic trees were selected to study their sensitivity to cold stress during Mediterranean winters and altitudinal distribution, to determine if antioxidant strategies and leaf phenology influence their ability to survive environmental constraints. On the other hand, lily flowers were selected to study photoinhibitory events taking place during flower opening and how phytohormones could regulate the timing of flower opening by interacting with photo-oxidative stress signals. Besides, sweet cherry fruits were also selected to better understand physiological events related to ripening onset and chloroplast dismantling with the production of antioxidants and hormonal signals involved. Our results show that timing and stage of organ development directly condition the outcomes of photoinhibition with regards of chloroplast status and antioxidants produced, with a general prevailing mechanism where reduced F_v/F_m triggers higher lipid peroxidation and parallel production of phytohormones and antioxidants that deal with increased photo-oxidative stress. In conclusion, photoinhibition does not compromise plant or organ

survival, but prompt the activation of signalling networks that promote metabolic changes to fine-tune the timing and severity of this process to allow acclimation and correct organ development.

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Abbreviations

Abscisic acid	**ABA**
Hydrogen peroxide	**H_2O_2**
Hydroxyl radical	**•OH**
Light harvesting complex	**LHC**
Lipid hydroperoxide	**LOOH**
Malondialdehyde	**MDA**
Non-photochemical quenching	**NPQ**
Photosystem I	**PSI**
Photosystem II	**PSII**
Polyunsaturated fatty acid	**PUFA**
Singlet oxygen	**$^1O_2$**
Superoxide anion radical	**$O_2•^-$**
Triplet state chlorophyll	**^{3}Chl***

INTRODUCTION

INTRODUCTION

1. Photoinhibition in plants

Light is a key element in nature, not only because it represents an unlimited source of energy, but because it also represents an environmental cue that strongly influences the structure of ecosystems and modulates life performance of most organisms. Photoautotrophs are the only entities that can use photons from light and convert them into chemical energy to produce biomass (Johnston *et al*., 2009). Oxygenic photoautotrophs were determining organisms to change atmospheric composition by the implementation of oxygenic photosynthesis, which provided molecular oxygen (O_2) and established an oxidizing environment that allowed aerobic metabolism, the evolution of complex multicellular organisms and shaped the biosphere we know today. The first record of significant increased levels in atmospheric O_2 known as the Great Oxidation Event (GOE) dates from ~2.2 – 2.4 billion years ago, according to geological records (Lyons, *et al*., 2014). However, it was not until ~600 – 800 million years ago that there was a second oxygenation event known as Neoproterozoic Oxidation Event (NOE), where atmospheric O_2 reached levels similar to the ones we find at present. In fact, this latest event has been linked to the origin of animals, major glaciations and disruptions in the carbon cycle (Sánchez-Baracaldo & Cardona, 2020). So, with this regard, oxygenic photosynthesis by using light energy to split water and release O_2, protons and electrons, with the concomitant fixation of carbon dioxide (CO_2) was, indeed, a major driver for the evolution of life on the Earth's surface. Even though there is still controversy in terms of evolutionary history of oxygenic phototrophs or the origin for the gain of photosynthetic apparatus, phylogenetic studies indicate that a group of Cyanobacteria (class *Oxyphotobacteria*) acquired photosynthetic genes throughout the Archean eon (Soo, *et al*., 2017; Cardona, *et al*., 2019). Subsequent endosymbiosis of a cyanobacteria with several eukaryotic hosts eventually led to the formation of chloroplasts,

cellular organelles with an internal three-dimensional network of infolded membranes named thylakoids and intermembrane space named lumen. Both eukaryotic algae and higher plants contain chloroplasts with the whole photosynthetic machinery that work as regulatory hubs to integrate environmental signals that allow acclimation to the environment and correct development (Foyer & Noctor, 2005; Gill & Nuteja, 2010; Mittler, *et al*., 2011).

Higher plants, along with oceanic cyanobacteria and algae, sustain the ~21% of atmospheric O_2 composition we find at present (Allen, *et al*., 2019). In modern vascular plants, the chloroplastic electron transport chain is composed by several protein complexes that through oxidation – reduction (redox) reactions allow linear electron flow to produce reducing power (NADPH) and chemical energy (ATP) to synthesize primary carbon skeletons from CO_2 assimilation during photosynthesis. Solar energy is harnessed by the light-harvesting complexes (LHCs) distributed along the thylakoid membranes of chloroplasts, being LHC-II the most abundant in vascular plants (Liu, *et al*., 2004). Channelled light energy creates electronic excitation in the peripheral area of the LHC that is transferred within ~100ps to the photochemical reaction centers of photosystems (PSs). Under favourable conditions, electronic excitation is converted to a stable charge-separation stage of chlorophyll dimers of PSI (P_{700}) and PSII (P_{680}) (Sundström, 2008; Cheng & Fleming, 2009). LHC-II along with the antenna proteins CP47 and CP43, funnel light energy to the reaction center of PSII and allow light-induced charge separation of chlorophylls into P_{680}^+ and pheophytin$^-$ (Pheo$^-$). A sequence of four redox steps powered by P_{680}^+ enables oxidative water splitting in the oxygen-evolving complex of PSII and gives O_2 and four protons as by-products (Renger, 2012; Kato *et al*., 2016). Subsequently, Pheo$^-$ transfers electrons from water splitting to the primary quinone electron acceptor Q_A and then to the secondary quinone electron acceptor Q_B, which after the uptake of two electrons, forms the plastoquinol (PQH_2) that is released into thylakoid membranes, with the concomitant

entry of two protons from the stroma to the thylakoid lumen (Kato *et al*., 2016). The cytochrome b_6f (cyt b_6f) complex catalyses the electron transfer from PQH_2 to plastocyanin (PC), whose soluble structure enables electron carry from cyt b_6f to PSI. LHC-I supplies PSI with electronic excitation from light absorption that allows charge-separation of P_{700}. The photo-oxidized side of PSI reacts with reduced PC in the thylakoid lumen and the electrons are transferred to the internal 4Fe-4S centers to ferredoxin, which generates reducing power for the reduction of $NADP^+$ to NADPH (Rochaix, 2011; Suga *et al*., 2016). The proton movement from the chloroplast stroma to the thylakoid lumen also generates a thylakoid proton motive force that enables ATP production, which together with NADPH, power carbon fixation in the Calvin-Benson cycle to synthesize primary carbon skeletons (Kramer *et al*., 2003).

Light is indeed essential to perform photosynthesis and sustain plant biochemistry. Nevertheless, absorbed light can also exceed plants photosynthetic capacity when there are limitations in carbon fixation that restrict electron flow throughout the chloroplast electron transport chain and disproportion the rapid turnover of these components. A lack of electron acceptors imbalance energy usage and induce photodamage of the reaction centers, specially to PSII that is highly sensitive to light-saturation. There is frequent photodamage of PSII due to high demand of electron transfer and the slower kinetics of CO_2 fixation, but it is usually counteracted by the rapid turnover of photodamaged D1 subunit in PSII by newly synthesized D1 protein in a process known as PSII repair cycle (Nickelsen & Rengstl, 2013). There is current discussion in the model associated to D1 photodamage considering direct damage of D1 protein through higher rates of PSII charge recombination by a higher proton motive force or a secondary damage of D1 protein with prior disruption of the manganese cluster of the oxygen-evolving complex (Li, *et al*., 2018). Either way, PSII photoinhibition occurs when damage rate of D1 protein exceeds the repair capacity by the inability of *de novo* synthesis of D1 and, consequently, excess excitation energy

accumulates in the reaction center of PSII (Takahashi, *et al*., 2007; Takahashi & Murata, 2008; Demmig-Adams & Adams, 2018). As it is further discussed in this dissertation, environmental factors as well as certain phases of plant development vary the intensity at which light is excessive and inactivates PSII, which also has different implications regarding plant acclimation or organ development. On the other hand, photoinhibition of PSI is less frequent and strongly relies on specific conditions of changing environments, such as fluctuating light or chilling stress (Havaux & Davaud, 1994; Suorsa, *et al*., 2012; Kono & Terashima, 2016). When electron carriers of PSI are overreduced, PSI is photoinhibited and there are important drops in CO_2 assimilation rates that greatly limit plant growth (Kaiser, 1976; Suorsa, *et al*., 2012). Since PSI repair is much slower than that of PSII when the core-complex is photodamaged, PSI photoinhibition has higher consequences for plant survival and accordingly, its avoidance is essential (Grieco, *et al*., 2012; Kono & Terashima, 2016). In fact, PSI is highly tolerant to excess excitation energy but very sensitive to electron excess from PSII when PSI has no electron acceptors available (Sonoike, 2011). Hence, primary photoinhibition of PSII constitutes a protective strategy to minimize photo-damage of PSI (Sonoike, 2011; Tikkanen, *et al*., 2014b; Tiwari, *et al*., 2016). However, photoinhibition of both PSs can compromise plants photosynthetic yield (Öquist, *et al*., 1992; Lima-Melo, *et al*., 2019) whilst severe photoinhibition may lead to a decline in plant growth and even mortality when sustained (op den Camp, *et al*., 2003; Triantaphylidès, *et al*., 2008).

2. Photo-oxidative stress and reactive oxygen species production in chloroplasts

High-energy quanta derived from an imbalanced production of ATP and NADPH at rates that do not meet metabolic demands induce photoinhibition of PSI and PSII, which prompt the production of reactive oxygen species (ROS) at both PSs reaction centers and within chloroplasts (Asada, 2006). ROS are highly reactive molecules that can generate photo-

oxidative stress in plant cells by inducing photodamage of essential biomolecules such as lipids, proteins or nucleic acids. Photo-oxidative stress potentially leads to cell death if ROS are not properly counteracted by photoprotective and detoxifying mechanisms. Nevertheless, as discussed throughout this dissertation, it has become more evident in the recent years the active role of ROS as redox sensors that facilitate chloroplast-to-nucleus communication through retrograde signalling and integrate environmental and developmental constraints when their production is fine-tuned by antioxidant systems (Foyer & Noctor, 2005; Takahashi & Murata, 2008; Rogers & Munné-Bosch, 2016; Dietz, *et al*., 2019).

Different types of ROS can be produced within the photosythetic complexes of thylakoid membranes in chloroplasts. PSII is the primary source of singlet oxygen ($^1O_2$), an extreme reactive form of excited oxygen that rapidly reacts with nearby macromolecules producing non-enzymatically peroxyl derivatives (Telfer, 2014; Foyer, 2018). In fact, $^1O_2$ is a short-lived ROS that lasts ~ 3 μs in aqueous solution (Egorov, *et al*., 1989), but its lifespan is shortened in cells, where $^1O_2$ only lasts around 200 ns (Gorman & Rodgers, 1992; Hatz, *et al*., 2007). Nevertheless, within this time, $^1O_2$ diffuses great distances in the order of hundred nanometres (Hatz, *et al*., 2007), although time and distance approximations strongly rely on the site and cell type where $^1O_2$ is produced (Telfer, 2014). Formation of $^1O_2$ derives from the production of triplet state chlorophylls (^{3}Chl*) at the LHC (Rinalducci, *et al*., 2004) as well as the reaction center of PSII when PC is over-reduced and excess light induces formation of excited triplet state ($^3P_{680}$*) that react with molecular oxygen and produce $^1O_2$ (Krieger-Liszkay, 2005; Asada, 2006). Due to close vicinity with its site of production, $^1O_2$ induces photodamage of D1 subunit in PSII and can impair D1/D2 repair cycle, as it interferes with the protein elongation factor G involved in newly synthesized D1 protein (Nishiyama, *et al*., 2006; Foyer, 2018). In addition, $^1O_2$ directly oxidizes polyunsaturated fatty acids (PUFA) of lipid membranes that contributes to cell oxidative photodamage (Triantaphylidès, *et al*.,

2008). There are different reactions involved in lipid peroxidation that arouse the production of lipid radicals (L• and LOO•) together with lipid hydroperoxides (LOOH). As $^1O_2$ induces an electrophilic reaction with lipid alkyl radicals with the addition of $^1O_2$ to the double bound of PUFA, only LOOH and endoperoxides are produced (Triantaphylides & Havaux, 2009; Pospíšil & Yamamoto, 2017). While LOOH are the primary products of lipid peroxidation, endoperoxides decompose into malondialdehyde (MDA) as secondary peroxidation products (Pospíšil & Yamamoto, 2017; Morales & Munné-Bosch, 2019). Besides, since α-linolenic acid is the most prominent PUFA in chloroplast membranes, by-products of lipid peroxidation produced during photo-oxidative stress mainly derive from α-linolenic (Murakami, *et al*., 2000; Triantaphylidès, *et al*., 2008). *In vivo*, LOOH are much less stable than MDA, but both can react and alter proteins, nucleic acids as well as other biomolecules increasing cellular damage. Moreover, chain reaction extension of lipid peroxidation occurs when L• and LOO• produced after ROS oxidation are not counteracted and react with neighbouring lipids, which exacerbates ROS action on membrane photodamage (Davey, *et al*., 2005; Gill & Nuteja, 2010). For this reason, primary and secondary lipid peroxidation are frequently employed as molecular markers of photo-oxidative stress (Pintó-Marijuan & Munné-Bosch, 2014; Morales & Munné-Bosch, 2019). Additionally, decomposition of LOOH and endoperoxides lead to the formation of triplet excited carbonyl (^{3}L=O*) and ground carbonyl, where ^{3}L=O* might undergo de-excitation or energy transfer, being the second an additional source for $^1O_2$ formation. Therefore, if LOOH and endoperoxides are not effectively reduced by antioxidants, photodamage to lipid membranes can also contribute to generate extra ROS and oxidative damage in plant cells (Miyamoto, *et al*., 2014; Pospíšil & Yamamoto, 2017). High reactivity of $^1O_2$ with biological molecules, has been indeed linked to cellular photo-oxidative damage *(Triantaphylidès, et al., 2008; Shumbe, et al.*, 2016) and programmed cell death (op den Camp, *et al*., 2003), although recent research also relates its production to acclimation stress responses

(Carmody, *et al*., 2016; Wang, *et al*., 2016; Shumbe, *et al*., 2017).

PSI is another site accountable for ROS production within thylakoid membranes. Superoxide anion radical ($O_2^{\bullet -}$) is the main ROS produced due to photoreduction of O_2 at the primary electron acceptor of PSI in the Mehler reaction (Mehler, 1951; Takahashi & Asada, 1982). Little attention has been paid to $O_2^{\bullet -}$ production since it has almost no reactivity to most biomolecules and is rapidly disproportionated in a protonated environment, with a half-life between 2 - 4 μs (Asada, 1999; Gill & Nuteja, 2010; Foyer , 2018). At low pH when protons are available $O_2^{\bullet -}$ is converted to its protonated form, hydroperoxyl radical (HO_2), whose diffusion rate is higher than that of $O_2^{\bullet -}$ and, unlike the later, can dissipate outside thylakoid membranes (Asada, 1999). Nevertheless, most $O_2^{\bullet -}$ produced at PSI is disproportioned into hydrogen peroxide (H_2O_2) and O_2, either spontaneously in presence of available protons in thylakoid membranes or *via* superoxide dismutase (SODs), such as CuZn-SOD, associated to PSI of intact chloroplasts (Gill & Nuteja, 2010; Dietz, *et al*., 2016). Further reduction of H_2O_2 into water takes place through the catalysed reaction of H_2O_2 and ascorbate by ascorbate peroxidase (APX) in the water-water cycle, where half of the electrons from water oxidation are used in the photoreduction of dioxygen and the other half in the regeneration of oxidized ascorbate (Asada, 1999). The water-water cycle is highly efficient to dissipate high-energy quanta at PSI and prevent H_2O_2 accumulation, which could lead to the formation of the highly toxic hydroxyl radical (•OH) in the presence of transition metals *via* Haber-Weiss or Fenton reactions (Mittler, 2002), which is a much more reactive ROS that can initiate lipid peroxidation and induce severe photodamage (Asada, 1999). Besides, H_2O_2 is a much more stable ROS than $O_2^{\bullet -}$ and $^1O_2$, with greater diffusion distances outside thylakoid membranes. It is less harmful than other ROS but can potentially oxidize enzyme thiol subunits, inducing photo-oxidative stress (Mittler, 2002; Gill & Nuteja, 2010). Indeed, accumulation of H_2O_2 can inactivate Calvin-Benson cycle enzymes (Strand, *et al*., 2015) and its production has been related to defence mechanisms of

programmed cell death after pathogen infection and also during senescence, among other physiological processes (Gill & Nuteja, 2010). However, its reactivity with thiol groups and its stability in plant cells also makes H_2O_2 suitable for downstream signalling transduction (Foyer & Noctor, 2005).

Other components of the chloroplast electron transport chain can also contribute to the production of $OH_2^{\bullet-}$ and H_2O_2 in chloroplasts, such as cyt *b*$_6$*f*, the plastid terminal oxidase and also the acceptor side of PSII, although these mechanisms of oxygen reduction seem to play minor roles in intact thylakoids (Dietz, *et al.*, 2016). Likewise, $^1O_2$ production by PSI is very limited, since the presence of O_2 does not shorten the lifetime of P_{700} triplet-state ($^3P_{700}$), therefore suggesting that $^1O_2$ is not produced at high rates by PSI (Sétif, *et al*., 1981). Nevertheless, Cazzaniga, *et al*., (2012) reported that $^1O_2$ at PSI seems to initiate $OH_2^{\bullet-}$ formation at the A_1 electron acceptor of PSI, consequently decreasing its activity under fluctuating light (Takagi, *et al*., 2016b).

3. Antioxidants involved in photoprotection and prevention of photo-oxidative stress in chloroplasts

To prevent photoinhibition and overcome photo-oxidative stress, plants have evolved a vast array of photoprotective mechanisms that allow plant survival in constant changing environments and plant specific requirements at different developmental processes. In this dissertation, special attention is drawn to photoprotective strategies implemented by chloroplastic pigments, including anthocyanin and carotenoids, as well as lipophilic antioxidants such as vitamin E, since they perform a dual role both preventing ROS formation and detoxifying ROS production. Nevertheless, it is important to mention that these antioxidants work closely with several other mechanisms. For instance, electron sinks or alternative flow of electrons are essential to minimise photoproduction of $O_2^{\bullet-}$ at PSI. For example, cyclic electron flow around PSI increases ATP production relative

to NADPH in order to balance the energy budget of the chloroplast and prevents overreduction of the acceptor side of PSI (Munekage, *et al*., 2004; Nawrocki, *et al*., 2019). Another example is photorespiration, which works as an electron sink, even though it also constitutes a source of H_2O_2 (Voss, *et al*., 2013; Takagi, *et al*., 2016a). In addition, the proton motive force generated through alternative electron sinks is required for non-photochemical quenching (NPQ) that prevents PSII photoinhibition (Makino, *et al*., 2002; Joliot & Johnson, 2011; Zaks, *et al*., 2012). Therefore, cyclic electron transport around PSI is essential to balance ATP/NADPH production and together with the water-water cycle, protect both PSs from overreduction of the linear electron transport elements. Likewise, alternative electron flow also occurs at PSII, where there is electron transfer from cyt b_{559} back to P_{680}^+ if there is low pH in the lumen. Nevertheless, even though this process avoids PSI photodamage, it also results in additional production of $O_2^{\bullet -}$ (Miyake & Yokota, 2001). Additionally, leaves and chloroplasts movement along with modifications of grana stacking also greatly contribute to avoid photoinhibition (Gamon & Pearcy, 1989; Huang, *et al*., 2014; Dutta, *et al*., 2017). Under non-detrimental conditions, PSII reaction centers distribute along the appressed region of thylakoid membranes within the grana stacks together with LHC-II, PQ and a fraction of cyt b_6f, while PSI localises at grana margins, in the unstacked region, along with another fraction of the cyt b_6f, ATP synthase, NAD(P)H dehydrogenase and PC that moves between regions (Albertsson, 2001; Pribil, *et al*., 2014). Under high-light conditions, an increased number of hydroxyl radicals can be detected in the grana stacks compared to the non-appressed region (Khatoon, *et al*., 2009), which makes thylakoid unstacking essential to reduce ROS production (Yamamoto, *et al*., 2014). Besides, reduction in grana diameter and modification of the stacked structure also facilitates PSII repair by higher accessibility to photodamaged D1 (Nixon, *et al*., 2005).

Furthermore, it is well established that redox cellular balance is tightly

controlled by enzymatic antioxidants including the already mentioned SOD, as well as catalases (CATs), different types of peroxidases, glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and thioredoxins (TRXs), whose main function is to scavenge ROS and control oxidative cascades (Gill & Nuteja, 2010). As previously stated, APX mediates dismutation of H_2O_2 produced from PSI using ascorbate as an electron donor for H_2O_2 reduction in chloroplasts but APX has a broader distribution within plant cells and can be found also in mitochondria, peroxisomes or the cytosol (Anjum, *et al*., 2016). After ascorbate oxidation, it is essential to restore redox balance and prolong the antioxidant potential of APX. Therefore, monodehydroascorbate derived from ascorbate oxidation is restored to ascorbic acid by MDHAR through the Foyer-Halliwell-Asada pathway and the ascorbic acid-glutathione cycle (Foyer & Halliwell, 1976; Noctor & Foyer, 1998; Asada, 1999). Indeed, overexpression of MDHAR or DHAR conferred higher tolerance to drought and ozone stress for tobacco transgenic plants (Eltayeb, *et al*., 2007) and protected plants from salt stress for transgenic rice (Sultana, *et al*., 2012). Moreover, upregulation and increased activity of enzymes related to ascorbate-glutathione cycle where found in apple leaves when exposed to heat stress (Ma, *et al*., 2008). Ascorbic acid and glutathione are the most prevalent antioxidants found in plant tissues, with an ubiquitous subcellular distribution and extensive metabolic functions (Smirnoff, 2000; Noctor*, et al*., 2012). Both antioxidants can directly react and scavenge ROS as well as reduce H_2O_2, either acting as cofactors or regenerating components of enzymatic reactions in charge of their reduction (Noctor & Foyer, 1998). Their role during photoinhibition and photo-oxidative stress has been extensively reviewed, just as their capacity to improve stress tolerance and benefit plant development (Noctor & Foyer, 1998; Smirnoff, 2000; Foyer, 2018).

Photoinhibition severity and the plausibility to induce plant cell damage greatly relies on the efficiency of photoprotective systems to either avoid

photoinhibition or overcome excess energy at PSs. Light shielding and dissipation of absorbed energy by NPQ are the two main strategies to escape from excess energy at PSs reaction centers, while quenching and scavenging ROS are essential to avoid radical formation (Mittler, 2002; Pintó-Marijuan & Munné-Bosch, 2014). Some antioxidants can play a dual role in plant photoprotection and contribute to ROS prevention, plus counteract peroxyl radicals derived from photo-oxidative stress and restore cellular redox balance. It is important to highlight that antioxidant activity occurs by reactions of hydrogen atom transfer or electron transfer and that composition of the surrounding media, determines the chance for these reactions. For instance, hydrophilic antioxidants such as anthocyanins are more prone to donate their hydrogen in an aqueous environment, whilst carotenoids and vitamin E that are lipophilic antioxidants will do so in the lipid fraction (Karadag, *et al*., 2009). Taken together, it seems important to relate their coordinated function during photoinhibition events and characterise their ability to recover and balance cellular redox status in developmental and acclimation processes. However, prior to show these possible interactions, individual features must be outlined.

3.1. Anthocyanins

Anthocyanins (of the Greek *anthos* = flower and *kianos* = blue) are hydrophilic non-photosynthetic pigments responsible of the impressive red to blue colours that one can find in nature. Since accumulation of these pigments is tissue- and time-dependant, anthocyanins were considered useless by-products for a very long time, as it was also the perception for most secondary metabolites (Gould, 2004). Nevertheless, these pigments derived from flavonoids have long been considered important to attract pollinators and to contribute with seed dispersal in flowers and fruits. Besides, their role in photoprotection may be critical at certain stages of development (Karageorgou & Manetas, 2006; Juvany, *et al*., 2012) and also fundamental to withstand environmental restrictions (Steyn, *et al*., 2009;

Landi, *et al*., 2014; Cotado, *et al*., 2018).

Anthocyanin biosynthesis derives from the shikimate pathway that triggers the formation of phenylalanine, the precursor of the phenylpropanoid pathway, and the constitution of flavonoids with the entry of three molecules of malonyl-CoA (Shirley, et al., 1995). It is important to note that both the enzymes phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) that catalyse phenylpropanoid and flavonoid biosynthesis, respectively, are rate limiting and susceptible to variations in environmental conditions and consequently, their activity is modified when there are certain constraints (Shvarts, *et al*., 1997; Wade, *et al*., 2001; Lillo, *et al*., 2008). Anthocyanins are the glycoside form of anthocyanidins that derive from dihydroflavonols and whose basic chemical structure consists of two aromatic rings bound by a heterocyclic ring. The number of hydroxylated groups and aliphatic or aromatic carboxylates on these rings gives rise to a vast array of anthocyanidin moieties that covers absorption spectra of the whole visible region and modulate their antioxidant properties (Kong, *et al*., 2003). However, not all plants can produce the wide range of anthocyanins, since their production depends upon flavonol hydroxylases and dihydroflavonol reductases that are species-specific (Tsuda, *et al*., 2004; Nakatsuka, *et al*., 2007). Glycosylation confers anthocyanins higher solubility to accumulate in vacuoles of epidermal cells where they are stored and also determines their biochemical properties. Acylation of these pigments with chromophores and aromatic organic acids is frequent in plants, which enhances their absorbance in UV regions, where natural anthocyanins absorb weakly, and increase photoprotection in these spectra (Ferreira Da Silva, *et al*., 2012; Rusistoni, *et al*., 2013). Moreover, depending on vacuolar pH, anthocyanins adopt different chemical structure conformation with specific absorption spectrum and stability (Brouillard & Dubois, 1977). Altogether makes anthocyanin function on photoprotection a subject of extensive debate as many factors condition their role (Steyn, *et al*., 2002; Gould, 2004; Hughes, 2011).

One of the generally known features about anthocyanins is that they are potent antioxidants in aqueous solutions with high antioxidant capacity, being the most widespread anthocyanin in plants kuromanin (cyanidin-3-glucoside), the one with highest oxygen radical absorbance capacity (Wang, *et al*., 1997). Their consumption has been proved to have health promoting effects (Zafra-Stone, *et al*., 2007; Crozier, *et al*., 2009) and hence, in the recent years, intensive research has been performed to obtain fruits and vegetables with increased anthocyanin content *(Butelli, et al., 2008; Xie, et al*., 2012; Espley, *et al*., 2013). In plant cells, anthocyanins can efficiently scavenge $O_2^{\bullet -}$ as well as H_2O_2. However, it remains elusive if such antioxidant properties belong to vacuolar or cytosolic tautomers of anthocyanins (Gould, 2004). Indeed, anthocyanin deficiency confers hypersensitivity to ROS-generating stresses in Arabidopsis (Xu, *et al*., 2017) and impairs seed viability and pollen tube growth in tomatoes with anthocyanin deficiency (Muhlemann, *et al*., 2018), which evidences the photoprotective role of anthocyanins in ROS and peroxyl detoxification. Higher relevance can be paid to these molecules if one considers that mechanisms to achieve photoprotection in anthocyanic cell vacuoles include not only detoxifying properties but also light quanta shielding.

Several research works have shown increases on anthocyanin contents in vegetative and reproductive organs triggered by stress conditions such as drought stress (Eppel, *et al*., 2013; Cotado, *et al*., 2018; An, *et al*., 2019), nitrogen deficiency (Diaz, *et al*., 2006), cold stress (Christie, *et al*., 1994; Gu, *et al*., 2015) or high-light stress (Merzlyak & Chivkunova, 2000; Ilk, *et al*., 2015), among others. In autumn, bright red leaves of several deciduous plants also owe their characteristic colour to increased anthocyanin accumulation that prevents from uncontrolled photodamage prior to seasonal senescence (Moore, 1965; Hoch, *et al*., 2001; Feild, *et al*., 2001; Anderson & Ryser, 2015). Besides, several studies have also highlighted the relevance of anthocyanins at early leaf development when light processing and carbon fixation mechanisms are not fully mature

(Hughes, *et al*., 2007; Van Den Berg & Perkins, 2007; Juvany, *et al*., 2012; Zhu, *et al*., 2016). Therefore, there is evidence that anthocyanins are photoprotective molecules that respond to changing environments. Indeed, anthocyanins modify both the quantity and quality of chloroplastic light incidence by absorbing blue-green light, which reduces light availability for chlorophylls (Smillie & Hetherington, 1999; Gould, *et al*., 2018). Moreover, their capacity to trap light excess and work as quanta sinks may depend upon anthocyanin localisation, as it was shown by Wang *et al*. (2012) who exposed seedlings of the purple top turnip 'Tsuda' to either irradiating blue, UV-A, UV-B or a combination of lights and observed differential spatial accumulation of anthocyanins regarding the employed light. The shielding effect results in lower light energy arriving to chloroplasts and as a result, lower photosynthetic efficiencies are achieved in light-limiting environments (Gould, *et al*., 2002). On the contrary, when light excess can induce photodamage, anthocyanins avoid inactivation of PSs, specially of PSII, without compromising photosynthesis (Pietrini, *et al*., 2002) thereby reducing ROS produced (Neill & Gould, 2003). Hence, it is important to determine which environmental conditions trigger specific accumulation of anthocyanins in plant organs, whether transient or sustained accumulation of these pigments entail detrimental physiological constraints during photoinhibition and establish if these antioxidants can restore redox cellular balance.

3.2. Carotenoids

Carotenoids represent a diverse group of tetraterpenoid pigments that comprise hundreds of characterised compounds that give spectacular yellow, orange and red colours with a widespread distribution in nature. Photosynthetic organisms like cyanobacteria, algae and plants, as well as some bacteria and yeast, can synthesize these pigments whose chemical structure derives from condensation of the 5-carbon (C_5) compound isopentenyl diphosphate (IPP) or its isomer dimethylallyl diphosphate

(DMAPP) and are the precursors of all isoprenoids (McGarvey & Croteau, 1995; Lichtenthaler, *et al*., 1997). In plants, biosynthesis of these precursors arise from either the cytosolic mevalonate (MVA) pathway or the chloroplastic methylerythritol 4-phosphate (MEP) pathway and, even though the latter is the most active for carotenoid formation, there is a balanced communication between both pathways (Lichtenthaler, *et al*., 1997; Rohmer, 1999; Rodríguez-Concepción & Boronat, 2002). Consecutive catalytic action of three IPP isomerases gives rise to the 40-carbon structure (C_{40}) named geranylgeranyl diphosphate (GGDP) that is the immediate precursor of carotenoids, as well as to gibberellins, phylloquinones and the chlorophyll phytol tail (Fraser & Bramley, 2004). The central chain of GGDP may carry cyclic end-groups which can be substituted with oxygen-containing functional groups. Based on their composition, carotenoids are divided into carotenes containing only carbon and hydrogen atoms, and oxocarotenoids (xanthophylls) which carry at least one oxygen atom (Stahl & Sies, 2003).

Even though animals cannot perform *de novo* synthesis of carotenoids, some of them accumulate these pigments, as it can be seen for example in crustaceans' exoskeleton or chicken yolk. Animals obtain these carotenoids from the diet and have different roles in sexual reproduction or animal fitness, among others. Dietary intake of carotenoids is essential for humans, since β-carotene acts as provitamin A that prevents from macular damage, while higher dietary uptake of these antioxidants is promoted to alleviate the incidence of chronic diseases (Fraser & Bramley, 2004). In plants, carotenoids can be found within the thylakoid membranes or accumulating in chromoplasts, which result from chloroplast differentiation in fruits and flowers (Ščepánková & Hudák, 2004; Lado, et al., 2015). In fact, large accumulations of carotenoids in the exocarp of some fruits as well as in flower corollas, give rise to the characteristic bright colours with functions in reproduction and to attract pollinators. Carotenoids also have a dual role in plant photoprotection, since they contribute to mitigate energy excess with thermal dissipation through NPQ by the xanthophyll

cycle and efficiently scavenge and quench $^1O_2$ (Demmig, *et al*., 1987; Triantaphylides & Havaux, 2009). Distribution of these carotenoids within LHCs and PSs is of paramount importance since it determines their function and efficiency, while they are also essential for assembly and structure stabilisation of PSs complexes. Current models of LHC-II allocate four carotenoid binding sites occupied by the xantophylls lutein, neoxanthin and violaxanthin, while two molecules of β-carotene are mainly bound to D1/D2 proteins in the reaction center of PSI and PSII (Pana, *et al*., 2019). Interconversion of violaxanthin to zeaxanthin by a two-step de-epoxidation is an essential protective function to dissipate excess energy as heat through NPQ, also referred as qE component (Demmig-Adams & Adams, 2018; Ruban, *et al*., 2012). The proton motive force generated across the thylakoid membrane through the linear electron flow and alternative cyclic electron flow, promote NPQ by protonation of the violaxanthin de-epoxidase involved in the violaxanthin conversion to zeaxanthin (Demmig, *et al*., 1987). Moreover, other antioxidants like ascorbate also interact with the violaxanthin de-epoxidase as reductants and its deficiency can limit the enzyme activity, which highlights the relevance of coordinated responses between different antioxidants to avoid over-reduction of the electron transport chain components (Niyogi, *et al*., 1998; Müller-Moulé, *et al*., 2002). The violaxanthin cycle is an effective photoprotective strategy since plants with genetic mutations on violaxanthin de-epoxidase that cannot convert violaxanthin to zeaxanthin are more sensitive to photoinhibition, especially under high light (Niyogi, *et al*., 1998). Likewise, several studies have evidenced that an increased de-epoxidation state (DPS) of the violaxanthin cycle greatly contributes to plant acclimation to several abiotic stresses and prevents from inactivation of PSII (Brugnoli, *et al*., 1994; Gulías, *et al*., 2002; Munné-Bosch & Peñuelas, 2003). On the other hand, lutein formation by de-epoxidation of lutein epoxide is another mechanism of NPQ but it has only been validated for a few species (Esteban, *et al*., 2009). In fact, due to close proximity between chlorophylls and lutein in LHC, this xanthophyll directly

contributes to chlorophyll triplet quenching (Mozzo, *et al*., 2007). Likewise, other xanthophylls like zeaxanthin that accumulate under high light also contribute to directly quench triplet state chlorophylls at specific monomeric antenna subunits, both in Lhcb4-6 of PSII and Lhca1-4 of PSI *(Dall'Osto, et al*., 2012). Besides, zeaxanthin that is also found at the inner protein/lipid interface of LHC-II can physically quench $^1O_2$ moving within complexes (Havaux, *et al*., 2007; Johnson, *et al*., 2007). On the other hand, neoxanthin regulates the availability of violaxanthin for NPQ by changing its affinity to its location at LHC-II and it also appears to be active against $O_2^{\bullet -}$ (Dall'Osto, *et al*., 2007; Wang, *et al*., 2017). Nevertheless, the main carotenoid to detoxify $^1O_2$ produced by high quanta energy in the reaction center is β-carotene (Telfer, 2002). The position of β-carotene within the reaction center of PSII makes it unfeasible to reach P_{680} and in so, is unable to quench its triplet excited state. Aside from its relevant role in $^1O_2$ inactivation, β-carotene also participates in PSII assembly and D1 repair cycle (D'Alessandro & Havaux, 2019). Moreover, chemical quenching of $^1O_2$ by β-carotene triggers the accumulation of β-carotene endoperoxides in PSII, specially under high light. From the photo-oxidation of β-carotene, a number of volatile short-chain compounds are produced, including β-cyclocitral that was found to be associated with retrograde signalling and induce the expression of $^1O_2$ responsive genes while increasing tolerance to photo-oxidative stress (Ramel, *et al*., 2012). Taking together the multiple roles of carotenoids not only for energy dissipation and ROS quenching, but also necessary in environmental sensing and signal transduction, their analysis is fundamental when discussing about photoinhibition and photo-oxidative stress in plants.

3.3. Vitamin E

Vitamin E comprises an array of lipid-soluble antioxidants that includes eight forms of tocochromanols whose chemical structure is composed by a polar chromanol head linked to a hydrophobic prenyl tail, completely

saturated for tocopherols and three unsaturated carbons at 3', 7' and 11' for tocotrienols. Considering the number and position of the methyl groups at the chromanol head, tocopherols and tocotrienols can be further divided into α, β, γ and δ isoforms, with very different biochemical and antioxidant properties (Müller, *et al*., 2010). Evans & Bishop (1922) were the first researchers to identify an essential factor for rat reproduction that years later was found to be α-tocopherol (Evans, *et al*., 1936; Fernholz, 1938; Karrer, *et al*., 1938). At present, there is evidence that a deficiency in tocopherols is associated to neurological disorders in humans and its supplementation is employed for treatment of non-alcoholic steatohepatitis, while they also positively influence the immune response and lower general inflammation (Khadangi & Azzi, 2019). Because humans cannot synthesize vitamin E, dietary uptake from fruits and vegetables is essential, although only α-tocopherol has been identified to be specifically required for body function since the α-tocopherol transfer protein (α-TTP) is the only import protein characterised and all the other analogues are rapidly metabolized (Khadangi & Azzi, 2019). In plants, early studies suggested that tocochromanols might not play an essential role in photoprotection since their rate constant for $^1O_2$ quenching was lower than that for carotenoids in solution (Di Mascio, *et al*., 1990) and plants with single mutations in chloroplastic tocopherol cyclase (*vte1*) or methyl transferase (*vte4*) did not compromise growth, chlorophyll content or photosynthetic quantum yield under standard conditions and were only slightly reduced during photo-oxidative stress (Porfirova, *et al*., 2002; Bergmüller, *et al*., 2003). However, more recent studies have evidenced the importance of vitamin E preserving PSII from photo-inactivation and protecting membrane lipids from photo-oxidation (Havaux, *et al*., 2005). Physical quenching and chemical scavenging of $^1O_2$ is one of the main features of tocochromanols, who work in coordination with other antioxidants like glutathione (Caretti, *et al*., 2002), xantophylls (Havaux, *et al*., 2005), β-carotene (Triantaphylides & Havaux, 2009) and ascorbic acid, the latter being responsible to recycle tocopheroxyl radicals (Smirnoff, 2000;

Kobayashi & DellaPenna, 2008), to prevent PSII from photo-inactivation.

As part of the present dissertation, we reviewed the latest research advances on vitamin E in plants to understand novel insights on metabolic pathways involved in tocochromanols biosynthesis, as well as new evidence for vitamin E transport within plastids and its diverse physiological roles in plant photoprotection, antioxidation and acclimation (see Annex 1, Vitamin E in Plants: Biosynthesis, Transport, and Function). For instance, it is a general believe that all different isoforms of vitamin E are supposed to be confined inside plastids, since the enzymes catalysing the final steps of tocopherols and tocotrienols biosynthesis are found at the chloroplast inner membrane and inside plastoglobuli. However, the recent discovery of a tocopherol binding protein in tomato plants (SlTBP), both in leaves and fruits, opens a new paradigm in the perception of tocopherol movement. In fact, even though this protein targets chloroplasts, other TBPs could be involved in extraplastidial transport that could explain unsolved questions such as accumulation of vitamin E in lipid bodies of seeds (Fisk, *et al*., 2006) and vacuoles of mesophyll cells (Rautenkranz, *et al*., 1994) or the presence of different proportions of vitamin E analogues in non-photosynthetic tissues (Fernandes, 2018; Gramegna, *et al*., 2019; Soba, *et al*., 2020). Moreover, recent findings on vitamin E biosynthesis show that chlorophyll recycling mediated by the phytol kinase VTE5 and the phytyl phosphate kinase VTE6 gives rise to phytyl-diphosphate as precursor for the tocopherols prenyl tail, alternative to *de novo* formation from GGDP of the MEP pathway (Valentin, *et al*., 2006; vom Dorp, *et al.*, 2015). Besides, VTE5 has also been characterised in tomato plants, where vte5-silenced plants (*SlVTE5*) experienced a dramatic reduction of up to 90% less tocopherol not only in leaves, but also in fruits whose quality was also greatly affected (Almeida, *et al*., 2016). The full description of this pathway is particularly significant considering that stress situations as well as several developmental processes in plants lead to chlorophyll degradation that can compromise light absorption and trigger ROS production in chloroplasts as it will be seen

for all three models of the present dissertation. Likewise, even though the chemical scavenging activity of vitamin E against membrane peroxyl radicals has long been known (Havaux, *et al*., 2005; Atkiston, *et al*., 2010), the exact mechanism to allow effective scavenging is still under study. A new theory describes high frequency of flip-flop movements of tocochromanols within lipid membranes as a convenient strategy to make peroxyl radicals more accessible and therefore, increase their protective efficiency in oxidized environments (Boonnoy, *et al*., 2018). On the other hand, it has been hypothesized by several authors that vitamin E can induce specific redox signatures derived from controlling ROS production or lipid peroxidation products that may be involved in chloroplast-to-nucleus signalling (Munné-Bosch, *et al*., 2007; Maeda, *et al*., 2008). New evidence supports this idea since α-tocopherol promotes the accumulation of 3'-phosphoadenosine 5'-phosphate (PAP), involved in the protection of microRNAs that induce nuclear gene expression and allow plant acclimation (Fang, *et al*., 2019). In that manner, tocochromanols may be modulating chloroplast retrograde signalling in coordination with other systems involved in the cellular redox balance and together operate in stress sensing and signalling. Considering all these features, special emphasis has been made in the present dissertation to set new evidence on vitamin E production and functions in very different models where chloroplast integrity is compromised.

4. Chloroplasts as hub regulatory sites to match environmental and developmental signals

Chloroplasts, by harbouring all photosynthetic machinery and several metabolic pathways related to the biosynthesis of plant photoprotective molecules and stress-related phytohormones, are sensitive sites for the perception of environmental and developmental cues that alter cellular redox balance and stimulate retrograde signalling to induce adaptive and developmental responses. Chloroplasts face multiple challenges throughout plant life cycle that demand a precise coordination of interorganellar

communication to preserve their functions in changing environments. There is considerable interest in the chloroplast-to-nucleus pathway since the exact mechanisms of signal transduction still remain elusive despite great efforts of multiple researchers to identify signalling molecules involved in retrograde communication. As previously highlighted, most environmental constraints result in high energy quanta at the reaction centers, although the means to achieve this situation can be diverse. Excessive light primarily triggers the production of ROS at both PSII and PSI, therefore it is simple to relate their production as the first elicitors that prompt retrograde signalling to regulate nuclear gene expression that allow plant acclimation or coordinated organ development (Gill & Nuteja, 2010; Rogers & Munné-Bosch, 2016). In fact, several studies have reported nuclear changes and higher acclimation upon ROS production, particularly when $^1O_2$ and H_2O_2 increase in a process known as oxidative burst (Gill & Nuteja, 2010; Foyer, 2018; Dietz, *et al*., 2019;). Other ROS-derived compounds that are produced from their oxidative action like β-cyclocitral (Ramel, *et al*., 2012; D'Alessandro & Havaux, 2019) or oxylipins (Weber, *et al*., 2004; Park, *et al*., 2013; Tikkanen, *et al*., 2014a) have also been pointed out as secondary elicitors to activate retrograde signalling pathways. Both ROS and ROS-derived compounds can forward the signal to the nucleus by redox changes in thiol groups, as well as inducing the release of Ca^{2+} that activate MAPK pathways or by crosstalk with other phytohormones that can physically interact with *cis* elements of nuclear genes encoding proteins that promote adaptive and developmental responses (Xia, *et al*., 2015; Choudhury, *et al*, 2017).

However, most of these studies where the role of ROS production is analysed have been devoted to understand specific responses derived from environmental constraints that allow plant acclimation, as it can be noted by the number of published reviews that one can find in literature (Mittler, 2002; Foyer & Noctor, 2005; Gill & Nuteja, 2010; Dietz, *et al*., 2016; Choudhury, *et al*., 2017; Foyer, 2018). Less attention has been paid to the possible regulatory role of retrograde signalling to coordinate developmental

processes in specific plant organs like fruits and flowers where, unlike leaves, chloroplasts are not retained but are dismantled or transformed several stages before senescence. In fact, during the development of some flowers and fruits there is a conversion of chloroplasts to other plastids types like chromoplasts or leucoplasts. As part of the present dissertation, we reviewed current knowledge on the contribution of photo-oxidative stress during leaf, flower and plant development to identify sensitive processes that could be subjected to this regulation, as it may have important economic implications in agriculture and horticulture (see Annex 2, Photo-oxidative stress during leaf, flower and fruit development). One of the main questions addressed in the review is that the timing and location of ROS production is important when comparisons are made between leaves, flowers and fruits because the nature of these ROS also determines their interactions and derived outcomes. Moreover, even though extensive research has been conducted to depict chromoplast formation with regard to carotenoid composition and enzymes involved in their biosynthesis both in flowers (Kishimoto & Ohmiya, 2006; Chiou, *et al*., 2010) and fruits (Fraser, *et al*., 1994; Kato, *et al*., 2004; Botella-Pavía & Rodríguez-Concepción, 2006; Diretto, *et al*., 2007), little is known about chloroplast to chromoplast transition, a subject that could arise many biotechnological agri-food applications as it is tightly related to organ shelf-life. On the other hand, from work performed on Arabidopsis mutants some specific proteins and transcription factors mediating retrograde signal pathways have been identified. That is the case of EXECUTER1 (EX1), a plastid protein that was overexpressed in *flu* mutants that produced higher levels of $^1O_2$ than the wild type after illumination. EX1 is supposed to be at the interface between plastids and the cytosol to mediate responses to $^1O_2$ (op den Camp, *et al*., 2003; Wagner, *et al*., 2004). Moreover, $^1O_2$ is believed to also interact through an EX1-independent pathway that involves activation of OXIDATIVE SIGNAL INDUCIBLE1 (OXI1), that can move across chloroplast membranes and induce the production of oxylipins that can mediate signal transduction (Shumbe, *et al*., 2016). In contrast, H_2O_2 also activates OXI1 but

transmits downstream signlalling through MAPK pathway (Rentel, et al., 2004). At present, no experiment has been conducted to identify the expression and activity of these proteins in other systems than leaves. As a matter of fact, only few studies have shown evidence of oxidative burst during ripening of fruits (Martí, et al., 2009; Kumar, et al., 2016) and flower opening (Chakrabarty, *et al*., 2007; Zhang, *et al*., 2007; Liu & Lin, 2013), which indicates that an integrated view on the relevance of photo-oxidative stress in other organs than leaves is needed and opens a framework to identify key events that contribute to a possible regulation of fruit ripening and flower development by retrograde signalling pathways.

5. Experimental models to study photoinhibition and photoprotection strategies

5.1. Leaf acclimation and development: *Pistacia lentiscus* L.

Overwintering has some advantages for evergreen plants because they can maximize carbon gain or improve nutrient use efficiency under nutritionally poor conditions (Öquist & Huner, 2003). Nevertheless, this process also constitutes a great challenge for plant physiological status since lowering temperatures affects membrane fluidity, protein and nucleic acid conformation, reduces the rate of biochemical reactions and induces gene expression reprogramming (Öquist & Huner, 2003). Besides, cold temperatures may also constraint photosynthetic rates because water and nutrient uptake is very limited and enzymatic activity is slower than in optimal temperatures. Thereafter, reduced photosynthesis under these conditions lowers the photoinhibition threshold and light saturates the reaction center of PSII at inferior energy quanta than in favourable conditions, which can trigger leaf photo-chilling (Silva-Cancino, *et al*., 2012; Míguez, *et al*., 2015). Mediterranean winters do not entail excessive drops in mean temperatures during winter but, unlike boreal regions, skies remain cloudiness during winter season. The combination of lowered temperatures and cloudless skies have forced Mediterranean perennial plants evolve different strategies

to avoid over-reduction of the electron transport chain components, which greatly influence their life performance. Photoinhibition of PSII may be one of the strategies to allow the preservation of photosynthetic structures during Mediterranean winters, as it entails different mechanisms of thermal dissipation to avoid high quanta energy at PSII and to prevent excessive production of ROS that could surpass the antioxidant system and compromise plant survival (Oliveira & Peñuelas, 2000; Sonoike, 2011; Tikkanen, *et al*., 2014b). Moreover, acclimation to adverse conditions in overwintering through a balance between photoinhibition and sustained photosynthetic rates may determine plant performance throughout the year and the capacity of these species to keep their ecological niches and influence their distribution. Taken together, it is important to analyse physiological changes taking place in overwintering for Mediterranean plants and assess to what extent leaf survival during the winter season compromises cellular redox status.

Mastic trees (*Pistacia lentiscus* L.) are perennial dioecious plants characteristic of the Mediterranean region. These angiosperms from the Anacardiaceae family can be found as part of the maquis vegetation in shrubberies and undergrowing in pine and holm-oaks forests forming bushes, although they can grow up to 10m in height (Maestre, *et al*., 2004; Nora, *et al*., 2015). Both the aerial part of *P. lentiscus* and its essential oil have long been used in traditional medicine for treatment of hypertension and for its diuretic properties due to high antioxidant content (Chryssavgi, *et al*., 2008). Leaves are composed with variable number in leaflets, always pair, and a structure strongly adapted to withstand large periods of drought characteristic of Mediterranean summers, with thick parenchima and strong cuticule to avoid water losses. In the Mediterranean basin, both male and female mastic trees flower between March and May giving two distinct characteristic flowers with red crolours for males and bright pink colours for females. Fructification usually starts in June and lasts until the end of August – beginning of September with small round fruits that change from green to

red and black colours during their ripening. *P. lentiscus* leaves produced at spring do not fall during winter and instead, they fall during August after a full year of development (Munné-Bosch & Peñuelas, 2003). Mastic trees are therefore a good example of Mediterranean plants that must cope with lowered temperatures and cloudless skies during winter, where PSII integrity might be compromised. In fact, several studies have already reported a decline of the maximum efficiency of PSII (F_v/F_m) in *P. lentiscus*during winter (Flexas, *et al*., 2001; Juvany, *et al*., 2014), even though photoprotective mechanisms have not been studied in plants growing in their natural habitat.

In consequence, the first chapter of the present dissertation analyses the involvement of winter photoinhibition in the life performance of *P. lentiscus* plants from the perspective of photo-oxidative damage and its implication on leaf development, plant acclimation and survival, while considering also specific patterns for antioxidants timescale production during the whole year and in an altitudinal gradient. Moreover, leaf phenology and its biological significance was also considered since the particular pattern of shoot renewal in mastic trees could greatly influence their plant stress responses.

5.2. Flower opening: *Lilium* L.A. var. Iitouwen

Flower development is a complex process that entails the orchestration of multiple regulatory pathways to external and internal cues that condition physiological and morphological changes taking place at different stages of organ development. From the floricultural industry worldwide, commercialisation of cut flowers represents the biggest market with a global trade worth around €15bn (International Trade Centre (ITC), 2020). Flower growers carefully pick the optimal stage for harvest, which can vary considering special features of each variety but usually coincides when the stem length meets the standards of wholesale market and less than one-third of the flowers from the inflorescence are open. In fact, the timing of harvest is essential for the flower market since growers must ensure that

flowers have no signs of deterioration when reaching the consumers. Because production of these flowers is distributed worldwide and great travelling distances are required for their distribution, flower shelf-life is particularly important. In this sense, much research has been conducted to depict biochemical and physiological events that lead to flower senescence, which determines flower vase life (van Doorn & Woltering, 2008; Rogers, 2013). However, less attention has been paid to other key developmental processes like flower opening, where there is great cell expansion in flower petals and a shift of flower corollas from source to sink, with conversion of starch to monosaccharides or sugar uptake from the apoplast (van Doorn & van Meeteren, 2003). Indeed, sugar uptake and the limits of flower resources have been considered as one of the many factors that contribute to determine vase life of cut flowers (Hoeberichts, *et al*., 2007; Singh, *et al*., 2008; Arrom & Munné-Bosch, 2012a), together with hormonal cross-talk (Koning, 1984; Arrom & Munné-Bosch, 2012b) and mechanisms to overcome ROS production in the late stages of flower development prior to programmed cell death (Rogers & Munné-Bosch, 2016). Therefore, controlling the timing of flower opening may be a promising strategy to implement flowers shelf-life.

Some flowers like those from the *Lilium* genus have corollas composed by tepals, with no difference between the outer and intern perianth whorls, both having petaloid structures. At the beginning of flower development, buds of lily flowers are closed, and the external petals exhibit green colours because of high retention of chlorophyll pigments. Considering different varieties of *Lilium*, the green hue of the outer tepals begin to fade prior or at the moment of flower opening, when other pigments such as anthocyanins (Nakatsuka, *et al*., 2009; Yamagishi, *et al*., 2010b) or carotenoids (Yamagishi, *et al*., 2010a) begin to accumulate giving rise to spectacular colourations to attract pollinators, but also consumers in the case of ornamental flowers. De-greening of lily tepals is related to chlorophyll degradation and chloroplast dismantling that contributes to the

physiological shift from source to sink during flower opening, since the corolla no longer contributes to the production of photoassimilates and begin a phase of intense metabolic activity (Van der Meulen-Muisers, *et al*., 2001; Arrom & Munné-Bosch, 2012). During this phase of physiological conversion, chloroplast integrity is compromised and therefore, ROS production from this organelle may arise. However, cell damage must be controlled by antioxidant mechanisms to allow full development of the flower so that the gyneceum is completely mature (van Doorn & Woltering, 2008). Moreover, as reviewed in Annex 2, chloroplast preservation is dependent upon a tight between phytohormones, nitrogen content and ROS, being ethylene and ROS promoting molecules for chloroplast conversion to other forms of plastids or contributing to their degradation, while gibberellins and nitrogen have been described as preventing molecules. Therefore, control of ROS production and hormonal levels in flowers prior to anthesis may prevent from a rapid dismantling of tepal chloroplasts and with that, possibly extend the flower vase life.

Lilium 'Litouwen' is a hybrid variety of *L. longiflorum* x Asiatic (LA) that shows severe de-greening in tepals during early stages of development. For this, in the second chapter of the present dissertation *Lilium* 'Litouwen' served as an excellent model to study photo-oxidative stress during tepal de-greening in flower opening. Moreover, since some commercial products that include phytohormones in their composition have shown positive effects to extend flower shelf life, the role of a gibberellin- and cytokinin-based product was analysed in terms of chloroplast maintenance and the role of these phytohormones in the control of flower opening.

5.3. Fruit ripening: *Prunus avium* L. var. Prime Giant

From a physiological point of view, fruit development encompasses all biochemical and structural changes taking place from fruit set to the end of fruit ripening, which includes different phases of growth and the onset of maturation. These physiological changes require the coordination of

metabolic and molecular signals that allow fruit softening from cell wall modifications, chlorophyll breakdown and pigment production, sugars accumulation and degradation of organic acids, as well as increased biosynthesis of secondary metabolites (Gapper, *et al*., 2013). The ripening process is highly relevant in commercial orchards, since the final organoleptic and nutritional quality strongly relies on correct on-tree development (Toscano, et al., 2019). In fact, most high-value compounds with beneficial properties for human health like unsaturated fatty acids or antioxidants, accumulate from maturation onwards (Díaz-Mula, *et al*., 2008; Ilahy, *et al*., 2011; Ornelas-Paz, *et al*., 2013). Ripening onset is characterised in most fruits by a considerably loss of chlorophylls due to chloroplast dismantling or conversion to chromoplasts (Kahlau & Bock, 2008; Lado, *et al*., 2015; Rodriguez-Concepcion, *et al.*, 2018). Recently, a new pathway for phytol formation from chlorophyll breakdown was described in Arabidopsis (Valentin, *et al*., 2006). As previously stated in section 3.3. and in the Annex 1 on new findings for vitamin E biosynthesis, the phytol recycling from chlorophyll degradation could participate in the production of tocopherols, also in fruits like tomato and olives (Almeida*, et al*., 2016; Georgiadou, *et al*., 2016). However, to date, no study has been conducted during on-tree development to determine whether this pathway in non-climacteric fruits like sweet cherries or if there is any phytohormone related to fruit ripening that could promote the activation of the phytol recycling pathway. Sweet cherries (*Prunus avium*) are stone fruits with high antioxidant properties that essentially belong to the hydrophilic fraction, since anthocyanins that accumulate in fruit cells vacuoles both at the exocarp and endocarp of the fruit, are the main antioxidants produced. Besides, other antioxidants like tocopherols have also been detected in cherry fruits at ripen stages. Because sweet cherries have low contents of carotenoids at harvest because they are degraded during ripening (McCune, *et al*., 2011; Tijero, *et al*., 2016), it may be interesting to understand how the biosynthetic pathway of vitamin E works in cherry fruits to try to increase the

lipophilic fraction of antioxidants, which could have health beneficial effects for consumers. Moreover, even though the ripening of sweet cherries relies on the coordinated work of different groups of phytohormones, abscisic acid (ABA) is the one that drives most of biochemical and structural changes related to fruit ripening in sweet cherries (Tijero, *et al*., 2016). Biosynthesis of this sesquiterpenoid phytohormone takes place from cleaveage of the carotenoid precursors violaxanthin and neoxanthin from the MEP pathway by the enzyme activity of NCED, which gives rise to the immediate precursor of ABA (Xiong & Zhu, 2003). In sweet cherries, ABA begins to accumulate at the onset of ripening when there is a change in the breaking colour, which is also coincident to the moment where tocopherols begin to accumulate (Tijero, *et al*., 2016). Therefore, ABA could promote the biosynthesis of tocopherols from chlorophyll breakdown when the chloroplast dismantling takes place and cherry fruits begin to ripen.

OBJECTIVES

OBJECTIVES

The present dissertation aims for a better understanding of the implication of photoinhibition and photo-oxidative events taking place at different intensities and timescales throughout plants lifespan. Moreover, because the significance of these events may be different considering the organ where they are occurring, it was important to determine if leaves, flowers and fruits of plants with ecological and commercial interest could have different susceptibility to photoinhibition and photo-oxidative stress considering their developmental stage.

To accomplish the general purpose of the present dissertation, specific objectives were defined:

- Evaluate the extent of photoinhibition in leaves of a Mediterranean plant with ecological relevance like mastic trees, as well as in flowers and fruits of economical important crops like lily flowers and sweet cherries.
- Determine if a common pattern of photoprotection is found for all different organs at different stages of development and whether production of photoprotective compounds modulates photo-oxidative stress.
- Assess if the extent of photo-oxidative stress compromises organ development and plant survival.
- Establish if chloroplast status conditions antioxidant mechanisms, as well as molecular and hormonal signals that regulate acclimatory and developmental processes through a coordinated response.

REPORT FROM THESIS DIRECTOR

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Prof. Sergi Munné Bosch, as Ph.D. supervisor of the thesis entitled "**Involvement of photoinhibition and redox balance in acclimation and developmental processes of plants**" presented by the Ph.D. candidate Paula Muñoz Roldán,

REPORTS on the impact factor and the participation of the Ph.D. candidate on every chapter and annexes included in this doctoral dissertation.

Chapter 1. Article "**Transient photoinhibition and photo-oxidative stress as an integral part of stress acclimation and plant development in a dioecious tree adapted to Mediterranean ecosystems**", submitted to the journal *Tree Physiology*, impact factor (2018) of 3.477. This study nicely illustrates the complexity of stress responses at various organization scales, from acclimation processes operating at the molecular level to responses at the population level. The candidate performed all samplings with the occasional help of other members of the research group, performed all biochemical and data analyses and wrote the manuscript. The candidate has shown an outstanding capacity of work, leadership in team work and great abilities for sample analysis in the laboratory. The candidate also demonstrated very good skills for data presentation and interpretation of results, showing an excellent intellectual maturity.

Chapter 2. Article "**Photoinhibition and photoprotection during flower opening in lilies**", published in the journal *Plant Science*, impact factor (2018) of 3.785. Using a simple but original experimental design, the role of photoinhibition and photoprotection in flower opening was studied, nicely illustrating the importance of chloroplasts in vase life extension. Results of this study attained the media due to the fact that they can be used to extend flower vase life. The candidate performed all samplings and biochemical analyses, prepared illustrations and figures, and performed statistical analyses. The candidate has shown an outstanding capacity of work, leadership in team work and great abilities for interpretation of results. The candidate demonstrated again very good skills for data presentation and interpretation of results, showing an excellent intellectual maturity.

Chapter 3. Article "**Abscisic acid triggers vitamin E accumulation from phytol recycling in "Prime Giant" sweet cherry fruits during ripening**", submitted to the journal *Food Chemistry*, impact factor (2018) of 5.399. This study shows for the first time that ABA can trigger vitamin E biosynthesis from phytol recycling from chlorophyll degradation. Interestingly, the study is performed in non-climacteric fruits of high interest not only for basic science but also for biotechnology. It is noteworthy the original experimental approach used and the difficulty of performing such experiments under field conditions. The candidate performed all treatments and samplings, biochemical analyses, statistical treatments and prepared figures and tables, aside from actively participating in the experimental design and interpretation of results. The candidate showed again a great capacity of work both in the field and in the laboratory, as well as an excellent use of HPLC and LC-MS/MS. The candidate also optimized extraction protocols and PCR conditions for the correct analysis of gene expression by RT-qPCR. The candidate participates in most of the conceptual work and shows excellent skills both for data interpretation and writing of the manuscript.

Annex 1. Article "**Vitamin E in plants: biosynthesis, transport, and function**", published in the journal *Trends in Plant Science*, impact factor (2018) of 14.006. The candidate participates in outlining the manuscript, writes a significant part of the manuscript and helps preparing all figures, showing an excellent intellectual maturity.

Annex 2. Article "**Photo-oxidative stress during leaf, flower and fruit development**", published in the journal *Plant Physiology*, impact factor (2018) of 6.305. The candidate writes a significant part of the manuscript and helps preparing several figures, showing an excellent intellectual maturity.

Sincerely yours,

Prof. Sergi Munné Bosch

RESULTS

Chapter 1

Transient photoinhibition and photo-oxidative stress as an integral part of stress acclimation and plant development in a dioecious tree adapted to Mediterranean ecosystems

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Transient photoinhibition and photo-oxidative stress as an integral part of stress acclimation and plant development in a dioecious tree adapted to Mediterranean ecosystems

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Abstract

Despite photoinhibition triggered by environmental changes is frequent in plants from Mediterranean-type ecosystems, scarce information exists on the extent of photo-oxidative stress during winter periods in several species, and in particular how sex, leaf phenology and altitudinal variation influence stress acclimation in dioecious plants. Here, photoinhibition and photo-oxidative stress markers were examined in mastic trees (*Pistacia lentiscus* L.) from a natural population growing in the Garraf Natural Park for a consecutive 12-month period (seasonal study), as well as in three populations naturally growing in the Montseny Natural Park, including the highest altitudes described for this species, during winter (altitudinal study). Furthermore, the influence of leaf phenology and sex on photoinhibition and photo-oxidative stress were evaluated in the Garraf Natural Park. Results indicated that mastic trees ssuffered photoinhibition during winter but not in summer, and that winter photoinhibition increased with elevation. In both cases, however, winter photoinhibition occurred transiently, and although it was accompanied by enhanced chlorophyll loss and malondialdehyde contents, no terminal damage to the photosynthetic apparatus occurred. Stress acclimation was achieved through biochemical adjustments in chloroplasts, characterized by anthocyanin shielding, increased de-epoxidation state of the xanthophyll cycle as well as tocopherol accumulation, and phenological adaptations, the latter allowing a complete resetting

of the physiological performance of leaves. Moreover, although females showed higher lipid peroxidation than males during both the coldest winter months and flowering at spring, this oxidative stress was mild and transient with no negative consequences for the physiology of plants. It is concluded that winter photoinhibition does not compromise shoot survival in mastic trees due to deployment of various biochemical and developmental adaptive mechanisms, from the activation of antioxidant defences to phenological adjustments, altogether playing a crucial role in acclimation and plant survival.

Keywords: altitudinal distribution, anthocyanins, carotenoids; dioecy; *Pistacia lentiscus* L.; phenology; photoprotection; vitamin E; winter photoinhibition.

Introduction

As photoautotrophic organisms, plants strongly rely on light to sustain their physiological functions and promote their correct development (Franklin 2009). During optimal environmental conditions, plants efficiently capture light to perform photosynthesis, which allows inorganic carbon assimilation and biosynthesis of organic carbon structures. Nevertheless, environmental conditions are constantly changing in nature, which lead to suboptimal photon usage and therefore, an imbalance of excitation energy in photosystems, generally known as photoinhibition (Demmig-Adams and Adams 1992, Asada et al. 2000). Light excess in chloroplasts can induce the production of reactive oxygen species (ROS) by electron transfer to ground oxygen (Triantaphylidès and Havaux 2009, Miyake 2010). Due to their oxidative nature, ROS can be harmful molecules for cell structures if their production is not avoided or efficiently counteracted. Mechanisms to overcome photoinhibition greatly vary on the timing and severity of this process and determine its transience or persistence. Movement of leaves, reorganisation of chloroplasts distribution and sunlight screening by phenolic compounds like anthocyanins, constitute fast and reversible strategies to prevent saturating light in photosystems (Steyn et al. 2002, Pintó-Marijuan and Munné-Bosch 2014). When light reaches chloroplasts, ROS formation is avoided through physical quenching of excited chlorophylls in the light harvesting complexes (LHC), by the involvement of the xanthophyll cycle that allow thermal energy dissipation (Thayer and Björkman 1990, Matsubara et al. 2007, Ruban 2016). If not avoided, ROS are quenched and scavenged by β-carotene or tocopherols (vitamin E) in the reaction centres and the lipid matrix of thylakoid membranes (Telfer et al. 2002, Havaux et al. 2005). When the rate of ROS production exceeds the capacity of these mechanisms, lipid peroxidation can occur. In these cases, lipid propagation in membranes can be prevented by tocopherols to avoid cell damage (Foyer and Shigeoka 2011, Muñoz and Munné-Bosch 2019). Moreover, ROS and antioxidant biochemistry is rather more complex, since ROS and antioxidants are also involved in cellular signalling and environmental sensing, essential for the

process of acclimation (Wagner et al. 2004, Foyer and Shigeoka 2011, Dietz et al. 2016, Mittler 2017, Muñoz and Munné-Bosch 2018).

Plants living in Mediterranean-type ecosystems are subjected to seasonal variations in climatic conditions and, consequently, potentially exposed to repeated periods of photoinhibition throughout the year. Evergreen Mediterranean plants have evolved to withstand seasonal climatic fluctuations without permanently losing their vegetative tissues. Nevertheless, according to the photo-protective mechanisms involved during photo-inhibition, susceptibility to environ-mental changes greatly differs among perennial plants. Even though Mediterranean winters do not entail an extreme drop in temperatures, decreases in the maximal efficiency of photosystem II (PSII) photochemistry (or F_v/F_m ratio) have been reported for several evergreen species (Martínez-Ferri et al. 2004, Silva-Cancino et al. 2012, Morales et al. 2016). Low temperatures affect plant physiological status by altering membrane fluidity as well as protein and nucleic acid conformation and influence cellular metabolism, indirectly through gene expression reprograming and directly reducing the rates of biochemical reactions. Besides, under low temperatures, water and nutrient uptake is also limited which, altogether, contributes to constraints in photosynthesis. As a consequence, altered photosynthetic rates from cold temperatures reduce the threshold at which light becomes saturating and leads to excess light stress at chilling conditions (Silva-Cancino et al. 2012, Míguez et al. 2015). Overwintering in evergreen plants confer some advantages such as maximizing carbon gain or improving nutrient use efficiency under nutritionally poor conditions. To maximise these benefits, perennial Mediterranean plants have indeed adopted the strategy of winter photoinhibition to cope with limited photosynthetic rates and avoid photosystem damage (Öquist and Huner 2003). However, some Mediterranean evergreen plants are chilling sensitive, incapable to cope with freezing temperatures, and do not tolerate ice formation within their tissues, a feature that can constraint the spatial distribution of these species, limiting both their

latitudinal and altitudinal distribution (Körner and Paulsen 2004, Rubio-Casal et al. 2010).

Pistacia lentiscus L. is a dioecious perennial plant that can be found across the Mediterranean basin in shrubberies and undergrowing in pine and holm-oaks forests. *P. lentiscus* is a very representative plant from the Mediterranean maquis vegetation (Maestre et al. 2004, Kozhoridze et al. 2015, Nora et al. 2015) and has sclerophyll composed leaves with thick parenchyma and cuticle structures, highly adapted to overcome drought and high temperatures characteristic from the Mediterranean climate. Unlike other shrubs from the same genus, *P. lentiscus* avoids leaf falling during winter periods and leaf senescence and abscission from old shoots takes place during the summer period, when new leaves are fully mature (Munné-Bosch and Peñuelas 2003). Overwintering constitutes a great challenge in terms of excess light dissipation and protection during chilling, and, in fact, some studies have already reported an impairment of PSII efficiency during cold periods in *P. lentiscus* (Flexas et al. 2001, Juvany et al. 2014), even though the transient nature of the process, their causes and the possible influence of phenology and dioecy have not been considered in detail thus far. As dioecious plants, it has been shown that the physiological performance of *P. lentiscus* is also influenced by sex (Barradas and Correia 1999, Correia and Diaz Barradas 2000, Ait Said et al. 2013). Higher demands of energy investment and resources allocation in female plants during flowering and fructification are important factors to explain a differential behaviour between male and female plants (Jonasson et al. 1997). Differential sex ratios and spatial distribution can be found for female and male mastic trees depending if they live in open forsaken agricultural areas or undergrowing forests (Barradas and Correia 1999). Moreover, *P. lentiscus* has a limited altitudinal distribution, with an usual range of distribution in the Iberian peninsula between 0 – 400m and very few exceptions of populations above 700 m (Martinez-Parras et al. 1985, Palacio et al. 2005). However, to our knowledge, no study has been conducted thus far to understand the physiological constraints that might entail this limited altitudinal distribution. With the aim of better

understanding stress acclimation mechanisms in this species, we evaluated seasonal variations in the extent of photoinhibition and photo-oxidative stress in a natural population growing in the Garraf Natural Park and during winter in three natural populations of the Montseny Natural Park growing at 360, 530 and 740 m above sea level (a.s.l.). The influence on leaf phenology and dioecy on photoinhibition and photo-oxidative stress was also considered.

Materials and methods

Plant material and samplings

In order to determine whether temperature fluctuations during annual seasonality could induce photoinhibition in the dioecious plant *Pistacia lentiscus* L., 20 female and 21 male plants were randomly selected (Figure 1a) from a natural population located in the Garraf Natural Park (Barcelona, Spain) at 480 m a.s.l. (41°16'56.61"N, 1°54'22.37"E). Samplings were performed during the second half of each month from October 2017 to September 2018 at solar midday. From October 2017 to July 2018, samples were collected from shoots formed during 2017; and from June to September 2018, samples were collected from shoots formed during 2018. Three composed leaves with south orientation from shoots of the upper part of the plant were sampled, the third leaflet of one of them taken to estimate the maximum efficiency of PSII (F_v/F_m), water contents and leaf morphology, while the other two were immediately frozen in liquid nitrogen and stored at -80°C until antioxidant and oxidative derivate analysis. Furthermore, the influence of leaf phenology and sex on photoinhibition and photo-oxidative stress were evaluated in the Garraf Natural Park. The influence of leaf phenology was evaluated considering the response of newly formed shoots during the next spring, the foliar physiology of which was evaluated between June and September 2018. The influence of dioecy was evaluated by examining the differences in the foliar physiology of males and females throughout the study period.

To study the influence of cold stress on photoinhibition and photo-oxidative stress considering an altitudinal gradient in *Pistacia lentiscus* L., the highest described population in Catalonia (Anthos, 2011) at 740 m a.s.l.

(41°44’53.63”N, 2°16’45.35”E), along with populations at 530 m a.s.l. (41°44’54.47”N, 2°16’11.39”E) and 360 m a.s.l. (41°44’45.32”N, 2°15’57.59”E) in the Montseny Natural Park (Barcelona, Spain) were selected. All samplings were performed on February 25th, 2019, so that a total of thirty individuals from each population were randomly selected and sampled following the same procedure described above.

Climatological data was collected from the weather stations situated at the Garraf Natural Park (El Rascler, 41°17’17.952”N, 1°54’27.9”E) and the Montseny Natural Park (Tagamanent, 41°44’51.396”N, 2°18’10.475”E; Servei Meteorologic de Catalunya, Generalitat de Catalunya, Spain).

Chlorophyll fluorescence, leaf mass per area and water status

The maximum efficiency of PSII photochemistry (F_v/F_m ratio) was determined by measures of chlorophyll fluorescence in the middle part of leaflets using a fluorimeter (MINI-PAM-II Photosynthesis Yield Analyzer, Walz, Effeltrich, Germany). Leaflets physiological traits were assessed estimating both leaf mass per area ratio (LMA) and leaf hydration (H). To calculate LMA, leaflets were weighed to estimate the fresh matter (FW) and scanned to analyse their area with Image J (Schneider et al., 2012). Later, to determine leaf hydration, samples were oven-dried at 80°C to constant weight to estimate the dry matter (DW) and H calculated as (FW-DW)/DW.

Foliar pigments and antioxidant molecules

To determine the content of foliar pigments along with antioxidant molecules, more specifically chlorophylls, carotenoids, tocopherols and total anthocyanins in leaflets of *P. lentiscus* L., 50 mg of leaflets grounded to dust with liquid nitrogen were extracted with 1.5 mL of cold methanol containing 0.01% butylated hydroxytoluene (BHT; Sigma-Aldrich, Steinheim, Germany), recovering supernatants of a two-step extraction with 30 min of cold sonication and centrifugation at 13000 rpm for 10 min at 4°C per step. From the extract, 400 µL were filtered through a 0.22 µm PTFE filter (Phenomenex, Torrance, CA, USA) to be analysed by high-performance liquid chromatography

(HPLC) as described by Muñoz et al. (2018) and 1mL acidified with 20 µL of hydrochloric acid and used to determine total anthocyanins spectrophotometrically as described by Gitelson et al. (2001).

Extent of lipid peroxidation

In order to estimate the extent of lipid peroxidation in leaflets of *P. lentiscus* L. leaves, both lipid hydroperoxides (LOOH) and malondialdehyde (MDA) contents were determined spectrophotometrically. For LOOH, 100 µL of leaflet extract were taken from the pigment and antioxidant extract described previously. From these, 50 µL were diluted with an equal volume of cold methanol with 0.01% BHT and 50 µL incubated with 10 mM triphenylphosphine (TPP) for 30 min at room temperature in darkness. After, 10 µL of each mixture were incubated by triplicate with FOX-2 reagent consisting of 25 mM sulfuric acid, 4mM BHT, 250µM ammonium iron (II) sulfate hexahydrate and 100µM xylenol orange from Bou et al. (2008) in a 96-well plate during 45 min and their absorbance measured at 560nm. Calculations were made using absorbance difference from methanol diluted and TPP incubated samples, using H_2O_2 calibration curve as a standard.

MDA determinations were based on the thiobarbituric acid (TBA)-reactive substances assay described by Hodges et al. (1999). To sum up, 100mg of tissue was ground in liquid nitrogen and extracted with 1mL 80% (v/v) ethanol containing 0.01% BHT using a vortex, 30 min ultrasonication followed by vortexing, 15 min ultrasonication, vortexing and centrifugation at 10000 rpm for 10 min at room temperature. The supernatant was recovered and placed in 5mL glass tubes. After that, samples were re-extracted twice more following the same procedure. Later, 1 mL of the recovered supernatant was incubated during 25 min at 95°C with TBA^- solution and another mL with TBA^+. MDA concentrations were estimated by measuring the absorbance at 440, 532 and 600nm in a 96-well plate with a spectrophotometer and following the equations described by Hodges et al. (1999) corrected by the length absorbance of the plate. All reagents were purchased at Sigma-Aldrich (Steinheim, Germany).

Statistical analysis

Statistical tests were performed with SPSS 20.0 statistical package. To determine if the month of the year, the shoot type or the plant sex influenced any of the parameters described above, mean values were tested by three-way ANOVA, being month, shoot and sex fixed factors. Tukey post hoc test was employed to determine statistical differences between month and interactions month-shoot and month-sex. Before the ANOVA, all parameters were correlated to test if repeated measurement analysis was necessary and applied if so. To estimate if the elevation influenced any parameter described above, mean values were tested by one-way ANOVA and Tukey post hoc test was also used to determine statistical differences between elevations.

Results

Photoinhibition in P. lentiscus plants: seasonal and altitudinal effects

The population studied in the Garraf Natural Park did not show spatial segregation between sexes, males and females being equally distributed in space (Figure 1a). Composed leaves, which were situated in the apical parts of the plants and therefore fully exposed to the sun (Figure 1c), were subjected to seasonal variations in air temperature, solar radiation and precipitation typical of the Mediterranean climate, with a relatively mild, cold winter and dry, warm summer (Figure 1b). The flowering period of *P. lentiscus* from this location was between April-May, while fruiting covered from June to September and new shoots of the year appeared in June, prior to abscission of the eldest leaves by the end of July. Additionally, to infer low temperature effects over an altitudinal range in photoinhibition and photo-oxidative stress in February, one of the coldest winter months, natural populations at 740, 530 and 360 m a.s.l. were also randomly selected at Montseny Natural Park.

Through the 12-month evaluation of the natural population at Garraf Natural Park, there were no differences in leaf biomass neither during winter, nor during differential shoot development (Figure 2). However, leaves developed in 2017 had higher LMA than leaves from 2018, even though the latest were fully mature. Moreover, leaves at the highest altitudes also had 16% and 7% greater LMA than the populations at 530

and 360 m a.s.l., respectively (Figure 2). On the other hand, water content of leaflets was assessed by RWC and H for leaflets of both experiments. While RWC showed progressive decrease over the monthly analysis, with its lowest values in summer and especially for leaves developed in 2017, that was not the case for the H index, which remained constant for 2017 shoots but showed twice the values for leaves of 2018 in June. Nevertheless, the H values of 2018 shoots gradually declined until similar to those of 2017 leaves before flowering (Figure 2). In altitude, RWC did not show significant variations and H was only different between populations at 740 and 530 m a.s.l., being 14% higher for the latest (Figure 2).

Greater differences where obtained for F_v/F_m values, with marked reductions over winter and at increasing altitudes (Figure 2). When mean temperatures ranged between 6-11°C from November to March (Figure 1b), F_v/F_m values dropped below the standardised photoinhibiton threshold of 0.75. The lowest values of 0.63 and 0.61 were registered in December and February, respectively, when minimum temperatures reached -1°C (some snowfalls occurred during February). With rising temperatures in April and May, F_v/F_m values gradually recovered and remained constant above 0.75 until the end of the experiment. No statistical differences were found between shoots developed in different years, as the recording of 2018 shoots started with fully-developed leaves and that of 2017 shoots ended before any the appearance of senescence symptoms. Moreover, F_v/F_m values below 0.75 were also recorded in February of the following year during the altitudinal study, showing that the plants growing at the highest altitude (at 740 m a.s.l.) were the most photoinhibited.

Relative to F_v/F_m values, during winter photoinhibition leaflets of both male and female mastic trees showed slight reductions in the total chlorophyll content, with their lowest values being observed in February (Figure 3). Inversely, chlorophyll a/b ratio increased during cold months because of greater reductions in chlorophyll b (Figure 3). Likewise, the most photoinhibited population at 740 m a.s.l. also showed the greatest reduction in chlorophyll content, while the highest chlorophyll a/b ratio was found for the lowest

population. No differences were found neither for total chlorophyll content, nor for chlorophyll a/b ratio between shoots developed in different years, even though the chlorophyll a/b ratio decreased after the flowering months and the beginnings of the fruiting period (Figure 3).

Photo- and antioxidant protection: anthocyanins, carotenoids and tocopherols

When temperatures started to decrease in November, total anthocyanin contents began to increase and their values remained high during the whole winter season, to later decline once temperatures warmed up around April (Figure 3). In fact, the highest anthocyanin contents were registered in December and February but were half-reduced by the end of June. Carotenoid contents also moderately increased during winter, but specific patterns were found for individual compouns (Figure 4). While lutein contents kept nearly constants throughout the study, β-carotene contents progressively incremented with cold and did not diminish with warmer temperatures. Indeed, the highest levels of carotenoids for 2017 shoots were achieved in May (Figure 4). Oppositely, neoxanthin contents steadily decreased from November to April (Figure 4). On the other hand, the total pool of xanthophyll cycle carotenoids (VZA) showed a particular dynamic during winter as their contents sharply increased when temperatures were below 0°C in December to later decrease in January, when temperatures were partially warmer. Afterwards their content increased again when temperatures were negative again, but VZA levels did not diminish with rising temperatures until May (Figure 4). Moreover, these increases were even sharper when calculated on a chlorophyll basis (Figure S3) being twice higher in December and February than in January. On the contrary, DPS levels increased in November and remained high until April, except in December, when DPS was reduced by 20%. Cool temperatures also induced increments of both in α- and γ-tocopherol contents (Figure 5), being particularly higher during winter periods, also when calculated on a chlorophyll basis, even though these increments were less marked than the

ones obtained for anthocyanins during the same period.

The content of these antioxidants showed some differences in an altitudinal gradient of *P. lentiscus* populations. Total anthocyanins of leaflets from the highest population at 470 m a.s.l. did not show statistical differences with the mid and lowest populations when given per leaf mass (Figure 3), but they were almost 20% higher when expressed per leaf area (Figure S1). Similar to the results of the winter period, total carotenoids did not show any significant difference with latitude, but when looking at specific carotenoids, the highest contents of lutein, β-carotene and VZA by chlorophyll unit were found at the highest elevation (Figure S3). At the same time, the population at 470 m a.s.l. also showed the lowest content of neoxanthin per chlorophyll (Figure S3), although it had the highest DPS (Figure 4). Tocopherol production showed a very specific profile at increasing altitude since the population at the highest altitude showed 40% and 50% more α-tocopherol than the populations at 360 and 530 m a.s.l., respectively. On the contrary, the population at 470 m a.s.l. accumulated 40% less γ-tocopherol than the other two populations (Figure 5). These differences were also greater when looking at the α-tocopherol content per chlorophyll unit, being almost twice and three times higher the content for the population at 470 m a.s.l. compared to the populations at 360 and 530 m a.s.l., respectively.

Winter and altitudinal influence on the degree of lipid peroxidation

Leaflets of *P. lentiscus* plants showed a differential degree of lipid peroxidation throughout the year conditioned by cold temperatures and altitude. During winter photoinhibition, endogenous contents of LOOH kept constant but were reduced when temperatures dropped below 0°C (Figure 6). Reductions in the contents of primary lipid peroxidation products were translated into higher contents of MDA, particularly during February and March (Figure 6), when the lowest temperatures were registered. In fact, March was the month with the highest foliar contents of MDA, being 30% higher than in November, when temperatures began to drop (Figure 1b). Considering the populations located at different altitudes, no differences were found for LOOH

content across the altitudinal range selected. However, MDA levels at 740 m a.s.l. were almost 30 and 50% higher than those analysed from leaflets of plants growing at 360 and 530 m a.s.l, respectively (Figure 6), showing an altitudinal gradient for MDA contents.

Sex-biased lipid peroxidation: transient increases of malondialdehyde in females

Sex-related effects on the the extent of lipid peroxidation and the accumulation of photoprotective molecules were analysed in February (when mean temperatures were the lowest), May (during flowering), and August (during the fruiting period). Females showed 20% higher contents of MDA than males in February (Table 1). Moreover, during flowering and fruiting, MDA levels were also higher for females than males, even though no differences were registered neither for F_v/F_m values, nor for LOOH contents (Table 1). As a matter of fact, no differences were found neither for male and females on antioxidant accumulation except during the fruiting period occurring in females in August, when the contents of lutein and β-carotene per chlorophyll unit were higher for males than females (Table 2). What is more, in August, decreases on total chlorophylls and a lower ratio of chlorophyll a/b were found along with reduced contents of neoxanthin for male plants (Table S1 and Table S2).

Influence of leaf phenology

Following the flowering period, the chlorophyll a/b ratio of leaflets developed in 2017 decreased, although no effects were recorded for F_v/F_m. Nevertheless, leaflets from 2017 restored total anthocyanins (Figure 3) and total carotenoids (Figure 4) from April to July to values similar to those obtained before the winter season. However, there was a 40% reduction in VZA contents from May to June, registering the lowest amounts in July for leaflets from 2017 (Figure 4). Oppositely, DPS levels increased by 25% from June to July While β-carotene and neoxanthin significantly increased their content in May, their levels started to decrease afterwards (Figure 4). When analysing different types of carotenoids on a chlorophyll basis, leaflets from shoots developed in 2018 showed higher contents than 2017 shoots until August when levels started to decrease and

achieved values similar to those of leaflets from 2017 shoots at the beginning of the experiment (Figure S3). Tocopherols from leaflets developed in 2017 registered the greatest increases after the flowering period reaching α- and γ-tocopherols three times higher contents than those of 2018 shoots in June and July (Figure 5).

Moreover, during the flowering months, LOOH contents were restored to similar values obtained before the cold period. However, since the beginning of fruiting, LOOH contents of leaflets from 2017 shoots sharply decreased, while LOOH levels remained low and stable in shoots developed during 2018 (Figure 6). Contrary to LOOH, MDA levels moderately recovered after cold temperatures and winter photoinhibition, but the contents of this compound did not fully restored and continued increasing for leaflets from 2017 shoots. Indeed, MDA levels only fully recovered with the formation of new shoots in 2018 (Figure 6).

Discussion

Winter and altitudinal photo-acclimation

Mid-low temperatures and scarce days of cloudiness are characteristic conditions of Mediterranean winters that favour excess excitation energy in the reaction centres of photosystems, particularly in PSII, due to restricted photosynthetic capacity that exacerbates over-reduction of the linear electron flow in thylakoid membranes (Demmig-Adams and Adams 1992, Oliveira and Peñuelas 2000). Throughout the seasonal evaluation of mastic trees at Garraf Natural Park, reductions in F_v/F_m below the standard photoinhibitory threshold of 0.75 were noticeable by the end of autumn, when mean temperatures began to drop below 15°C in November. Besides, the lowest F_v/F_m values were registered in the coldest months (December 2017 and February 2019). Moreover, results from the altitudinal gradient at Montseny Natural Park that were also evaluated in February, showed that all three populations selected were photoinhibited, being the highest population at 740 m a.s.l. the most sensitive. These results show that cold temperatures largely influence light saturation of PSII in mastic trees, especially when minimum temperatures are below 0°C, which is in agreement

with previous studies showing lower photosynthetic rates of *P. lentiscus* plants in winter and at high altitudes (Flexas et al. 2001) while others also reported a decline on F_v/F_m over the cold season, even though the noted values were above 0.75 (Ait Said et al. 2013, Juvany et al. 2014). Moreover, lower contents of total chlorophylls and increased chlorophyll a/b ratio were found for *P. lentiscus* plants when temperatures were reduced and in the population at the highest elevation, which suggests that photoinhibition induced photobleaching and reductions in the size of LHC (Evans 1988). In this context, primary and secondary lipid peroxidation was expected to increase, particularly when plants were more photoinhibited, since rates of ROS production were likely to be higher. Nevertheless, lipid hydroperoxides showed the lowest values in December, February and March, when PSII was highly inactivated and no differential pattern was found on the altitudinal gradient. On the other hand, a progressive increment in MDA levels was found since the beginning of the cold period in November and did not cease until the flowering season in April, when there was a slight reduction in MDA accumulation. Moreover, higher contents of MDA were also found for the highest population at 740 m a.s.l. and there was an altitudinal pattern. MDA has long been related to photo-oxidative damage in plants, since its formation results from the endoperoxide production after lipid membrane peroxidation by ROS arisen from light saturation of reaction centers (Pospíšil and Yamamoto 2017, Morales and Munné-Bosch 2019). However, several studies have reported MDA increments derived from different environmental stresses that did not compromise leaf or plant survival when its production was transient (Zhang et al. 2010, Ellouzi et al. 2014, Morales et al. 2016). Moreover, Weber et al. (2004) identified abiotic stress-related genes responsive to MDA in *Arabidopsis thaliana*, highlighting the regulatory role of MDA production in chloroplast retrograde signalling. For *P. lentiscus* plants, even though there was an additive monthly accumulation of MDA, the formation of this compound did not put in jeopardy plant survival, thus suggesting this is another example in which MDA might play a regulatory role towards stress acclimation, in this

case most likely leading to a programmed process of leaf senescence (Munné-Bosch and Peñuelas 2003).

Different photoprotective strategies were found to work together to control photoinhibition and ROS production in monthly and altitudinal photo-acclimation. Anthocyanins played an important role in photoprotection of mastic trees during the cold periods as their accumulation was increased when PSII was photochemically inactivated. Likewise, a gradual increase and gradient-dependent accumulation of anthocyanin content was found in the altitudinal experiment, being mastic trees at the highest and the most photoinhibited population, the one that had higher contents on total anthocyanins. Anthocyanins have long been related to cold stress and several studies have reported higher transcription of anthocyanin biosynthesis-related genes when plants are exposed to low temperatures (Ilk et al. 2015, Schulz et al. 2015). Moreover, it has also been stated that transition to shorter days from autumn to winter and decreased red/far-red ratios of light induce anthocyanin accumulation (Zhang et al. 2016, Wang et al. 2019). However, the means to sense cold and induce anthocyanin accumulation is still under debate, except that cold perception leads to elevated cytosolic calcium (Knight and Knight 2012). Vacuolar anthocyanin accumulation in epidermal cells protect photosystems from excessive light by absorbing in the blue-green spectra, along with the UV-spectra when they are acylated to chromophores or aromatic organic acids (Ferreira da Silva et al. 2012, Gould et al. 2018). In this sense, *P. lentiscus* plants with higher anthocyanin accumulation may protect photosystems by filtering light and prevent uncontrolled photodamage. At the same time, DPS had a relevant role in overwintering and at the altitudinal range, as there were increased values in both conditions. DPS, a measure of violaxanthin to zeaxanthin conversion by de-epoxidation is highly related to non-photochemical quenching (NPQ) by thermal dissipation at LHC and prevents PSII inactivation (Demmig-Adams and Adams 1996). Therefore, additionally to the anthocyanin-mediated light shielding, the xanthophyll cycle may be alleviating energy excess in PSII with higher DPS during overwintering and in altitudinal acclimation of *P. lentiscus*.

Moreover, neoxanthin, which is the carotenoid precursor for abscisic acid biosynthesis (Nambara and Marion-Poll 2005), showed its lowest values both during cold periods and at the highest studied altitude. It is important to note that defence mechanisms to quench and scanvenge ROS production were also active during winter photoinhibition, being the proportion of tocopherols of paramount importance. For instance, contents of α- and γ-tocopherols increased in mastic trees during the coldest months, especially on a chlorophyll basis. Nevertheless, α-tocopherol seems to play a relevant role in altitudinal acclimation, since the contents of its precursor, γ-tocopherol was reduced in the population at 740 m a.s.l. and almost all vitamin E was found in the form of α-tocopherol. Tocopherols have a dual role in ROS detoxification, since they can both perform physical quenching and chemical scavenging of singlet oxygen, as well as inhibit the propagation of lipid peroxidation (Muñoz and Munné-Bosch 2019). Singlet oxygen produced at PSII is highly relevant to integrate external cues in chloroplasts, since its production is one of the main signals when there are environmental constraints and is involved in retrograde signalling to mediate plant acclimation (op den Camp et al. 2003, Wagner et al. 2004). A tight control of singlet oxygen production by antioxidants, such as tocopherols, has been proven to be essential to regulate its function (Rastogi et al. 2014, Carmody et al. 2016). In mastic trees, the production of tocopherols may regulate the extent of singlet oxygen production when coupled to additional mechanisms to avoid excessive energy and altogether, allow acclimation to overwintering and adapt to altitudinal restrictions. Therefore, the coordinated participation of these photoprotective strategies seems essential to withstand cold temperatures and could be contributing to the spatial distribution of *P. lentiscus* altitudinally, even though other factors such as plant competition might also have a strong influence.

Female sensitivity to photoinhibition and photo-oxidative stress

Dioecious plants present sexual dimorphism between male and females that distinguish both sexes in terms of reproductive characters and vegetative traits that affect their physiology and life

performance (Barrett and Hough 2013, Juvany and Munné-Bosch 2015). Higher resource allocation in female plants during reproductive efforts has been pointed out as an important trait that may compromise their behaviour under abiotic or biotic stress (Obeso 2002, Harris and Pannell 2008, Juvany and Munné-Bosch 2015, Tonnabel et al. 2017), including in *P. lentiscus* plants (Jonasson et al. 1997, Correia and Diaz Barradas 2000, Ait Said et al. 2013, Seyedi et al. 2019). In the present study, while no differences were found between sexes in the severity of PSII inactivation nor in the production of primary products of lipid peroxidation, higher levels of MDA were found for female mastic trees, especially at full bloom and during the fruiting period. Indeed, females produced lower contents of lutein and β-carotene per chlorophyll unit during the fruiting period in August and could be more sensitive to environmental constraints than males. Nevertheless, females also showed higher chlorophyll contents and lowest chlorophyll a/b ratio during fruiting, which could be associated to higher photosynthetic rates to meet carbon demands for fruit development (Jonasson et al. 1997, Seyedi et al. 2019).

Shoot renewal as an effective strategy to restore the system

In perennial plants there is no leaf abscission prior to winter periods and leaves can remain attached from one to several years, depending on the species. In mastic trees, shoots that start to develop by the end of March generally fall during summer of the following year, although leaf phenology can show strong local variations (Jonasson et al. 1997, Munné-Bosch and Peñuelas 2003). Similarly, the mean lifespan of *P. lentiscus* plants is generally around 25-50 years but there are rare examples with more than 200 years. Carbon and nutrient translocation from older leaves to new leaves is essential to develop fully expanded leaves in *P. lentiscus* (Diamantoglou and Kull 1988) but in order to do so, it is necessary that chloroplast dismantling takes place in a time-controlled manner (Munné-Bosch and Peñuelas 2003, Juvany et al. 2012). Indeed, it is important to emphasize the beneficial mechanism of resetting shoots in *P. lentiscus* at the cellular oxidative level, which could be of paramount

importance to better understand negligible or negative senescence in plants (Munné-Bosch 2018). In the yearly analysis of mastic trees in Garraf Natural Park, F_v/F_m values and chlorophyll contents of the leaflets developed during 2017 were restored after winter photoinhibition. However, none of the photoprotective mechanisms activated during this period could restore MDA contents to those shown prior to cold temperatures. Tocopherols greatly increased after full-bloom above levels acquired at overwintering, DPS was also greatly promoted and neoxanthin production enhanced, particularly during June and July, which were the months before senescence. Taken together, this scenario responds to a very frequent pattern of leaf senescence (García-Plazaola and Becerril 2001, Munné-Bosch and Peñuelas 2003) but it has little to do with the physiological status of fully expanded leaves that were also present in June and July and that were newly developed in 2018. Interestingly, leaves developed in 2017 persisted until new leaflets from 2018 shoots acquired chlorophyll contents, leaf areas and H values similar to those at the beginning of the experiment in 2017 and resulted in a reset at oxidative status level, since both LOOH and MDA were substantially reduced. Therefore, shoot renewal is important to alleviate oxidative stress during plant development along the years and is undoubtedly a means for achieving stress adaptation and long life in *P. lentiscus*.

Conclusions

Mastic trees showed strong photoinhibition at cold temperatures during overwintering and at high elevations that did not compromise shoot survival, at least in part because of the activation of photoprotective mechanisms of light shielding through anthocyanins, along with higher DPS and increased tocopherol content, particularly that of α-tocopherol, that might help provide an adequate oxidative balance and plant acclimation. Females of mastic trees showed higher sensitivity to stressful environmental conditions showing enhanced production of MDA, even though no reductions of F_v/F_m were registered, which emphasizes the high adaptation of female plants to withstand photo-oxidative stress. On the other hand, the analysis of natural populations of *P.*

lentiscus plants puts in relevance the importance of shoot renewal on evergreen long-lived plants as a mechanism to fully restore the system at the cellular level.

Supplementary data

Supplementary Data for this article are available at Tree Physiology Online.

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Conflict of interest

None declared.

Authors' contribution

P.M. and S.M.-B. designed the study. P.M. and A.C. performed experiments and analysed the data. P.M. wrote the manuscript with the help of S.M.-B.

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Table 1. Maximum quantum efficiency of PSII photochemistry (F_v/F_m) and lipid peroxidation, estimated as the contents of primary (hydroperoxide, LOOH) and secondary (malondialdehyde, MDA) lipid peroxidation products in leaflets of female and male mastic trees during winter (blue), flowering (grey) and fruiting (orange). Data show the mean±SE of n=20 females and n=21 males. Results of the one-way ANOVA are shown. NS, Not significant

		February		May		August	
		Mean ± SE	P_value	Mean ± SE	P_value	Mean ± SE	P_value
F_v/F_m	Female	0,613 ± 0,014	NS	0,783 ± 0,006	NS	0,757 ± 0,007	NS
	Male	0,601 ± 0,011		0,769 ± 0,008		0,744 ± 0,008	
LOOH (μmol · g DW^{-1})	Female	48,244 ± 3,033	NS	81,349 ± 10,404	NS	22,078 ± 3,113	NS
	Male	52,030 ± 3,437		63,654 ± 6,729		21,653 ± 2,249	
MDA (nmol · g DW^{-1})	Female	250,416 ± 16,831	**0,018**	256,038 ± 9,236	**< 0,001**	133,552 ± 10,231	**0,021**
	Male	193,481 ± 15,685		154,782 ± 12,527		102,015 ± 8,303	

Table 2. Antioxidant protection per unit of chlorophyll (Chl) in female and male mastic trees during winter (blue), flowering (grey) and fruiting (orange). Data show the mean±SE of n=20 females and n=21 males. Results of the one-way ANOVA are shown. NS, Not significant; Chl, chlorophyll; VZA, xanthophyll cycle pool.

		February		May		August	
		Mean ± SE	P_value	Mean ± SE	P_value	Mean ± SE	P_value
Total anthocyanins (mmol . mol Chl^{-1})	Female	583,370 ± 47,735	NS	335,991 ± 6,679	NS	314,173 ± 4,247	NS
	Male	624,573 ± 71,487		338,646 ± 8,424		318,186 ± 3,780	
VZA (mmol . mol Chl^{-1})	Female	90,857 ± 7,513	NS	92,266 ± 10,964	NS	101,464 ± 12,010	NS
	Male	93,294 ± 8,252		86,753 ± 7,374		122,102 ± 8,523	
Lutein (mmol . mol Chl^{-1})	Female	458,115 ± 12,189	NS	316,616 ± 4,188	NS	563,727 ± 34,403	**0,031**
	Male	445,249 ± 12,973		320,463 ± 8,585		688,604 ±52,376	
β-carotene (mmol . mol Chl^{-1})	Female	129,066 ± 5,897	NS	137,853 ±3,887	NS	217,436 ± 12,370	**0,046**
	Male	120,400 ± 4,889		133,276 ± 4,118		266,826 ± 16,954	
Neoxanthin (mmol . mol Chl^{-1})	Female	31,723 ± 1,651	NS	33,527 ± 1,157	NS	39,994 ± 1,493	NS
	Male	32,844 ± 1,418		36,351 ± 0,623		36,462 ± 1,287	
α-tocopherol (mmol . mol Chl^{-1})	Female	266,802 ± 26,440	NS	287,964 ± 38,795	NS	180,462 ± 14,819	NS
	Male	287,163 ± 41,190		258,618 ± 24,210		195,811 ± 13,529	
γ-tocopherol (mmol . mol Chl^{-1})	Female	85,157 ± 12,997	NS	21,957 ± 9,450	NS	23,920 ± 3,063	NS
	Male	76,497 ± 11,244		15,134 ± 5,521		25,738 ± 3,408	

FIGURE LEGENDS

Figure 1. a) Spatial distribution of female and male mastic tress (*P. lentiscus*) in the studied population at the Garraf Natural Park (NE Spain) show absence of spatial sex segregation. **b)** Climatological conditions, including mean monthly maximum, average and minimum temperatures, mean monthly global solar radiation and accumulated monthly precipitation as recorded in the weather station situated next to the studied population in the Garraf Natual Park. Note that plants were exposed to typical Mediterranean climatic conditions, with a relatively cold, but wet winter, and a hot, dry summer, especially during July and August. Bars shown in the inlets correspond to the climatological data obtained during samplings of February in three populations found at different elevation in the Montseny Natural Park. **c)** Detail of an example of the leaflets measured during the study.

Figure 2. Physiological stress indicators, including leaf biomass, leaf area, leaf mass per area ratio (LMA), relative water content (RWC), leaf hydration (H) and maximum efficiency of PSII photochemistry (F_v/F_m) of mastic trees (*P. lentiscus*) growing under Mediterranean field conditions. Results of two independent experiments performed at the Garraf Natural Park and Montseny Natural Park are shown in the main graphs and the inlets, respectively. Highlighted months correspond to the period where mean temperatures in the Garraf Natural Park where below 15°C (blue), plants were flowering during spring (grey) and the fruiting period (orange). Green bars shown in the inlets correspond to the data obtained during February in three populations found at different elevation in the Montseny Natural Park. Data show the mean±SE of n=41 for the seasonal variations study and n=30 for the altitudinal study at the Garraf and Montseny Natural Parks, respectively. Results of two- and one-way ANOVAs, respectively, are shown in the corresponding inlets. Different letters indicate significant differences between months or elevations ($P<0.05$) using Tukey post hoc tests and asterisks indicate significant differences between spring 2017 and 2018 shoots in the Garraf Natural Park study. A red dashed line indicates the threshold for photoinhition ($F_v/F_m < 0.75$). NS, Not significant.

Figure 3. Chlorophyll (Chl) a+b, Chl a/b ratio and total anthocyanin contents in leaflets of mastic trees (*P. lentiscus*) growing under Mediterranean field conditions. Results of two independent experiments performed at the Garraf Natural Park and Montseny Natural Park are shown in the main graphs and the inlets, respectively. Highlighted months correspond to the period where mean temperatures in the Garraf Natural Park where below 15°C (blue), plants were flowering during spring (grey) and the fruiting period (orange). Green bars shown in the inlets correspond to the data obtained during February in three populations found at different elevation in the Montseny Natural Park. Data show the mean±SE of n=41 for the seasonal variations study and n=30 for the altitudinal study at the Garraf and Montseny Natural Parks, respectively. Results of two- and one-way ANOVAs, respectively, are shown in the corresponding inlets. Different letters indicate significant differences between months or elevations (P<0.05) using Tukey post hoc tests and asterisks indicate significant differences between spring 2017 and 2018 shoots in the Garraf Natural Park study. A red dashed line indicates the threshold for photoinhition (F_v/F_m < 0.75). NS, Not significant.

Figure 4. Photoprotection exerted by carotenoids, as indicated by the contents of total carotenoids, lutein, xanthophyll cycle pool (VZA), β-carotene and neoxanthin, and de-epoxidation state of the xanthophyll cycle (DPS) in leaflets of mastic trees (*P. lentiscus*) growing under Mediterranean field conditions. Results of two independent experiments performed at the Garraf Natural Park and Montseny Natural Park are shown in the main graphs and the inlets, respectively. Highlighted months correspond to the period where mean temperatures in the Garraf Natural Park where below 15°C (blue), plants were flowering during spring (grey) and the fruiting period (orange). Green bars shown in the inlets correspond to the data obtained during February in three populations found at different elevation in the Montseny Natural Park. Data show the mean±SE of n=41 for the seasonal variations study and n=30 for the altitudinal study at the Garraf and Montseny Natural Parks, respectively. Results of two-way and one-way ANOVAs, respectively, are shown in the corresponding inlets. Different letters indicate significant differences between months or elevations (P<0.05) using Tukey post hoc tests and asterisks indicate

significant differences between spring 2017 and 2018 shoots in the Garraf Natural Park study. A red dashed line indicates the threshold for photoinhition (F_v/F_m < 0.75). NS, Not significant. DPS was calculated as Z+0.5A/VZA, where Z and A are zeaxanthin and antheraxanthin, respectively.

Figure 5. Contents of α-tocopherol and its precursor, γ-tocopherol, in leaflets of mastic trees (*P. lentiscus*) growing under Mediterranean field conditions. Results of two independent experiments performed at the Garraf Natural Park and Montseny Natural Park are shown in the main graphs and the inlets, respectively. Highlighted months correspond to the period where mean temperatures in the Garraf Natural Park where below 15°C (blue), plants were flowering during spring (grey) and the fruiting period (orange). Green bars shown in the inlets correspond to the data obtained during February in three populations found at different elevation in the Montseny Natural Park. Data show the mean±SE of n=41 for the seasonal variations study and n=30 for the altitudinal study at the Garraf and Montseny Natural Parks, respectively. Results of two- and one-way ANOVAs, respectively, are shown in the corresponding inlets. Different letters indicate significant differences between months or elevations (P<0.05) using Tukey post hoc tests and asterisks indicate significant differences between spring 2017 and 2018 shoots in the Garraf Natural Park study. A red dashed line indicates the threshold for photoinhition (F_v/F_m < 0.75). NS, Not significant. DPS was calculated as Z+0.5A/VZA, where Z and A are zeaxanthin and antheraxanthin, respectively.

Figure 6. Contents of primary (hydroperoxides, LOOH) and secondary (malondialdehyde, MDA) lipid peroxidation products in leaflets of mastic trees (*P. lentiscus*) growing under Mediterranean field conditions. Results of two independent experiments performed at the Garraf Natural Park and Montseny Natural Park are shown in the main graphs and the inlets, respectively. Highlighted months correspond to the period where mean temperatures in the Garraf Natural Park where below 15°C (blue), plants were flowering during spring (grey) and the fruiting period (orange). Green bars shown in the inlets correspond to the data obtained during February in three populations found at different elevation in the Montseny Natural Park. Data show the mean±SE of n=41 for the seasonal variations

study and n=30 for the altitudinal study at the Garraf and Montseny Natural Parks, respectively. Results of two- and one-way ANOVAs, respectively, are shown in the corresponding inlets. Different letters indicate significant differences between months or elevations (P<0.05) using Tukey post hoc tests and asterisks indicate significant differences between spring 2017 and 2018 shoots in the Garraf Natural Park study. A red dashed line indicates the threshold for photoinhition (F_v/F_m < 0.75). NS, Not significant.

Figure 1

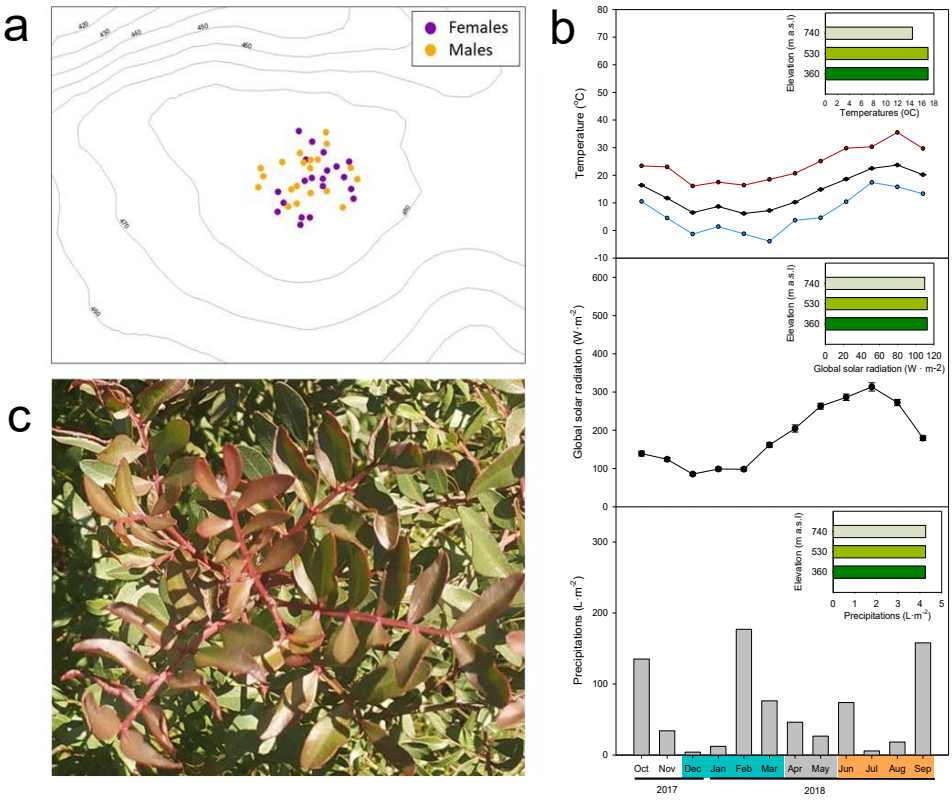

a
Females
Males
b
Temperature (°C)
Elevation (m a.s.l)
740
530
360
Temperatures (oC)
Global solar radiation (W · m-2)
Precipitations (L·m-2)
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
2017
2018
c

Figure 2

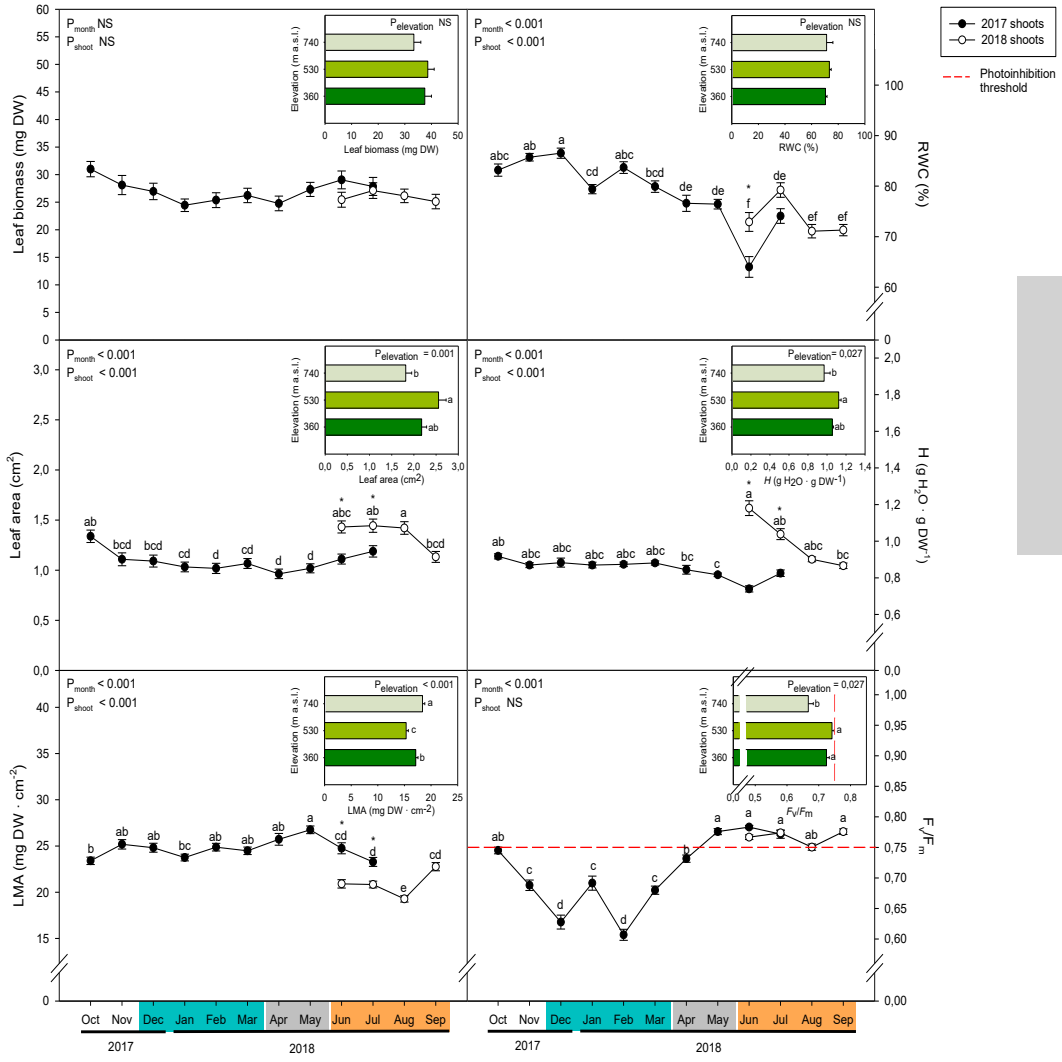

2017 shoots
2018 shoots
Photoinhibition threshold
Leaf biomass (mg DW)
RWC (%)
Leaf area (cm²)
H (g H2O · g DW-1)
LMA (mg DW · cm-2)
Fv/Fm
Pmonth NS
Pshoot NS
Pmonth < 0.001
Pshoot < 0.001
Pelevation NS
Pelevation = 0.001
Pelevation = 0,027
Pelevation < 0.001
Elevation (m a.s.l.)
Leaf biomass (mg DW)
RWC (%)
Leaf area (cm2)
H (g H2O · g DW-1)
LMA (mg DW · cm-2)
Fv/Fm
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep
2017
2018

Figure 3

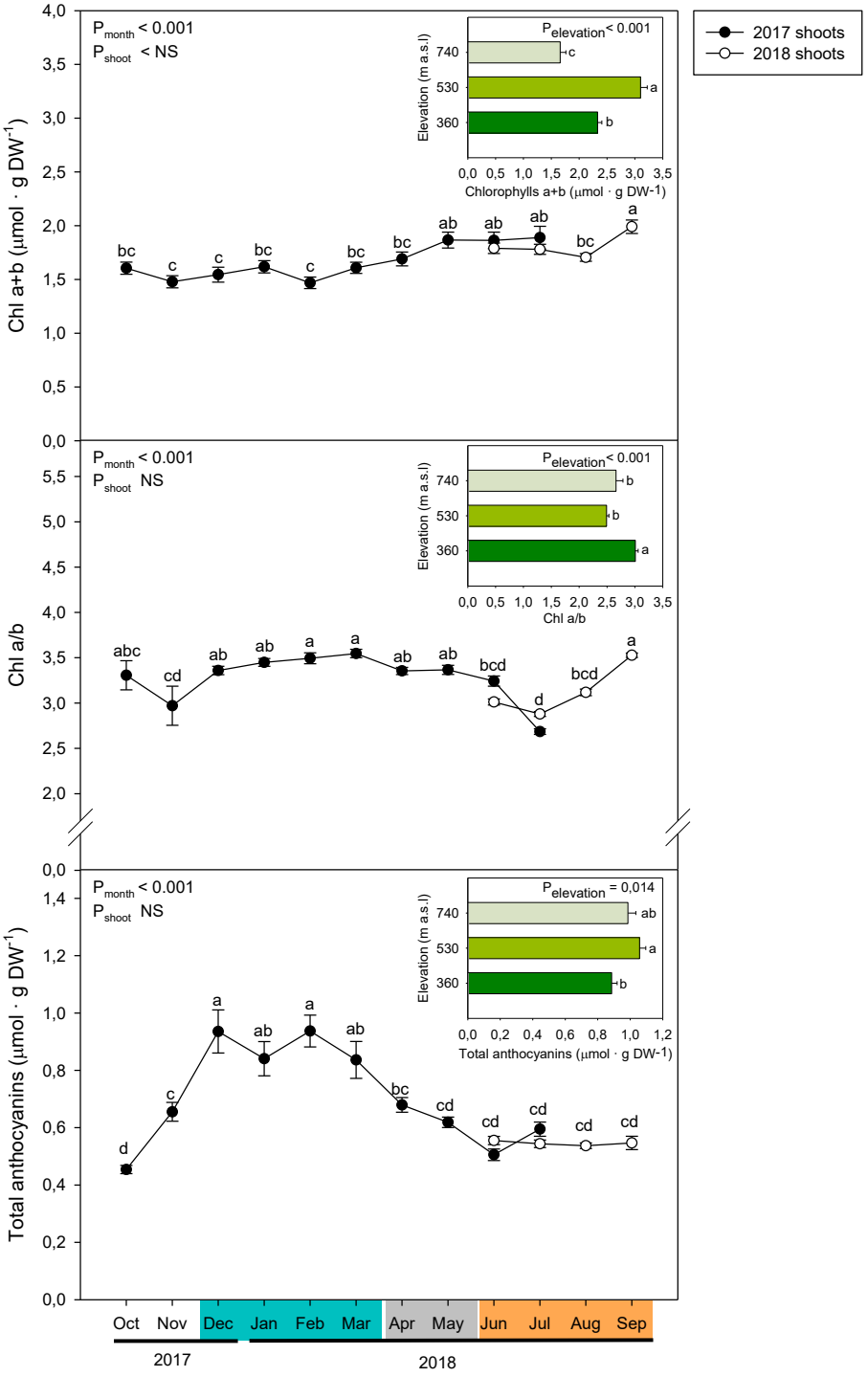

2017 shoots
2018 shoots
Chl a+b (µmol · g DW-1)
Pmonth < 0.001
Pshoot < NS
Pelevation < 0.001
Elevation (m a.s.l)
Chlorophylls a+b (µmol · g DW-1)
Chl a/b
Pmonth < 0.001
Pshoot NS
Pelevation < 0.001
Chl a/b
Total anthocyanins (µmol · g DW-1)
Pmonth < 0.001
Pshoot NS
Pelevation = 0,014
Total anthocyanins (µmol · g DW-1)
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep
2017
2018

Figure 4

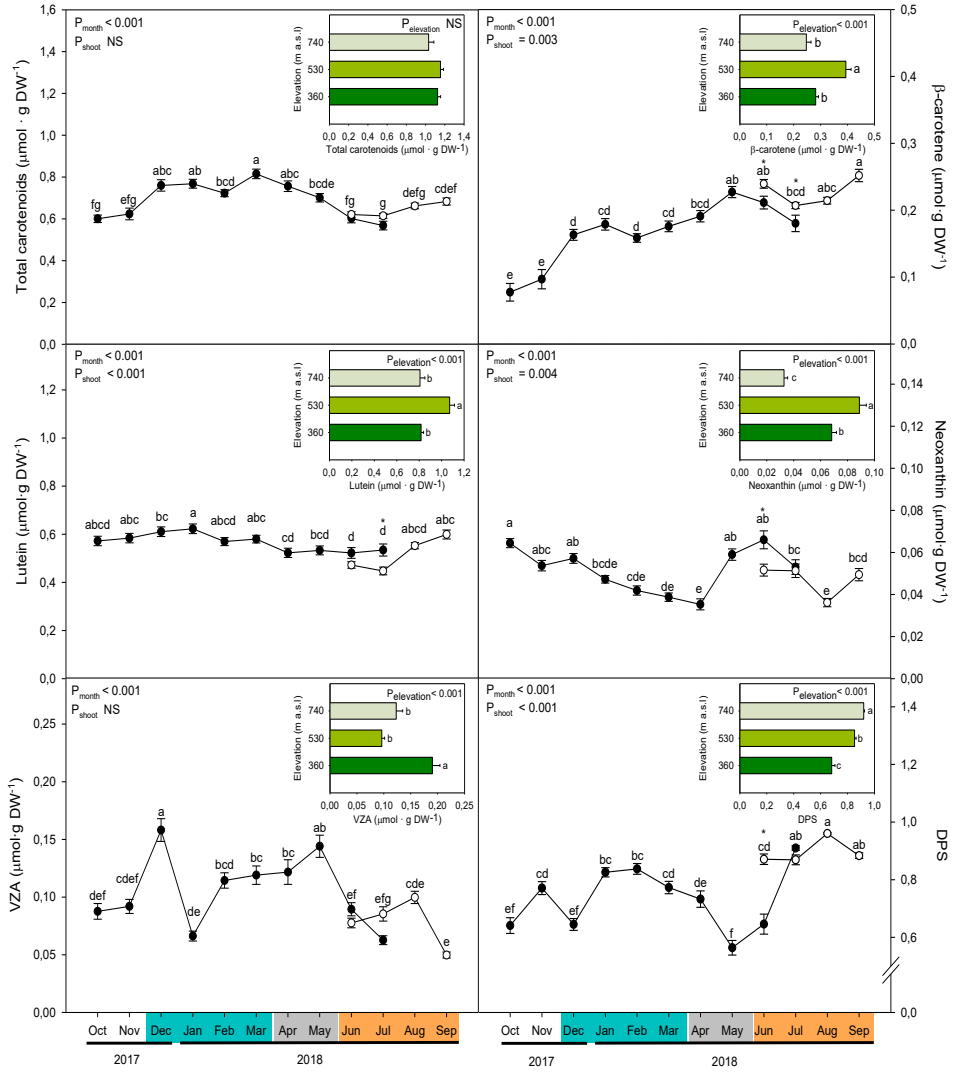

2017 Shoots
2018 Shoots
Total carotenoids (µmol · g DW-1)
β-carotene (µmol·g DW-1)
Lutein (µmol·g DW-1)
Neoxanthin (µmol·g DW-1)
VZA (µmol·g DW-1)
DPS
Elevation (m a.s.l)
Total carotenoids (µmol · g DW-1)
β-carotene (µmol · g DW-1)
Lutein (µmol · g DW-1)
Neoxanthin (µmol · g DW-1)
VZA (µmol · g DW-1)
DPS
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep
2017
2018

Figure 5

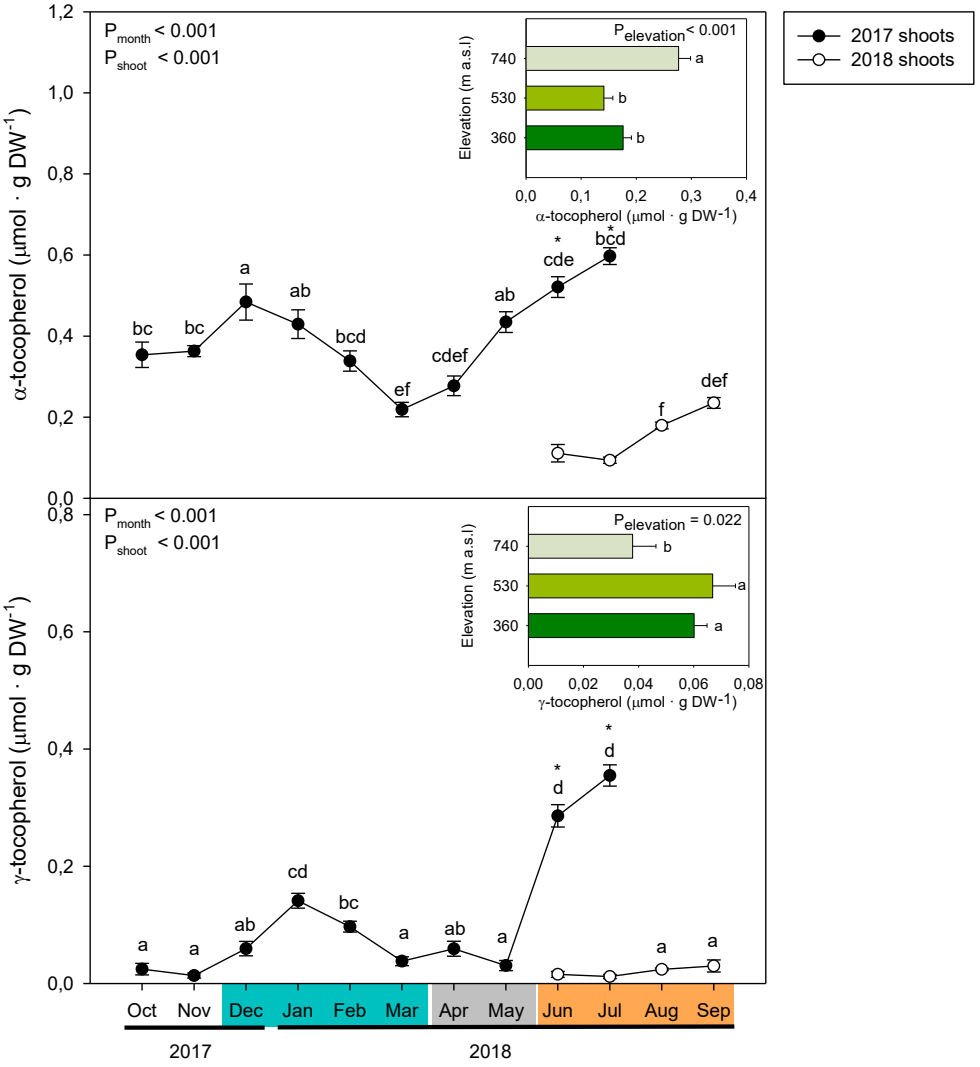
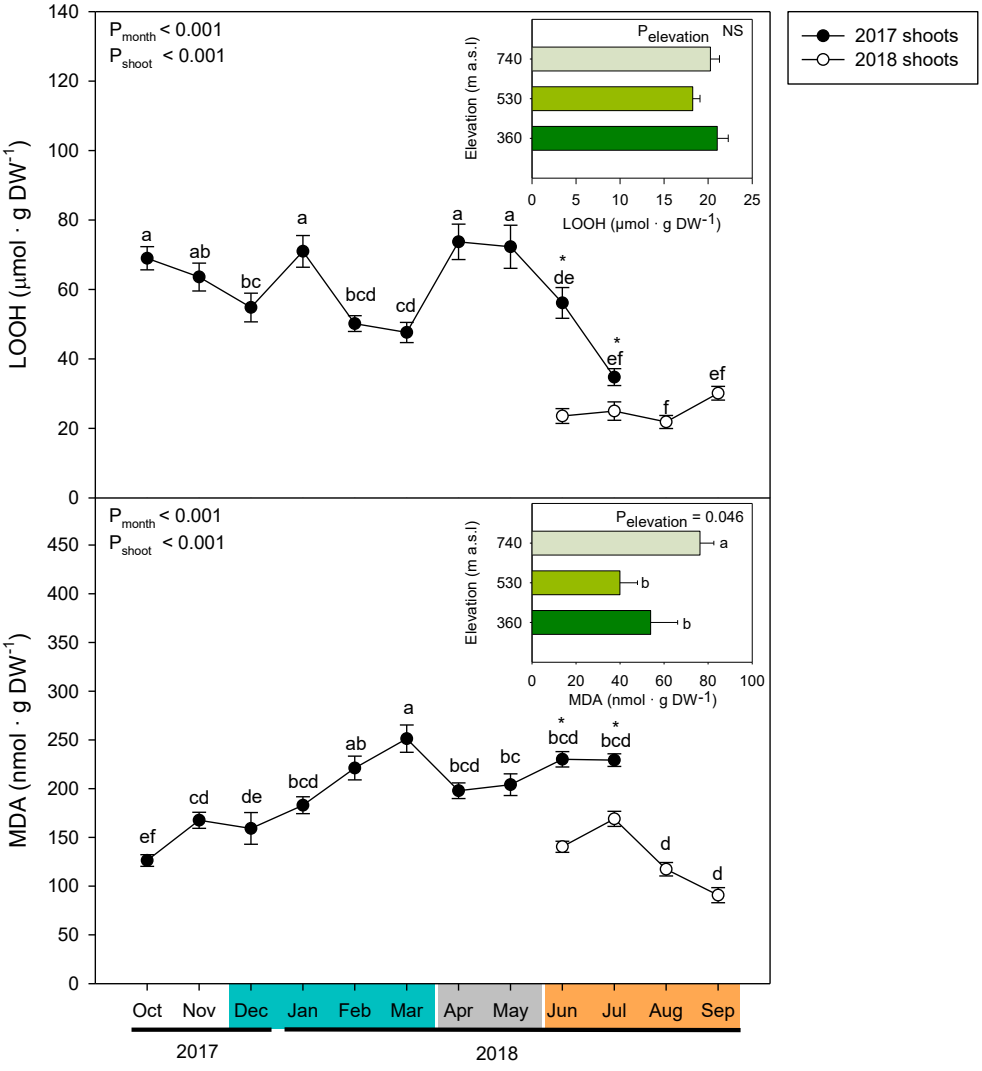

2017 shoots
2018 shoots
α-tocopherol (µmol · g DW-1)
P month < 0.001
P shoot < 0.001
P elevation < 0.001
Elevation (m a.s.l)
740
530
360
α-tocopherol (µmol · g DW-1)
γ-tocopherol (µmol · g DW-1)
P month < 0.001
P shoot < 0.001
P elevation = 0.022
γ-tocopherol (µmol · g DW-1)
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
2017
2018

Figure 6


2017 shoots
2018 shoots
P month < 0.001
P shoot < 0.001
LOOH (µmol · g DW-1)
P elevation NS
Elevation (m a.s.l)
740
530
360
LOOH (µmol · g DW-1)
P month < 0.001
P shoot < 0.001
MDA (nmol · g DW-1)
P elevation = 0.046
MDA (nmol · g DW-1)
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep
2017
2018

SUPPLEMENTAL FIGURES LEGENDS

Figure S1: Seasonal and altitudinal variations in foliar pigments (chlorophylls and anthocyanins) contents in mastic trees. Highlighted months correspond to the period where mean temperatures where below 15°C (Blue), flowering period (Grey) and fruiting period (Orange). Data show the mean±SE of n=41 for the annual dynamics and n=30 for populations at various altitudes. Results of two-way ANOVA are shown in the inlets. Different letters indicate significant differences between months or elevation ($P<0.05$) using Tukey post hoc tests and asterisks indicate significant differences between shoot types (see text for details). NS, Not significant; Chl, chlorophyll.

Figure S2: Seasonal and altitudinal variations in the carotenoid content per leaf area unit in mastic trees. Highlighted months correspond to the period where mean temperatures where below 15°C (Blue), flowering period (Grey) and fruiting period (Orange). Data show the mean±SE of n=41 for the annual dynamics and n=30 for populations (see text for details). Results of two-way ANOVA are shown in the inlets. Different letters indicate significant differences between months or elevation ($P<0.05$) using Tukey post hoc tests and asterisks indicate significant differences between shoots. NS, Not significant.

Figure S3: Seasonal and altitudinal variations in the carotenoid content per chlorophyll in *P. lerntiscus*. Highlighted months correspond to the period where mean temperatures where below 15°C (Blue), flowering period (Gray) and fruiting period (Orange). Data shows the mean±SE of n=41 for the annual dynamics and n=30 for populations various altitudes. Results of two-way ANOVA are shown in the inlets. Different letters indicate significant differences between months or elevations ($P<0.05$) using Tukey post hoc tests and asterisks indicate significant differences between shoots. NS, Not significant;

Figure S4: Ratio of tocopherol content per leaf area of mastic trees during annual development and altitudinal distribution. Highlighted months correspond to the period where mean temperatures where below 15°C (Blue), flowering period (Gray) and fruiting period (Orange). Data shows the mean±SE of n=41 for the annual

dynamics and n=30 for altitudinal populations. Results of two-way ANOVA are shown in the inlets. Different letters indicate significant differences between months or elevations ($P<0.05$) using Tukey post hoc tests and asterisks indicate significant differences between shoots. NS, Not significant.

Figure S5: Seasonal and altitudinal variations in the tocopherol content per chlorophyll in *P. lerntiscus*. Highlighted months correspond to the period where mean temperatures where below 15°C (Blue), flowering period (Grey) and fruiting period (Orange). Data show the mean±SE of n=41 for the annual dynamics and n=30 for the altitudinal study. Results of two-way ANOVA are shown in the inlets. Different letters indicate significant differences between months or elevations ($P<0.05$) using Tukey post hoc tests and asterisks indicate significant differences between shoots. NS, Not significant.

Figure S1:

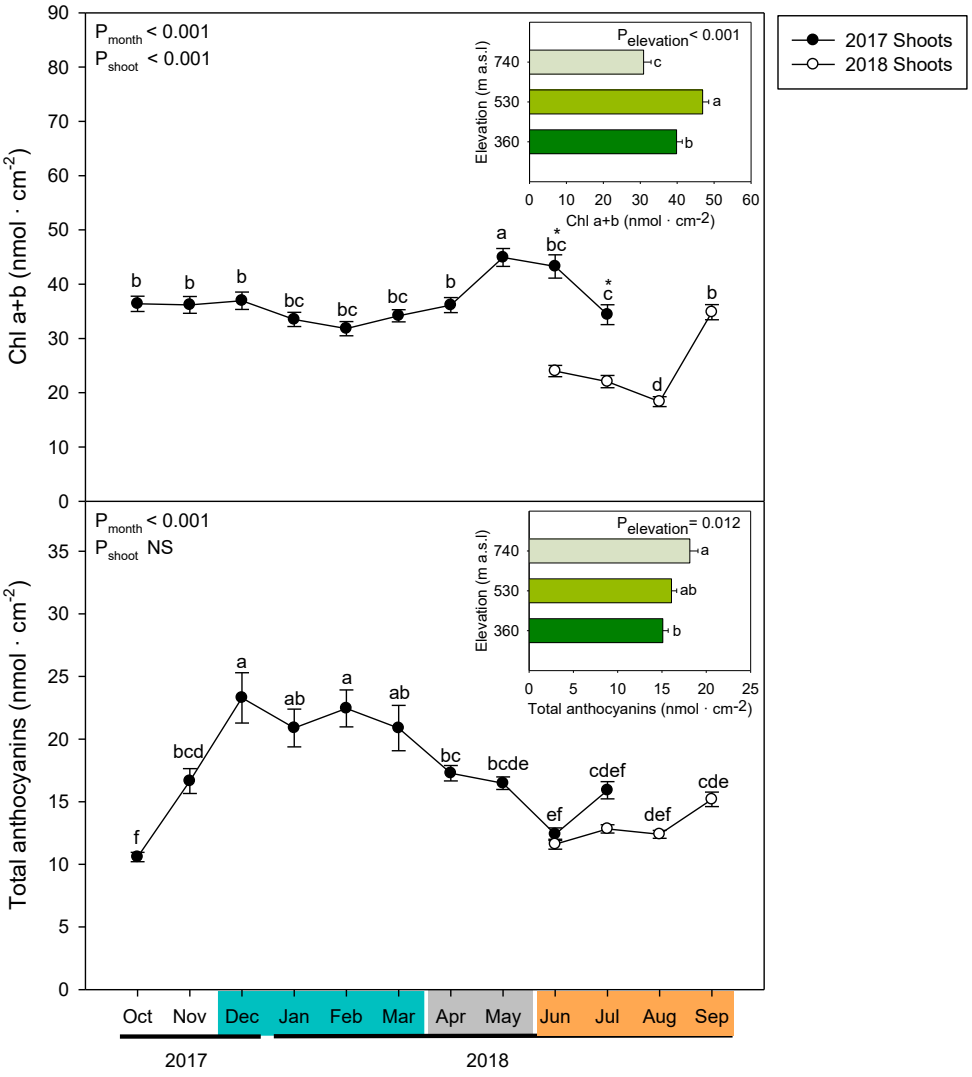

P month < 0.001
P shoot < 0.001
Chl a+b (nmol · cm-2)
P elevation < 0.001
Elevation (m a.s.l)
740
530
360
Chl a+b (nmol · cm-2)
2017 Shoots
2018 Shoots
P month < 0.001
P shoot NS
Total anthocyanins (nmol · cm-2)
P elevation = 0.012
Total anthocyanins (nmol · cm-2)
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep
2017
2018

Figure S2:

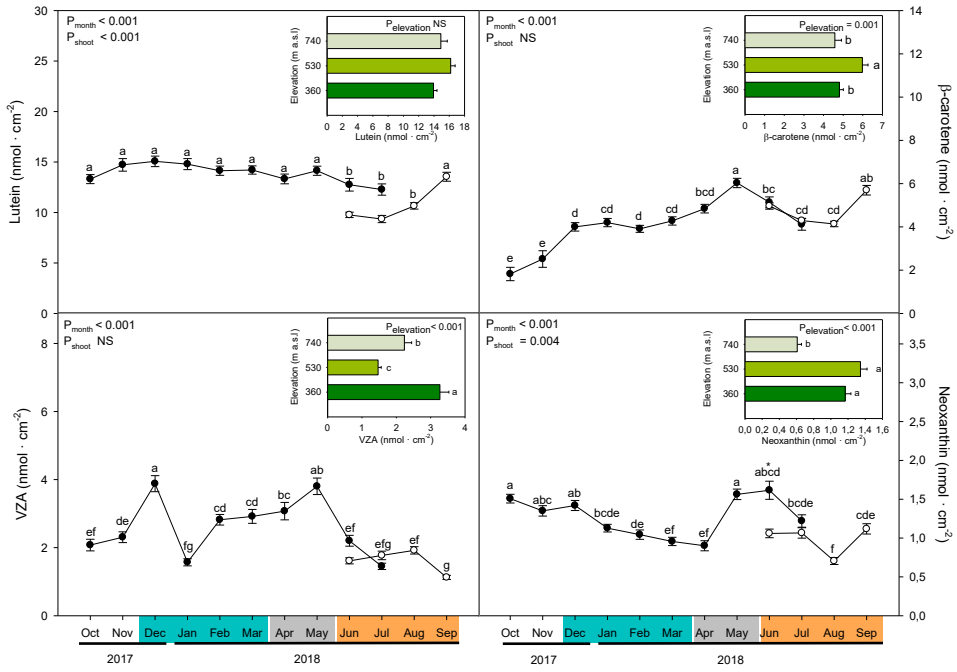

2017 Shoots
2018 Shoots
Lutein (nmol · cm-2)
β-carotene (nmol · cm-2)
VZA (nmol · cm-2)
Neoxanthin (nmol · cm-2)
Pmonth < 0.001
Pshoot < 0.001
Pelevation NS
Pmonth < 0.001
Pshoot NS
Pelevation = 0.001
Pmonth < 0.001
Pshoot NS
Pelevation< 0.001
Pmonth < 0.001
Pshoot = 0.004
Pelevation< 0.001
Elevation (m a.s.l)
Lutein (nmol · cm-2)
β-carotene (nmol · cm-2)
VZA (nmol · cm-2)
Neoxanthin (nmol · cm-2)
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep
2017
2018

Figure S3:

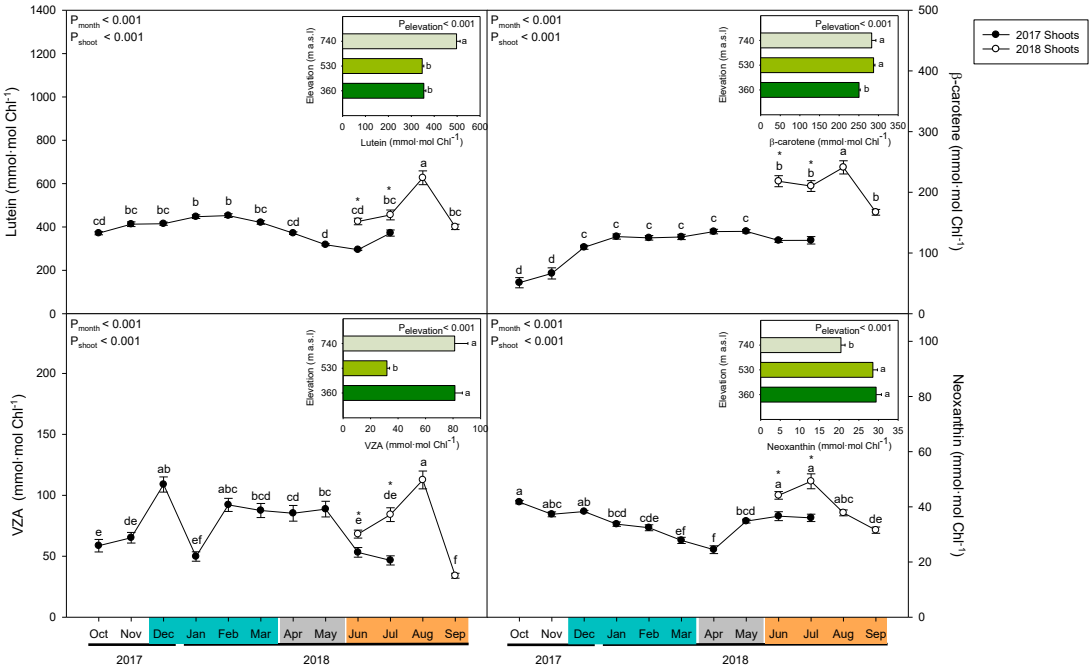

Lutein (mmol·mol Chl-1)
β-carotene (mmol·mol Chl-1)
VZA (mmol·mol Chl-1)
Neoxanthin (mmol·mol Chl-1)
Pmonth < 0.001
Pshoot < 0.001
Pelevation< 0.001
Elevation (m a.s.l)
2017 Shoots
2018 Shoots
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep
2017
2018

Figure S4:

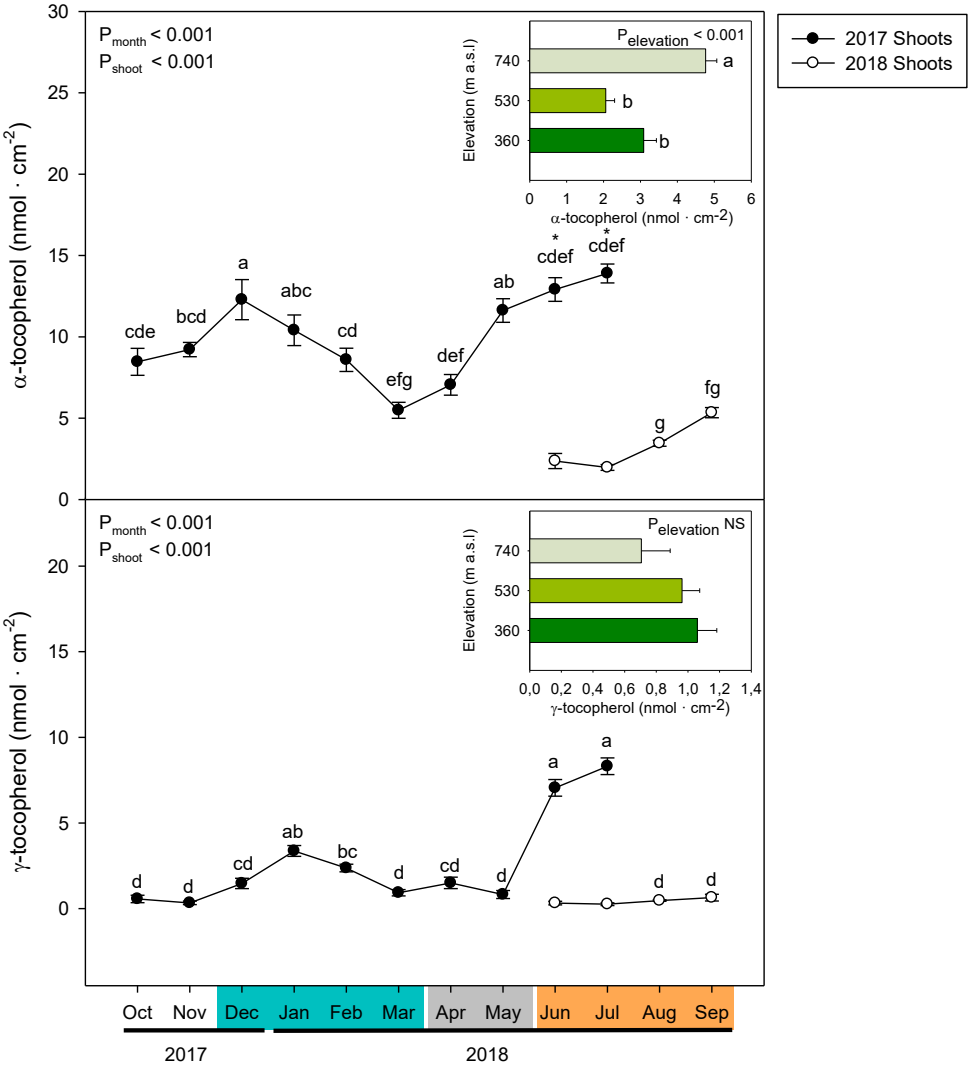

2017 Shoots
2018 Shoots
α-tocopherol (nmol · cm-2)
γ-tocopherol (nmol · cm-2)
Pmonth < 0.001
Pshoot < 0.001
Pelevation < 0.001
Pelevation NS
Elevation (m a.s.l)
740
530
360
α-tocopherol (nmol · cm-2)
γ-tocopherol (nmol · cm-2)
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep
2017
2018

Figure S5:

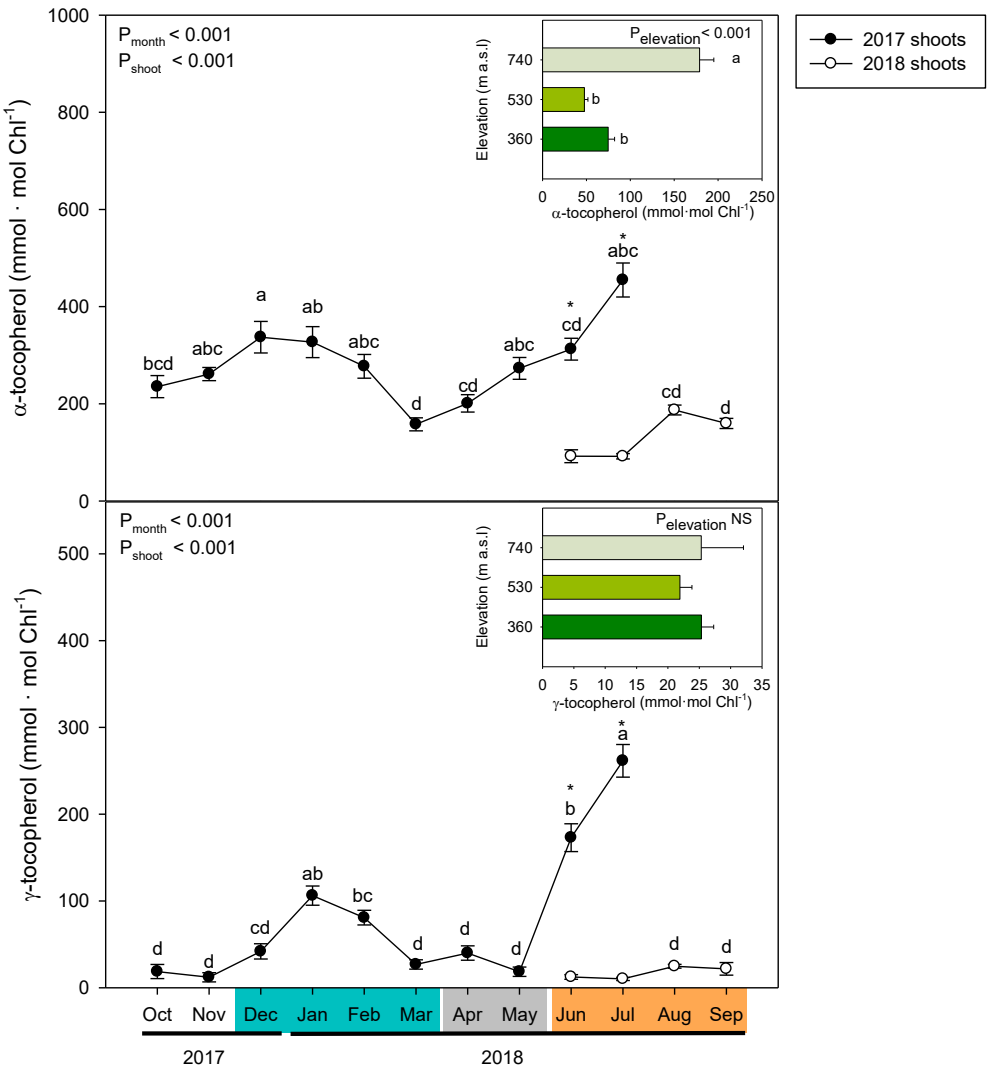

2017 shoots
2018 shoots
α-tocopherol (mmol · mol Chl-1)
Pmonth < 0.001
Pshoot < 0.001
Pelevation < 0.001
Elevation (m a.s.l)
α-tocopherol (mmol·mol Chl-1)
γ-tocopherol (mmol · mol Chl-1)
Pelevation NS
γ-tocopherol (mmol·mol Chl-1)
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep
2017
2018

Table S.1: Pigments and antioxidant content in female and male mastic trees during winter (Blue), flowering (Gray) and fruiting (Orange). Data shows the mean±SE of n=20 for female *P. lentiscus* plants and n=21 for male P. lentiscus plants. Results of the one-way ANOVA are shown. NS, Not significant; Chls, chlorophylls; VZA, summary of violaxanthin, zeaxanthin and antheraxanthin.

		February		May		August	
		Mean ± SE	P_value	Mean ± SE	P_value	Mean ± SE	P_value
Chl a+b (µmol . g DW $^{-1}$)	Female	1,250 ± 0,059	NS	1,668 ± 0,091	NS	1,032 ± 0,052	**0,025**
	Male	1,303 ± 0,076		1,717 ± 0,099		0,865 ± 0,053	
Chl a/b	Female	3,769 ± 0,120	NS	3,470 ± 0,057	NS	1,508 ± 0,102	**0,012**
	Male	3,833 ± 0,134		3,335 ± 0,067		1,112 ± 0,121	
Total anthocyanins (µmol . g DW $^{-1}$)	Female	0,945 ± 0,071	NS	0,599 ± 0,025	NS	0,535 ± 0,013	NS
	Male	0,929 ± 0,086		0,637 ± 0,027		0,539 ± 0,014	
VZA (µmol . g DW $^{-1}$)	Female	0,111 ± 0,010	NS	0,143 ± 0,014	NS	0,097 ± 0,009	NS
	Male	0,116 ± 0,010		0,148 ± 0,014		0,102 ± 0,007	
DPS	Female	0,867 ± 0,025	NS	0,567 ± 0,031	NS	0,954 ± 0,005	NS
	Male	0,812 ± 0,027		0,553 ± 0,042		0,966 ± 0,003	
Lutein (µmol . g DW $^{-1}$)	Female	0,565 ± 0,021	NS	0,528 ± 0,031	NS	0,555 ± 0,021	NS
	Male	0,567 ± 0,026		0,539 ± 0,023		0,550 ± 0,017	
β-carotene (µmol . g DW $^{-1}$)	Female	0,160 ± 0,009	NS	0,228 ± 0,012	NS	0,214 ± 0,007	NS
	Male	0,155 ± 0,010		0,225 ± 0,012		0,217 ± 0,007	
Neoxanthin (µmol . g DW $^{-1}$)	Female	0,040 ± 0,003	NS	0,056 ± 0,004	NS	0,041 ± 0,003	**0,011**
	Male	0,043 ± 0,003		0,063 ± 0,004		0,032 ± 0,003	
α-tocopherol (µmol . g DW $^{-1}$)	Female	0,326 ± 0,031	NS	0,441 ± 0,036	NS	0,181 ± 0,011	NS
	Male	0,351 ± 0,040		0,429 ± 0,037		0,177 ± 0,013	
γ-tocopherol (µmol . g DW $^{-1}$)	Female	0,103 ± 0,015	NS	0,034 ± 0,014	NS	0,024 ± 0,003	NS
	Male	0,091 ± 0,011		0,027 ± 0,011		0,024 ± 0,007	

Table S.2: Pigments and antioxidant content per leaf area in female and male mastic trees during winter (Blue), flowering (Gray) and fruiting (Orange). Data shows the mean±SE of n=20 for female *P. lentiscus* plants and n=21 for male P. lentiscus plants. Results of the one-way ANOVA are shown. NS, Not significant; Chls, chlorophylls; VZA, summary of violaxanthin, zeaxanthin and antheraxanthin.

		February		May		August	
		Mean ± SE	P_value	Mean ± SE	P_value	Mean ± SE	P_value
Chl a+b (nmol . cm $^{-2}$)	Female	30,640 ± 1,565	NS	44,220 ± 2,294	NS	20,525 ± 1,275	**0,019**
	Male	32,710 ± 2,181		45,311 ± 2,534		16,409 ± 1,214	
Total anthocyanins (nmol . cm $^{-2}$)	Female	22,528 ± 1,719	NS	15,953 ± 0,698	NS	12,657 ± 0,466	NS
	Male	22,372 ± 2,413		16,976 ± 0,723		12,151 ± 0,420	
VZA (nmol . cm $^{-2}$)	Female	2,719 ± 0,226	NS	3,825 ± 0,367	NS	1,927 ± 0,188	NS
	Male	2,882 ±0,227		3,831 ± 0,339		1,905 ± 0,122	
Lutein (nmol . cm $^{-2}$)	Female	13,875 ± 0,606	NS	13,982 ± 0,745	NS	11,013 ± 0,538	NS
	Male	14,195 ± 0,701		14,223 ± 0,560		10,355 ± 0,361	
β-carotene (nmol . cm $^{-2}$)	Female	3,911 ± 0,224	NS	6,044 ± 0,311	NS	4,260 ± 0,209	NS
	Male	3,851 ± 0,243		5,959 ± 0,314		4,076 ± 0,147	
Neoxanthin (nmol . cm $^{-2}$)	Female	0,975 ± 0,070	NS	1,473 ± 0,090	NS	0,820 ± 0,061	**0,010**
	Male	1,095 ± 0,101		1,656 ± 0,103		0,609 ± 0,058	
α-tocopherol (nmol . cm $^{-2}$)	Female	8,151 ± 0878	NS	11,872 ± 1,123	NS	3,583 ± 0,259	NS
	Male	9,002 ± 1,133		11,375 ± 0,956		3,300 ± 0,264	
γ-tocopherol (nmol . cm $^{-2}$)	Female	2,481 ± 0,357	NS	0,943 ± 0,401	NS	0,487 ± 0,059	NS
	Male	2,258 ± 0,277		0,705 ± 0,270		0,445 ± 0,070	

Chapter 2

Photoinhibition and photoprotection during flower opening in lilies

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Photoinhibition and photoprotection during flower opening in lilies

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ABSTRACT

Although most studies to extend vase life in cut flowers have focused on flower senescence thus far, flower opening is a complex process of major biological significance in the determination of flower commercialization. In order to better understand flower opening, this study evaluated to what extent photoinhibition and photo-oxidative stress are associated with tepal de-greening during flower opening in lilies (*Lilium* "Litouwen"). We estimated the degree of photoinhibition, the capacity for photo- and antioxidant protection, and the extent of lipid peroxidation at four flower opening stages, from closed flowers to anthesis. Additionally, we evaluated to what extent and by which mechanisms related to photo- and antioxidant protection, Promalin® (a combination of gibberellins and cytokinins) delays flower opening. Results showed that chlorophyll content decreased progressively during flower opening, while a sharp decrease of the maximum PSII efficiency (*Fv/Fm* ratio) was observed just before anthesis. Moreover, content of secondary lipid peroxidation products (malondialdehyde and jasmonic acid) increased just before anthesis, which was preceded by an enhanced production of primary lipid peroxidation products (lipid hydroperoxides). While both tocopherols and tocotrienols (vitamin E) increased during flower opening, β-carotene and xanthophyll content decreased sharply, which may be associated with the sharp decline in the *Fv/Fm* ratio before anthesis. Flowers treated with Promalin®, which showed delayed opening, experienced transient increases of lipid hydroperoxide and jasmonic acid contents at early stages of flower opening, together with reduced vitamin E and malondialdehyde contents just prior to anthesis. We conclude that the extent of photoinhibition, the capacity of photo- and antioxidant protection and the production of primary and secondary products of lipid peroxidation are finely controlled in a time-dependent manner to allow a correct development of lily flowers.

1. Introduction

In recent years, there has been a big effort to better understand the physiology behind the complex process of flower development, above all, because its market generates an annual turnover of around €3.5 billion in Europe [1]. For this reason, besides colour variation studies, most research has focused on preserving a visually attractive corolla for longer periods, in other words, expanding the vase life of cut flowers either by avoiding senescing symptoms like wilting or preventing early flower abscission. This process not only depends upon variety, cultivation methods, and storage and transport conditions, but also rests on a tight control of flower physiological status [2,3]. For this reason, several studies have concentrated on characterizing hormones responsible for unleashing flower senescence; like ethylene in ethylene-sensitive flowers [4], or abscisic acid in ethylene-insensitive flowers [5]. Moreover, other hormones like cytokinins [6,7], gibberellins [8,9], auxins [10] and jasmonates [11] also influence flower senescence in a cross-talk regulated manner. Likewise, carbohydrate levels have been reported to influence flower longevity and constitute a limiting factor at early stages of flower development [12], along with the interaction between these sugars and certain hormones regulating the life span of cut flowers [13,14]. Furthermore, adverse water relations are also a key factor influencing early petal or tepal wilting because of xylem blocking [15].

Oxidative stress has been traditionally regarded as a harmful process leading to flower senescence, usually associated with programmed cell death in petals or tepals [16,17]. However, recent research points out that reactive oxygen species (ROS) and therefore a transient oxidative stress may be exerting an essential signalling role in senescing flowers, activating mechanisms that control the activation of nutrient remobilization and programmed cell death in a time-regulated manner [18]. Unfortunately, however, very little is known about the putative role of oxidative stress in other processes of flower development, such as corolla de-greening and flower opening, despite these processes being key determinants of flower appearance and anthesis, two key traits that are determinant in flower commercialisation.

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There are some types of flowers, like petunias [19], lilies [20] and orchids [21] that contain functional chloroplasts in petals and tepals at the beginning of flower development, when they are still closed and green. De-greening starts just before or during flower opening because of a loss in chlorophyll content, associated with the dismantling of the photosynthetic apparatus [22]. This process can generate photoinhibition in corolla chloroplasts because of an excess of light energy that will eventually lead to the production of ROS, such as singlet oxygen ($^1O_2$) or hydrogen peroxide (H_2O_2). Although this kind of photo-oxidative stress during petal or tepal de-greening might be comparable to that suffered by leaves, little research has been performed in flowers thus far [22].

In this study, we aimed to evaluate the degree of photoinhibition, the capacity of photo- and antioxidant protection, and the extent of lipid peroxidation during the early stages of flower development, from closed flowers to anthesis, in the tepals of an ethylene-insensitive flower, *Lilium* "Litouwen". This variety is a *L. longiflorum* x Asiatic (LA) hybrid that shows severe de-greening in tepals during early stages of development, hence serving as an excellent model to study photo-oxidative stress during tepal de-greening. Furthermore, we also evaluated the putative cross-talk between hormones and oxidative stress during flower opening in Promalin®-treated lilies.

2. Materials and methods

2.1. Plant material, treatments and samplings

Lilium "Litouwen" (L.A., *longiflorum x* Asiatic hybrid) bulbs were purchased from MapiFloricultura, S.L. (Madrid, Spain) and grown in a greenhouse at the University of Barcelona (Barcelona, Spain). Eighty bulbs of lily flowers where transferred to 3 L pots (one bulb per pot) with a substrate composed of peat:perlite:vermiculite (2:1:1, v/v/v) situated 10 cm apart from each other. Bulbs were cultivated from 16th December 2016 to 23rd February 2017, irrigated with half concentration Hoagland solution [23] every day, with a mean daily temperature and relative humidity of 19.8 ± 0.1 °C and 49.2 ± 0.5%, respectively, maximum diurnal photosynthetically-active photon flux density of 900 μmol $m^{-2} s^{-1}$ and a photoperiod of 14 h/10 h (light:darkness).

Lily inflorescences with 1 m stems were cut on 23rd February when most flowers were at stage I and at least one flower was at stage II of development (see Fig. 1 for a description of flower developmental stages). Cut inflorescences, with a distribution of six inflorescences per vase, where immediately placed in 1.5 L vases with 1 L of tap water, which was replaced every 24 h to prevent the proliferation of algae and microorganisms.

In a first experiment, we evaluated the degree of photoinhibition, the capacity of photo- and antioxidant protection and the extent of lipid peroxidation during flower opening in lilies. With this aim, flowers at stage I were marked and outer tepals collected at stage I (0 h) and after 1d, 5d, 7d and 8d of flower development, which corresponded to stages I, II-a, II-b and III, respectively (see Fig. 1 for details; note that stages were assigned following Arrom and Munné-Bosch [24], with some modifications).

In a second experiment, we evaluated to what extent and through which mechanisms related to photo- and antioxidant protection, Promalin®, a combination of gibberellins – $GA_4 + GA_7$ – and cytokinins – benzyladenine -, may influence flower opening. With this purpose, Promalin®-treated flowers were compared to those treated with water (controls), and samples of outer tepals of both groups were collected at stage I (following a 1d treatment) and at Stage II-b (initial flower opening corresponding to 7d and 9d treatment for controls and Promalin®, respectively). The exact hormonal composition of the vase in Promalin®-treated flowers, which was measured by LC–MS/MS as described [25], was the following: 1.07 mg/L GA_4, 0.07 mg/L GA_7 and 0.75 mg/L benzyladenine.

For both experiments and at each sampling point, the outer tepals of eight flowers from different vases were collected at predawn (1 *h* before sunrise) between 23th February and 4th March 2017. Temperature and relative humidity, which were measured with a thermohygrometer *in situ*, stayed nearly constant with values between 14.5–15.5 °C and 43-54%, respectively, during samplings. During the study period, the maximum diurnal photosynthetically-active photon flux density was kept between 350-1000 μmol $m^{-2} s^{-1}$ with a photoperiod of 14 h/10 h (light:darkness). One outer tepal was immediately used to estimate the maximum efficiency of photosystem II photochemistry (*Fv*/*Fm* ratio), water content, and fresh and dry weight, while the other two outer tepals were immediately frozen in liquid nitrogen *in situ* and stored at -80 °C for biochemical analyses. All samples were collected at predawn (1 h before sunrise) to avoid the potential effects of variations in light intensity between sampling days.

Furthermore, around 200 flowers from six vases (three per treatment), with six inflorescences per vase, were selected to evaluate the effect of Promalin® on flower opening. The percentage of opened/closed and senescing/non-senescing flowers treated with water (Control, 95 flowers) and Promalin® (86 flowers) was evaluated at the start, and after 10d and 20d of treatment. Closed flowers were defined as those found at stages I and II-a, while opened flowers included those found at stage II-b and onwards in development. Non-senescent flowers included those found from stages I to IV, while senescent ones included those found at stages V and VI, all descriptions following Arrom and Munné-Bosch [24].

Fig. 1. Developmental stages during flower opening in *Lilium* "Litouwen". Stages were selected from closed flower to anthesis based on Arrom and Munné-Bosch [24], but including two additional stages. From left to right; Stage I – 0d (Flower closed with green tepals and fully compacted organs at the beginning of experiment), Stage I – 1d (Flower closed similar to the previous stage but after 1-day monitoring), Stage II-a – 5d (Flower closed with fully mature brown stamens, first visual loss of green colour, just before opening after 5-day monitoring), Stage II-b – 7d (Initial flower opening with whitish tepals after 7-day monitoring) and Stage III – 8d (Flower anthesis with white tepals) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

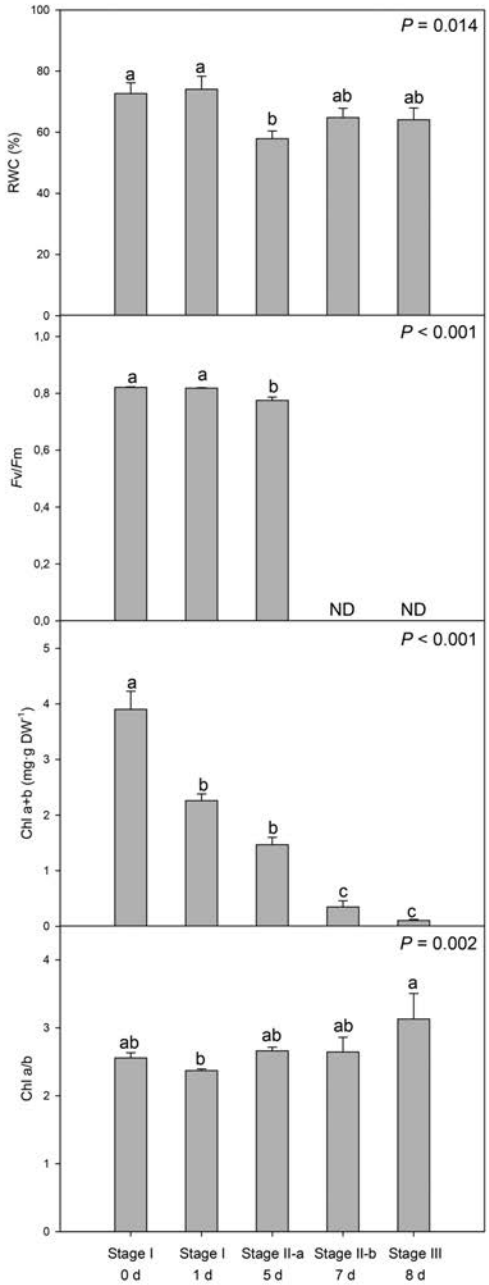

Fig. 2. Relative water content (RWC), maximum efficiency of photosystem II photochemistry (*Fv*/Fm ratio), chlorophyll (Chl) a + b and Chl a/b ratio in tepals at various flower opening stages in *Lilium* "Litouwen". Data shows the mean ± SE of $n = 8$. Results of one-way ANOVA are shown in the inlets. Different letters indicate significant differences between stages ($P < 0.05$) using Tukey post hoc tests. NS, Not significant.

2.2. Chlorophyll fluorescence and tepal water content

The maximum efficiency of PSII photochemistry (*Fv*/Fm ratio) was determined by measuring chlorophyll fluorescence in the middle part of the tepal using a fluorimeter (mini-PAM Photosynthesis Yield Analyzer, Walz, Effeltrich, Germany). To estimate the relative water content (RWC), tepals were immediately weighed to estimate the fresh matter (FW), later immersed in distilled water at 4 °C for 24 h to estimate the turgid matter (TW) and then oven-dried at 80 °C to constant weight to estimate the dry matter (DW). RWC was calculated as 100 *x* (FW-DW)/(TW-DW).

2.3. Estimation of lipid peroxidation products

The extent of lipid peroxidation was estimated by measuring the amount of primary and secondary lipid peroxidation products, including the contents of lipid hydroperoxides (LOOH), malondialdehyde (MDA) and jasmonic acid (JA). For LOOH measurements, tepals (200 mg) were ground to a powder in liquid nitrogen and repeatedly extracted with 2 mL of methanol containing 0.01% butylated hydroxytoluene (BHT, w/v). The extraction was performed in two steps of vortexing, followed by 30 min ultrasonication (Bransonic Ultrasonic Bath, Emerson Industrial Automation, Danbury, CT, USA), vortexing and 10 min centrifugation at 4 °C and 13,000 rpm. Supernatants were pooled, and 1 mL of the extract was transferred to a 2 mL Eppendorf tube with 5% polyvinylpolypyrrolidone (PVPP, 110 µm particle size) and vortexed until PVPP was dissolved. Samples were then ultrasonicated for 20 min, vortexed again and centrifuged at 4 °C and 13,000 rpm for 20 min. The supernatant was then recovered and used for LOOH determination as described by Bou et al. [26], with some modifications. Briefly, two aliquots of the extract were incubated for 30 min in darkness in 96 multi-well plates, one marked as positive (+), consisting of a mixture of 50µL of sample and 50µL of cold methanol with 0.01% BHT, and the other marked as negative (-), consisting of a mixture of 50µL of sample and 50µL of 10 mM triphenylphosphine. After that, 10 µL of + and – samples were incubated for 45 min at room temperature with 190 µL of freshly prepared FOX-2 reagent consisting of 25 mM sulfuric acid, 4 mM BHT, 250µM ammonium iron (II) sulfate hexahydrate and 100µM xylenol orange. A standard curve from 3.75µM to 100µM of H_2O_2 was also incubated with the samples. Measurements were made at 560 nm and determination was performed using the absorbance difference between (+) – (-) samples, using the H_2O_2 calibration curve as a standard.

MDA determinations were based on the thiobarbituric acid (TBA)-reactive substances assay described by Hodges et al. [27]. To sum up, 100 mg of tissue was ground in liquid nitrogen and extracted with 1 mL 80% (v/v) ethanol containing 0.01% BHT using a vortex, 30 min ultrasonication followed by vortexing, 15 min ultrasonication, vortexing and centrifugation at 10 000 rpm for 10 min at room temperature. The supernatant was recovered and placed in 5 mL glass tubes. After that, samples were re-extracted twice more following the same procedure. Later, 1 mL of the recovered supernatant was incubated during 25 min at 95 °C with TBA^- solution and another mL with TBA^+. MDA concentrations were estimated by measuring the absorbance at 440, 532 and 600 nm with a spectrophotometer and following the equations described by Hodges et al. [27]. All reagents were purchased at Sigma-Aldrich (Steinheim, Germany).

Jasmonic acid (JA) was extracted from 100 mg of tepals ground in liquid nitrogen as described by Müller and Munné-Bosch [25]. Sample tissue was spiked with the isotopically labelled internal standard and then extracted with 250 µl of cold methanol with 0.01% glacial acetic acid (v/v) using ultrasonication for 30 min, vortex and centrifugation (13 000 rpm for 15 min at 4 °C). Supernatant was then collected and the pellet re-extracted with 250 µl of methanol with 0.01% (v/v) glacial acetic acid. Supernatants were combined, centrifuged (13 000 rpm for 5 min at 4 °C) and filtered through a 0.22 µm PTFE filter (Phenomenex,

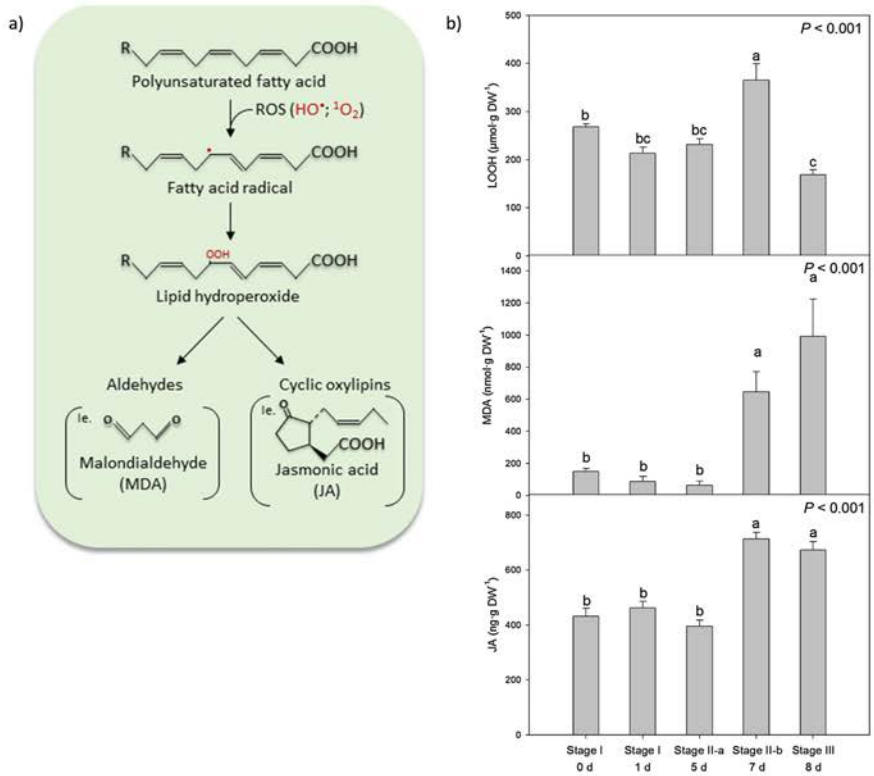

Fig. 3. (a) Formation of primary and secondary lipid peroxidation products in biological membranes. Peroxidation of polyunsaturated fatty acids (PUFA), such as linolenic acid (18:3), gives rise to fatty acid radicals, which, in turn, can convert into lipid hydroperoxides (LOOH). These primary products of lipid peroxidation can give rise to a number of secondary lipid peroxidation products, including among others, aldehydes like malondialdehyde (MDA) and the cyclic oxylipin, jasmonic acid (JA). (b) Contents of primary (LOOH) and secondary (MDA and JA) lipid peroxidation products in tepals at various flower opening stages in *Lilium* "Litouwen". Data shows the mean ± SE of $n = 8$. Results of one-way ANOVA are shown in the inlets. Different letters indicate significant differences between stages ($P < 0.05$) using Tukey post hoc tests. NS, Not significant.

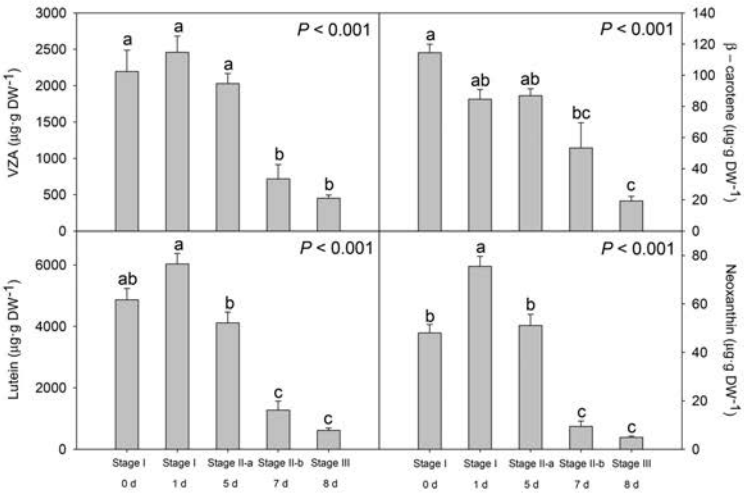

Fig. 4. Contents of the xanthophyll cycle pool (VZA), lutein, neoxanthin and β-carotene at various flower opening stages in *Lilium* "Litouwen". Data shows the mean ± SE of $n = 8$. Results of one-way ANOVA are shown in the inlets. Different letters indicate significant differences between stages ($P < 0.05$) using Tukey post hoc tests. VZA represents the sum of violaxanthin plus zeaxanthin and antheraxanthin. NS, Not significant.

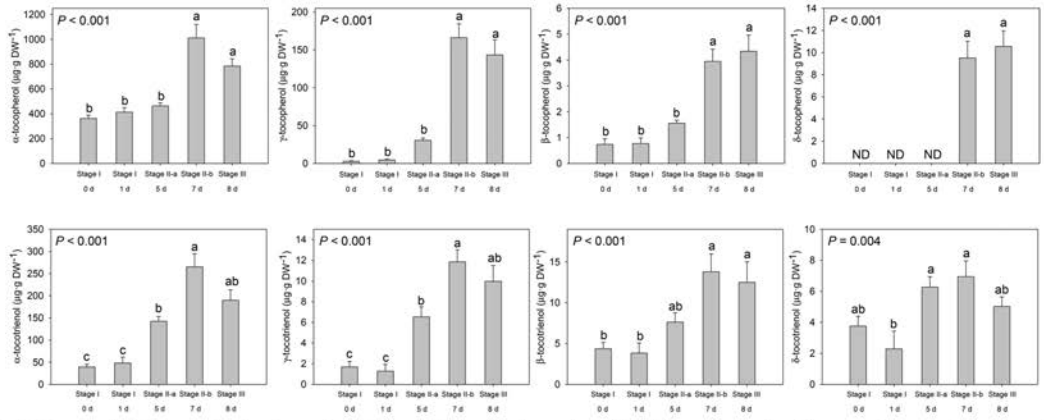

Fig. 5. Contents of all four tocopherols and tocotrienols (vitamin E) found in the tepals of *Lilium* "Litouwen" at various flower opening stages. Data shows the mean ± SE of $n = 8$. Results of one-way ANOVA are shown in the inlets. Different letters indicate significant differences between stages ($P < 0.05$) using Tukey post hoc tests. NS, Not significant.

Torrance, CA, USA) to be analyzed using a UHPLC/ESI–MS/MS system. The LC system consisted of an Aquity UHPLC TM System (Waters, Milford, MA USA) and samples (5 μl) were first separated on a C18 Kinetex column (50 × 2.1 mm, 1.7 μm; Phenomenex, Macclesfield, UK). MS/MS experiments and detection were performed on an API3000 triple quadruple mass spectrometer (PE Sciex, Concord, Ont., Canada) by using multiple reaction monitoring (MRM) in negative ion mode. The optimized MS/MS conditions were determined in infusion experiments using a purified standard and its isotopically labelled internal standard. MRM transitions were used as described by Müller and Munné-Bosch [25]. Quantification was performed by a ten-point calibration curve including an isotopically labelled JA internal standard using Analyst™ software (PE Sciex, Concord, Ont., Canada).

2.4. Capacity for photo- and antioxidant protection

The capacity for photo- and antioxidant protection was estimated by measuring various chloroplastic antioxidants (carotenoids, tocopherols and tocotrienols). Carotenoids, including carotenes and xanthophylls, together with chlorophylls, and vitamin E compounds were extracted from tepals (200 mg) by grinding the tissue to a powder in liquid nitrogen and repeated extraction with 2 mL of methanol containing 0.01% (w/v) BHT. The extract was then filtered through a 0.22 μm PTFE filter (Phenomenex, Torrance, CA, USA) to be analysed by high-performance liquid chromatography (HPLC).

Carotenoids were separated by injecting 80 μL of the extract in a Dupont non-endcapped Zorbax ODS-5-lm column (250 mm long, 4.6 mm i.d.; 20% carbon, Teknokroma, Sant Cugat, Spain) at 30 °C at a flow rate of 1 mL min^{-1} for 38 min. The solvent mixture for the gradient and detection at 445 nm (diode array detector, HP1100 Series HPLC System, Agilent Technologies, Santa Clara, CA, USA) was performed as described by Munné-Bosch and Alegre [28]. Quantification was based on the results obtained from the absorbance at 445 nm and compared with those of calibration curves made with authentic standards of chlorophyll (Chl) a, neoxanthin, violaxanthin and β-carotene (Sigma-Aldrich, Steinheim, Germany). All other pigments were quantified by obtaining the peak area to pmol ratio per injection being 15.5 for antheraxanthin, 18.41 for zeaxanthin, 16.59 for lutein and 19.27 for Chl b.

Tocopherols and tocotrienols were separated following Amaral et al. [29] by injecting 10 μL in an HPLC integrated system that consisted on a Jasco PU-2089 Plus pump, a Jasco AS-2055 Plus auto-sampler (Jasco, Japan) and a FP-1520 fluorescence detector (Jasco, Japan). All vitamin E forms were separated on an Inertsil 100 A (5 μm, 30 250 mm, GL Sciences Inc., Japan) normal-phase column operating at room temperature. The flow rate was 0.7 mL·min^{-1}. The mobile phase was a mixture of *n*-hexane and *p*-dioxane (95.5:4.5 v/v). Detection was carried out at an excitation of 295 nm with emission at 330 nm. Quantification was based on the results obtained from the fluorescence signal and compared with those of calibration curves made with authentic standards (Sigma-Aldrich, Steinheim, Germany).

2.5. Statistical analysis

Statistical tests were performed with SPSS 20.0 statistical package. To estimate the effect of flower opening mean values were tested by one-way ANOVA and Tukey post hoc test to determine statistical differences between stages. To determine whether flower opening is influenced by Promalin®, mean values were tested by two-way ANOVA, being treatment one fixed factor and developmental stage the other fixed factor. Tukey post hoc tests were also used to determine statistical differences between treatments at specific developmental stages.

3. Results and discussion

3.1. Photoinhibition during flower opening

We characterised four developmental stages from closed flowers to anthesis (Fig. 1). During these selected stages, lily flower tepals did not present any significant differences in RWC, but a very sharp and significant decrease in *Fv/Fm* values (Fig. 2). The sharp decrease in the *Fv/Fm* ratio between stages II-a and II-b, from above 0.75 to non-detectable values, clearly indicates a loss of functionality in photosynthetic electron transport, at least at the PSII level. This has also been observed in other flowers, although corollas can maintain some PSI activity and still keep Chl content at detectable amounts [30]. In our study, PSI activity was not measured but the reductions in *Fv/Fm* values were accompanied by a progressive reduction in Chl content (Fig. 2). Interestingly,

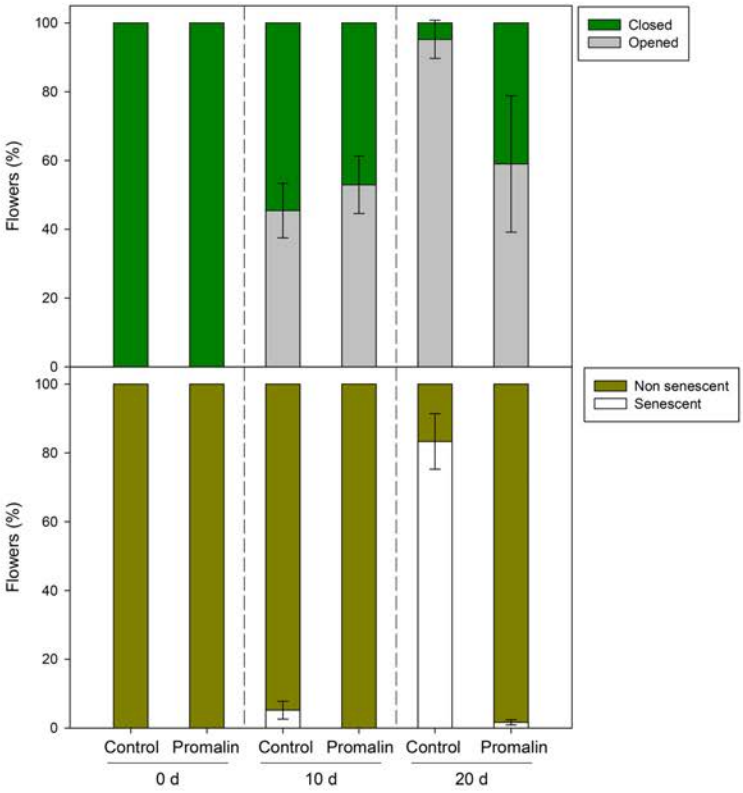

Fig. 6. Percentage of opened and senescing *Lilium* "Litouwen"flowers treated with water (Control) and Promalin® for 20 days. Closed flowers include flowers at stage I or stage II-a, while opened flowers include those found at stage II-b and onwards. Non-senescent flowers include those found from stages I to IV, while senescent ones include those found at stage V and VI, following Arrom and Munné-Bosch [24]. Data show the mean ± SE of $n = 3$ vases for each treatment, including six inflorescences per vase, and a total of 95 flowers for Controls and 86 flowers for Promalin®. Statistical differences were observed at 20d of treatment (Open/Closed flowers, $P = 0.035$; and Non-senescing/Senescing flowers, $P < 0.001$).

there was a first significant half-reduction in Chl content from initial to 1-d monitoring at stage I, followed by a second reduction of about 24% from stage II-a to stage II-b, coincident with the fall in *Fv/Fm* values. Therefore, it appears that photosynthetic pigment antennae start to be dismantled just before photoinhibition of PSII occurs followed by severe degradation of both PSII reaction centers and antennae. These results are in agreement with another study performed in tepals of *Lilium longiflorum* showing a reduction in PSII maximum quantum yield, along with a decrease in Chl content [20]. Moreover, the Chl a/b ratio increased by 15% at flower anthesis (between stages I and III; Fig. 2), which is consistent with a slight decrease of pigment antennae relative to reaction centers. It appears, therefore, that lily tepals present PSII photoinhibition at very early stages of development, most particularly just prior to flower anthesis.

3.2. Lipid peroxidation during flower opening

Dismantling of the photosynthetic apparatus implies a production of ROS due to an energy excess in the reaction centres of thylakoid membranes. Some of these ROS, like $^1O_2$ and $HO^{\bullet}$, have the potential to oxidise chloroplast membrane polyunsaturated fatty acids (PUFA) such as linolenic acid (Fig. 3a). Lipid peroxidation gives rise to the formation of PUFA derivatives like LOOH which can convert into aldehydes, such as MDA, or cyclic oxylipins, such as JA, among others [31,32]. Petals (or tepals) of several flowers showed increased lipid peroxidation and higher levels of MDA during the latest stages of flower development, which has been associated with senescence-related programmed cell death [3,18]. In our study, lily tepals showed a 40% increase of LOOH content at stage II-b relative to stage I, that is during initial flower opening, followed by a 80% reduction at stage III relative to stage II-b (at flower anthesis, Fig. 3b). LOOH content increased in parallel with those of MDA and JA at Stage II-b, MDA increasing again at stage III, while JA remained stable (Fig. 3b). These results are suggestive of an oxidative burst suffered during tepal de-greening and disassembly of the photosynthetic apparatus during flower opening, without compromising the viability of the flower corolla. In this respect, it should be noted that all open flowers completed their developmental program, thus indicating that enhanced lipid peroxidation did not compromise flower development. Lipid peroxidation products, including JA but also MDA, might therefore serve a signaling role during corolla de-greening and flower opening in lilies, an aspect that warrants further investigation. Although it has already been shown that MDA can serve a signalling role in stress responses [31], nothing is known about its putative signalling role in plant developmental processes. The role of lipid peroxidation during flower opening might also partly be seen as a cause of cellular degradation occurring later in flower development, since there is already substantial disorganization of internal cellular structure in some lilies just after anthesis as a sign of senescence start [33]. Flower

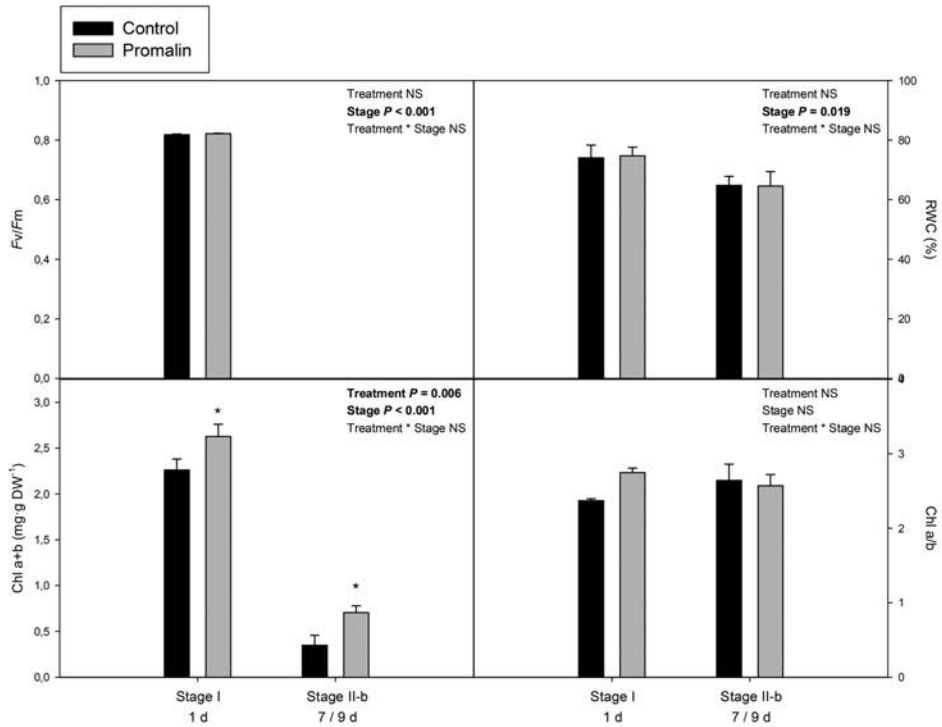

Fig. 7. Relative water content (RWC), maximum efficiency of photosystem II photochemistry (*Fv*/*Fm* ratio), chlorophyll (Chl) a + b and Chl a/b ratio in tepals of Promalin®-treated flowers and controls at 24 h of treatment (stage I) and at initial flower opening (stage II-b) in *Lilium* "Litouwen". Data shows the mean ± SE of $n = 8$. Results of two-way ANOVA are shown in the inlets. Asterisks indicate significant differences between treatments ($P < 0.05$) using Tukey post hoc tests. NS, Not significant.

development has indeed also been seen as a parallel to leaf development and the increase in lipid peroxidation during de-greening could be seen as support for the start of a developmental program of the organ terminating in its death [18,22].

3.3. Photo- and antioxidant protection during flower opening

Lipophilic antioxidants have the ability to prevent and modulate the propagation of lipid peroxidation by scavenging ROS and lipid peroxyl radicals [34,35]. The major lipophilic antioxidants found in chloroplasts are (i) carotenoids, which are essential for photosynthesis and photoprotection, particularly xantophylls, which are mostly located in the light-harvesting antennae, and β-carotene, which may additionally be located in the thylakoid matrix; and (ii) tocochromanols (tocopherols and tocotrienols, also known as vitamin E), which are associated with the chloroplast membrane, where they are synthesized, thylakoid membranes, where they exert a protective antioxidant role, or plastoglobuli, where they accumulate as a reservoir in close contact with thylakoids [18,34].

Although carotenoids tend to accumulate in the chromoplasts of some varieties of *Lilium* [20,36,37], we found a large decrease of carotenoids in tepals of *Lilium* "Litouwen" during early development (Fig. 4). The xanthophylls cycle pool (VZA) and lutein contents suffered a sharp decrease (65–69%) between stages II-a and II-b, together with those of β-carotene, which achieved their minimum levels at stage III ($19 \pm 3\ \mu g \cdot gDW^{-1}$). Neoxanthin content, recently reported for its antioxidant properties against photo-oxidative stress [38], peaked at stage I – 1d, but then decreased, particularly between stages II-a and II-b. Therefore, lily tepals did not accumulate carotenoids during flower opening, rather these compounds decreased in parallel to reductions in the *Fv*/*Fm* ratio, thus further supporting the contention of a general dismantling of the photosynthetic apparatus during tepal de-greening and flower opening in *Lilium*. PSII photoinhibition appears therefore to be associated with a general degradation of carotenoids in tepal chloroplasts of this lily variety. This is consistent with the protective role of β-carotene and other carotenoids against $^1O_2$-induced photoinhibition in chloroplasts [38,39].

In contrast, vitamin E content, which has also been shown to protect the photosynthetic apparatus from photo-oxidative stress [34,40], increased during early development in lily tepals (Fig. 5). Interestingly, tepals of *Lilium* "Litouwen" not only contained tocopherols, as reported previously for other lily varieties [24], but also tocotrienols, thus extending our current knowledge on the occurrence of these secondary metabolites in reproductive tissues [41]. *Lilium* "Litouwen" tepals showed an increase in all forms of tocopherols and tocotrienols, particularly starting at stage II-a for tocotrienols and stage II-b for tocopherols (Fig. 5), once chlorophyll loss has already started (Fig. 2). The most abundant vitamin E form was α-tocopherol with its highest value at initial flower opening (with values around $1\ mg \cdot gDW^{-1}$). This was followed by α-tocotrienol and γ-tocopherol (with values around 265 and $166\ \mu g \cdot gDW^{-1}$, respectively). All other forms were present at amounts below $15\ \mu g \cdot gDW^{-1}$ but followed a similar pattern to that

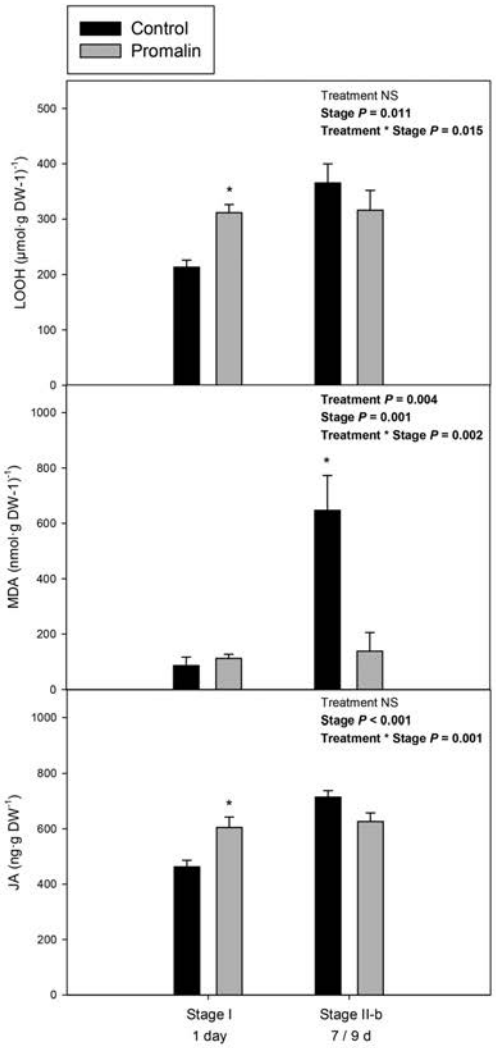

Fig. 8. Contents of primary (LOOH) and secondary (MDA and JA) lipid peroxidation products in tepals of Promalin®-treated flowers and controls at 24 h of treatment (stage I) and at initial flower opening (stage II-b) in *Lilium* "Litouwen". Data shows the mean ± SE of $n = 8$. Results of two-way ANOVA are shown in the inlets. Asterisks indicate significant differences between treatments ($P < 0.05$) using Tukey post hoc tests. NS, Not significant.

showed by the major vitamin E forms. Although vitamin E content rose at flower opening, the biosynthesis of these antioxidants was not sufficient to prevent MDA formation, thus probably indicating a significant part of these compounds accumulated in plastoglobuli, rather than in thylakoid membranes. Accumulation of vitamin E in plastoglobuli may simply result from Chl loss, as the phytol released from Chl degradation and the geranylgeraniol not used for Chl biosynthesis during tepal degreening may instead be diverted to tocopherol and tocotrienol accumulation, respectively. Further research is however needed to better understand the possible role of vitamin E, as well as its relative distribution between chloroplast membranes, thylakoids and plastoblobuli, during tepal de-greening and flower opening in lilies.

3.4. Cross-talk between hormones and lipid peroxidation during flower opening

Promalin®, a combination of GA_{4+7} and benzyladenine, is a commercial product used to delay flower opening and expand the vase life of flowers, including diverse *Lilium* varieties [7,42,43]. Here, we found that Promalin® significantly delayed flower opening in lilies (Fig. 6). To establish some mechanisms related to a possible cross-talk between hormones and lipid peroxidation products in the control of flower opening, we evaluated possible differences between treatments (Promalin® *vs.* controls) at two developmental stages: stage I after 1d of application and at stage II-b at 9d and 7d of application, respectively. We had to wait two additional days for sampling Promalin®-treated plants in order for the study to be fully comparable in terms of developmental stages. We could not find any statistically significant difference for *Fv/Fm*, RWC or Chl a/b ratios, but a slightly smaller Chl degradation was observed in flowers treated with Promalin® compared to controls (Fig. 7), which is consistent with the phenotype observed (that is a slower de-greening in Promalin®-treated tepals).

Interestingly, time-dependent changes in the accumulation of primary and secondary lipid peroxidation products differed between Promalin®-treated flowers and controls. LOOH and JA contents were higher at stage I, while MDA content was reduced at stage II-b in Promalin®-treated flowers compared to controls (Fig. 8). A first Promalin®-induced oxidative burst resulted in enhanced lipid peroxidation, in terms of LOOH and JA accumulation at 24 h of application during stage I, which was translated into a lower MDA accumulation in Promalin® treated lilies at stage II-b. This suggests that LOOH and JA accumulation may serve a signalling role associated with a slower degreening, while reduced MDA content at stage II-b might be the result of a reduced velocity in flower opening. It should be noted that, in this plant species, as soon as flowers open they quickly enter senescence [33]; therefore, higher MDA content at anthesis could make the tissue more sensitive to accelerated senescence, as it occurred in controls compared to Promalin®-treated flowers. Although MDA accumulation may not necessarily be negative for flower opening, our results suggest that it could mark the pace of flower development in lilies, an aspect that undoubtedly needs further study.

The capacity for photo- and antioxidant protection was only slightly influenced by hormonal treatment. The carotenoid content remained nearly unchanged (Fig. 9), while those of vitamin E, in particular γ- and δ- tocopherols along with γ-tocotrienol (Fig. 10), decreased in Promalin®-treated flowers compared to controls. Since this was observed in parallel with significant changes in MDA content in Promalin®-treated flowers, these results support the contention that (i) a significant part of the vitamin E that accumulates in tepals may be found in plastoglobuli, therefore not exerting a protective role against lipid peroxidation in thylakoid membranes, and (ii) hormones may influence the extent of lipid peroxidation by mechanisms not related to the photo- and antioxidant protection. It should be noted that cytokinins and gibberellins may protect membranes from lipid peroxidation through other mechanisms, such as a direct inhibition of lypoxygenase, either at the gene expression or enzyme activity levels, as it occurs in other plant systems [44,45]. Interestingly, γ- and δ- tocopherols along with γ-tocotrienol, and either α- or β-tocopherol/tocotrienol, decreased in Promalin®-treated flowers compared to controls (Fig. 10). This suggests a specific effect of hormones on vitamin E biosynthesis, both downstream of γ- and δ- tocopherols/tocotrienols and at the level of γ-tocopherol methyltransferase regulation, which is the responsible for the conversion of γ- and δ-tocopherol/tocotrienol to their corresponding α and β homologues [41], an aspect that warrants further investigation.

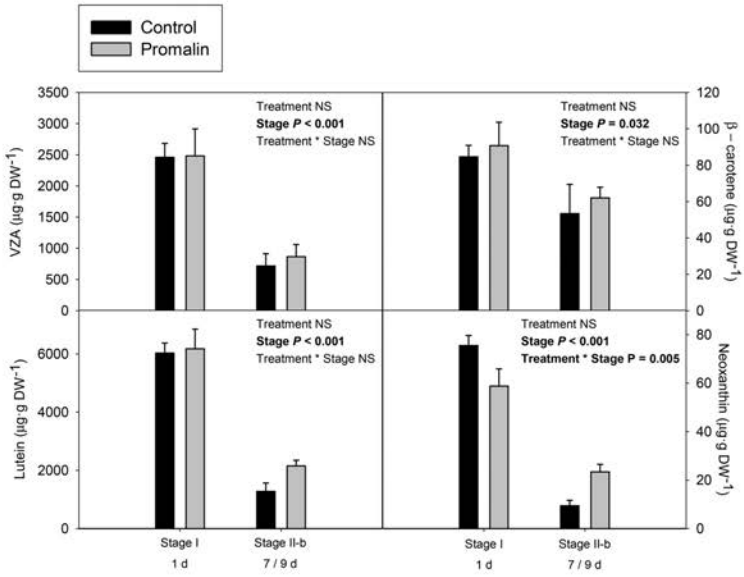

Fig. 9. Contents of the xanthophyll cycle pool (VZA), lutein, neoxanthin and β-carotene in tepals of Promalin®-treated flowers and controls at 24 h of treatment (stage I) and at initial flower opening(stage II-b) in *Lilium* "Litouwen". Data shows the mean ± SE of $n = 8$. Results of two-way ANOVA are shown in the inlets. Asterisks indicate significant differences between treatments ($P < 0.05$) using Tukey post hoc tests. NS, Not significant.

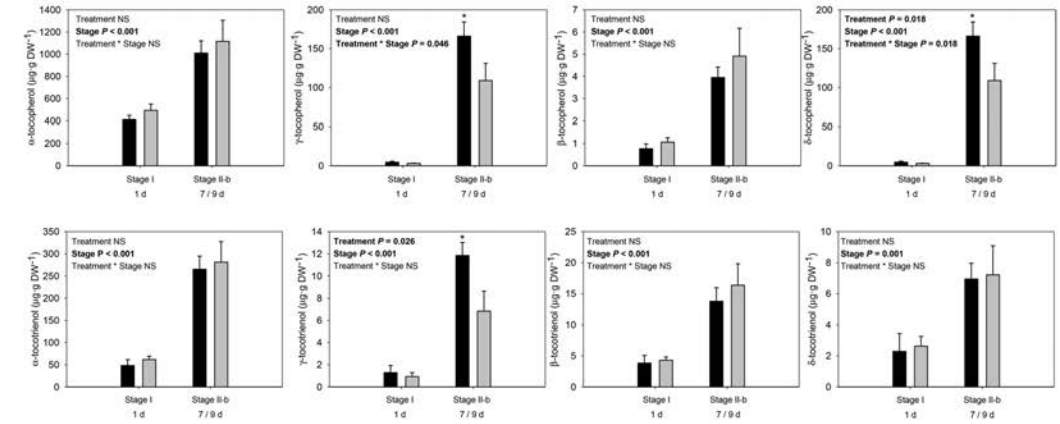

Fig. 10. Contents of all four tocopherols and tocotrienols (vitamin E) found in tepals of Promalin®-treated flowers and controls at 24 h of treatment (stage I) and at initial flower opening (stage II-b) in *Lilium* "Litouwen". Data shows the mean ± SE of $n = 8$. Results of two-way ANOVA are shown in the inlets. Asterisks indicate significant differences between treatments ($P < 0.05$) using Tukey post hoc tests. NS, Not significant.

4. Conclusions

We conclude that (i) the extent of photoinhibition, the capacity of photo- and antioxidant protection and the production of primary and secondary products of lipid peroxidation is finely controlled in a time-dependent manner during tepal de-greening and flower opening, (ii) enhanced photoinhibition and photo-oxidative stress occurs after the start of tepal de-greening and just prior to anthesis, and (iii) Promalin® may exert a delaying effect on flower opening through a cross-talk between hormones and lipid peroxidation in lilies.

Acknowledgements

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Chapter 3

Abscisic acid triggers vitamin E accumulation from phytol recycling in 'Prime Giant' sweet cherry fruits during ripening

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ABSTRACT

Vitamin E is a group of lipophilic antioxidants whose dietary intake from fruits and vegetables is essential for human health. Here we hypothesized that abscisic acid (ABA) could promote the activation of the phytol recycling pathway enhancing the production of tocopherols in sweet cherries, which shows color de-greening fruit ripening. Hence, ABA was exogenously applied prior to ripening during on-tree development and contents of chlorophylls, anthocyanins, lipid hydroperoxides and tocopherols analyzed. Moreover, the expression of key tocopherol biosynthesis genes was determined, including *PacVTE5*, *PacVTE6*, *PacHPPD*, and *PacHPT*. Results showed that exogenous ABA stimulated the production of both α- and γ-tocopherols, immediately after ABA application remaining higher than in controls at harvest. Furthermore, ABA application, which promoted hydroperoxide formation and chlorophyll degradation, enhanced *PacVTE5* and *PacVTE6* expression, particularly during the first 24h. In conclusion, phytol recycling appears to be an active pathway of tocopherol biosynthesis and could be a good target to increase vitamin E contents in sweet cherries.

Keywords: Abscisic acid; Fruit ripening; Phytol recycling; Sweet cherry; Tocopherol; vte5; vte6

Highlights

- ABA stimulates vitamin E accumulation in “Prime Giant” sweet cherries during ripening
- Phytol recycling from chlorophyll degradation is an active pathway for vitamin E biosynthesis
- *PacVTE6* appears to play a major role in tocopherol biosynthesis in sweet cherries

1. Introduction

Fruits are good sources of nutraceutical and high value compounds that contribute to a healthy and balanced diet. Fruit ripening is strongly dependent upon environmental and endogenous signals that coordinate physiological and morphological changes taking place during the ripening process and strongly condition the final fruit quality, including its antioxidant properties. Vitamin E encompasses a group of lipophilic antioxidants present in many fruits, particularly those with a high lipid profile like almonds, avocados or mangoes (Lu *et al.*, 2009; Ornelas-Paz *et al.*, 2007). Due to their amphipathic molecular structure comprising a chromanol head and a prenyl tail with different degrees of saturation (tocopherols saturated and tocotrienols with three unsaturations), vitamin E is usually located in lipid membranes, where they give structural support and protect from oxidative damage by preventing the propagation of lipid peroxidation (Muñoz and Munné-Bosch, 2019). Out of the eight compounds conforming vitamin E (four isoforms α-, β-, γ- and δ- for each form of tocopherol and tocotrienol), α-tocopherol is the most prominent in fruits (Burns *et al.*, 2003) while γ-tocopherol is mostly found in seeds (Urvaka *et al.*, 2019). Interestingly, the human body has a selective liver protein for vitamin E absorption named α-tocopherol transfer protein (α-TTP) and in consequence, α-tocopherol contents are enhanced in plasma levels above all other isoforms, which are rapidly metabolized. In fact, tocopherol deficiency in humans induces severe neurological disorders which are usually associated to disorders in α-TTP. On the other hand, dietary intake of vitamin E is also promoted due to its antioxidant properties and because it also has beneficial effects on the immune system function (Khadangi and Azzi, 2018).

In plants, tocopherol deficiency has a great impact on their sensitivity to high light, since its main function is to protect PSII from the production of reactive oxygen species (ROS) prompted in photoinhibitory events when absorbed light exceeds the thylakoid electron chain capacity (Havaux *et al.*, 2005; Muñoz and Munné-Bosch, 2019). Vitamin E (physically) quenches and (chemically) scavenges singlet oxygen ($^1O_2$) formed in PSII thus preventing

photo-oxidative damage (Triantaphylidès and Havaux, 2009; Muñoz and Munné-Bosch, 2019). Likewise, vitamin E also has a significant role in non-photosynthetic tissues like flowers or fruits, where their high antioxidant capacity and specificity enables higher tolerance to oxidative stress characteristic of the late stages of organ development (Muñoz and Munné-Bosch, 2018). Nevertheless, not all fruits accumulate vitamin E within their tissues substantially enough to contribute to a beneficial health promoting effects. For instance, vitamin E contents in stone fruits such as sweet cherries have been estimated to be a small fraction of their total antioxidant content (Tijero *et al.*, 2016; Mirto *et al.*, 2018; Bastos *et al.*, 2015). Therefore, improvements on the lipophilic fraction of antioxidants in sweet cherry fruits would be highly relevant for human consumption, since these fruits are devoid of carotenoids at harvest and most of its antioxidant capacity comes from hydrophilic antioxidants like anthocyanins.

Sweet cherries (*Prunus avium* L.) are non-climacteric fruits highly appreciated by consumers owing their organoleptic and nutritional properties (Crisosto *et al.*, 2003). To reach the desired quality at harvest, it is important that cherries undergo a controlled process of ripening that includes fruit softening, promotion of metabolic pathways related to flavor and nutrient composition, and finally, exocarp and mesocarp de-greening by chlorophyll breakdown while anthocyanin accumulation takes place. The complexity of this process at the molecular level requires the coordinated action of elicitors that induce physiological and metabolic changes during fruit ripening. In this sense, phytohormones are plant growth regulators actively participating in fruit ripening (Rademacher, 2015; Kumar *et al.*, 2013), and the ripening process of non-climacteric fruits like sweet cherries depend on the production of abscisic acid (ABA) (Teribia *et al.*, 2016). This sesquiterpenoid hormone is involved in cell wall modifications, accumulation of sugars through starch degradation and the regulation of anthocyanin biosynthesis, as it has been demonstrated in climacteric fruits such as tomatoes and non–climacteric fruits like strawberries (Kumar *et al.*, 2013; Leng *et al.*, 2013). In sweet cherries, exogenous application

of ABA induced anthocyanin biosynthesis and an increase in the SSC/TA ratio, thereby promoting fruit ripening (Luo *et al.*, 2014).

It was not until very recently that tocopherol biosynthesis was described to share the MEP pathway for *de novo* formation of geranylgeranyl diphosphate (GGDP) as the precursor of phytyl diphosphate (phytyl-DP) which, together with homogentisate derived from the shikimate pathway, gives rise to the biosynthesis of tocopherols by the catalytic action of homogentisate phytyl transferase (HPT, also named *vte2*) (Havaux *et al.*, 2005). However, a new pathway has been recently described for the production of phytyl-DP from chlorophyll degradation and phytol recycling that depends on the consecutive action of a phytol kinase (VTE5) and a phytyl phosphate kinase (VTE6) that give rise to free phytyl-DP for tocopherol production in chloroplasts (Valentin *et al.*, 2006; vom Dorp *et al.*, 2015). Even though this alternative pathway for tocopherol biosynthesis has been described for leaves and fruits of climacteric fruits like tomatoes (Almeida *et al.*, 2016) and olives (Almeida *et al.*, 2016), no evidence exists for the existence of this pathway in non-climacteric fruits like sweet cherries. Moreover, no compound has yet been described to elicit the production of vitamin E from this pathway for any fruit, despite this might be an excellent approach to enhance the endogenous contents of this antioxidants in several fruits.

Since ABA is the main phytohormone involved in the ripening of sweet cherries upon the maturity stage and tocopherols begin to accumulate from this stage onwards during normal ripening of fruits in orchard trees (Tijero *et al.*, 2016), we hypothesized that ABA could potentially trigger the production of tocopherols in cherry fruits, which undergo a de-greening process during ripening without chloroplast conversion to chromoplast. Hence, the aim of this study was to identify whether ABA could promote tocopherol biosynthesis during on-tree development of sweet cherries and determine if the phytol recycling was an active pathway accounting for the total tocopherol content in cherry fruits. Likewise, to control other physiological implications that could arose ABA application, fruit biomass, total chlorophylls, total

anthocyanins and lipid hydroperoxides were also analyzed at each experimental time point.

2. Materials and methods

2.1. Experimental design, treatments and samplings

Experiments were performed on 16 randomly selected trees from an exploited orchard (at Partida Vall del Sector III, Lleida, NE Spain) using the commercial variety 'Prime Giant'. The exocarp of cherry fruits attached on eight trees were sprayed with 10mL of ABA 10^{-5} M just prior to the fruit ripening onset, 50 days after full bloom (between stages II and III, *sensu* Teribia et al., 2016). Likewise, a control treatment with the same number of trees was also run with exogenous application of 10mL of distilled water. Samplings were performed at 0 hours (h), 4h, 1 day (d), 5d, 9d and 11d after exogenous application between 9 and 10 a.m. local time with an average temperature of 18 ± 4°C. Six fruits per tree from a total of eight trees were randomly sampled at each time point for each treatment (control and ABA) during the experiment. Samples for biomass estimation were stored in the dark until arrival to the laboratory at the University of Barcelona, while cherries for biochemical and genetic evaluation were immediately frozen in liquid nitrogen in the field and stored at -80°C in the laboratory upon arrival and until analysis. Each tree was considered as a single replicate at each time point of measurements for each treatment.

2.2. Fruit biomass, pigments and lipid hydroperoxides estimation

Fruit biomass was estimated by weighing individual cherry fruits without the stem in a lab scale for each treatment and afterwards, oven dried at 96°C to obtain dry weights.

For the analysis of chloroplastic pigments chlorophylls and anthocyanins were estimated by extracting 200 mg of the grounded sample with 1mL of cold methanol using 30 min of sonication and 30 s of vortexing, before and after the sonication of each sample. Extracts were centrifuged at 600g for 10 min at 4°C and the supernatant of each sample was recovered and kept at 4°C. The pellet was re-extracted with 1mL of methanol following the same procedure. Supernatants were pooled and chlorophylls and anthocyanins were

determined spectrophotometrically (Cecil Aquaris CE7400, Cecil Instruments, Cambridge, UK). First, chlorophylls were analyzed as described by Lichtenthaler (1987) and thereafter extracts were recovered and acidified by adding 1% HCl and total anthocyanins measured at 530 nm using the molar extinction coefficient of cyanidin-3-glucoside as a reference.

Lipid hydroperoxides (LOOH) were determined as described by Muñoz *et al*. (2018) with some modifications. In short, 50 mg of grounded cherries were extracted with two consecutive re-extractions of 350µL methanol containing 0.01% butylated hydroxytoluene (BHT, w/v). Pooled supernatants were then mixed with 5%PVPP (w/v) and further sonicated for 20 min (using vortexing for 10s before and after sonication). Afterwards, samples were centrifuged at 600g for 10 min at 4°C and the supernatant of each sample recovered and analysed using the FOX-2 assay as described by Muñoz et al. (2018).

2.3. ABA analysis by UHPLC- ESI-MS/MS

ABA contents in treated cherries were determined as described by Teribia *et al*. (2016). Briefly, 100 mg of grounded samples were extracted with 200 µL methanol:isopropanol:acetic acid, 50:49:1 (v/v/v) and deuterium-labelled internal standards of ABA (d6-ABA) were added prior to ultrasonication and vortexing (Branson 2510 ultrasonic cleaner, Bransonic, USA) for 30 min. After centrifugation, the pellet was re-extracted with 200uL of the extraction buffer without the internal standard and after centrifugation, the collected supernatants were merged and filtered through a 0.22 µm PTFE filter (Waters, USA) before analyses. Phytohormone levels were analysed by UHPLC-ESI–MS/MS as described (Teribia *et al.*, 2016). Quantification was made considering recovery rates for each sample by using the deuterium-labelled internal standards.

2.4. HPLC analysis of vitamin E

Analysis of vitamin E forms was performed as described by Muñoz *et al*. (2018). 200 mg of the grounded sample were extracted as explained for pigment analysis. Then, tocopherols and tocotrienols were analyzed using a

HPLC system that consisted on a Jasco PU-2089 Plus pump, a Jasco AS-2055 Plus auto-sampler (Jasco, Japan) and a FP-1520 fluorescence detector (Jasco, Japan). All vitamin E forms were separated on an Inertsil 100 A (5 μm, 30 250 mm, GL Sciences Inc., Japan) normal-phase column operating at room temperature. The flow rate was 0.7 $mL \cdot min^{-1}$. The mobile phase was a mixture of *n*-hexane and *p*-dioxane (95.5:4.5 v/v). Detection was carried out at an excitation of 295 nm with emission at 330 nm. Quantification was based on the results obtained from the fluorescence signal and compared with those of calibration curves made with authentic standards (Sigma-Aldrich, Steinheim, Germany).

2.5. RNA extraction

Total RNA from sweet cherries was extracted as previously described by Gambino *et al.* (2008) with some modifications. The whole cherry was grounded with liquid nitrogen until powder and extracted with pre-heated (65°C) CTAB buffer (2% CTAB, 2.5% PVP-40, 2M NaCl,100 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0 and 2% of β-mercaptoethanol added just before use) with an adjusted volume of 9 mL per g of fruit. Each sample was divided into separated Nalgene™ Oak Ridge 50 mL PTFE centrifuge tubes so that the top volume of CTAB buffer was of 25 mL. Samples were immediately incubated for 10 min at 65°C and an equal volume of chloroform:isoamyl alcohol (24:1; v/v) was added. After consecutive inversion and centrifuge at 11000 *g* for 10 min at 4°C, the upper layer of the supernatant was carefully recovered in a new Oak centrifuge tube. Thereafter, a second wash and re-extraction was performed with an equal volume of the chloroform:isoamyl alcohol mixture. After vortexing and centrifugation at 11000 g for 10 min at 4°C, the supernatant was recovered in a new Oak tube and 1/3 of LiCl (9M) added for RNA precipitation. After 4h of cold incubation at 4°C, vortexing and centrifugation at 21000 *g* for 20 min at 4°C was repeated, the supernatant was discarded and the pellet resuspended with 5 mL of SSTE buffer 10 mM Tris–HCl pH 8.0, 1 mMEDTA pH 8.0, 1% SDS, 1M NaCl) pre-heated at 65°C. An equal volume of the chloroform:isoamyl alcohol mixture was then added and samples were centrifuged at 11000 g for

10 min at 4°C. All supernatants from the same sample were pooled and transferred to a new Oak tube and the RNA was precipitated with 0.7 volumes of cold isopropanol and immediately centrifuged at 21,000g for 15 min at 4°C. The pellet was twice-washed with ethanol (70%), dried and resuspended in 50µL of diethyl pyro-carbonate (DEPC)–water.

Quantification of untreated RNA was performed with NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) and RNA was then treated with DNase I (Invitrogen) according to manufacturer's instruction. Afterwards, DNase treated RNA was quantified with a Qubit 4 Fluorometer (Thermo Fisher Scientific, Massachusetts, USA). RNA integrity was assessed with a 1% agarose gel in TAE buffer and three samples from each treatment and each time point were analyzed with 2100 Bioanalyzer system (Agilent Technologies, California, USA). Thereafter, RNA was reverse transcribed to cDNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to manufacturer's instructions.

2.6. Primer design

Design of specific primers for *P. avium* L. var. Prime Giant was based on Blastp searches of nucleotide sequences of VTE5, VTE6, HPT and HPPD from close-related species to *P. avium* from the Rosaceae family, mainly from *Fragaria vesca* and *Prunus mume*, along with *Arabidopsis thaliana* and *Solanum lycopersicum* sequences, to identify conserved regions for amplification. Additionally, sequences of *P. avium* were obtained for NCED and the housekeeping ACT from the Rosaceae database (https://www.rosaceae.org). Sequences were aligned using MUSCLE package available in MEGA-X software with default parameters (Tamura *et al.*, 2007). The oligonucleotide primer sequences and accessions for the genes annotated sequences are shown in Suppl. Table 1.

2.7. Gene expression quantification

RT-qPCR was performed on cDNA using the Roche LightCycler® 480 Instrument II (Roche, Basel, Switzerland) and LightCycler® 480 SYBR Green I Master (Roche). The RT-qPCR reactions were conducted by heating at 95ºC for 10 min followed by

45 cycles of 95ºC for 10 s, 60ºC for 20 s and 72ºC for 30 s with a final reaction volume of 10µL. For each sample, RT-qPCR was conducted with *PacHPT*, *PacHPPD*, *PacVTE5*, *PacVTE6* and *PacNCED* specific primers, and a parallel reaction with *PacACT* primers as the internal reference gene. Cp values and dissociation curves were analyzed after RT-qPCR reactions with the LightCycler® 480 Software, Version 1.5 (Roche, Basel, Switzerland) and RT-qPCR products were further analyzed with 2% (w/v) agarose gel. After primer efficiency testing, expression levels for each gene were calculated with the 2−ΔΔCT method and standardized with the Cp values of each gene at 0h.

2.8. Statistical analysis

Statistical tests were performed with SPSS 20.0 statistical package. To estimate the effect of ABA exogenous application, mean values were tested by two-way ANOVA and Duncan post hoc test to determine statistical differences between treatments.

3. Results and discussion

Due to reported health beneficial properties of a balanced diet and a regular dietary intake of vitamin E, great efforts have been made to enhance the contents of this antioxidant in fruits and vegetables, where vitamin E is naturally found and synthetized, especially in the form of α-tocopherol. Here, we show that ABA may be triggering tocopherol production in cherry fruits while contributing to its biosynthesis through the newly described pathway of phytol recycling.

3.1. Abscisic acid accumulation and fruit ripening

ABA application in fruits of *P. avium* L. var Prime Giant before the onset of fruit ripening induced high accumulation of this phytohormone immediately after its application, as shown by higher endogenous contents of ABA at 4h after treatment and sustained levels of ABA up to 5d after its application (Figure 1). In fact, cherry fruits treated with 10^{-5}M ABA had over 40% higher contents of ABA than control fruits. Nevertheless, after 5d ABA contents restored and kept similar values to those found for control-treated fruits until the end of the experiment. Likewise, after ABA application LOOH contents sharply increased up to twice the contents of control fruits and

remained high throughout the experiment (Figure 1). It is important to note that control plants were also treated with the same technique but using water instead of ABA, hence the reported increases ought to be associated to triggering effects of ABA in sweet cherries. The interrelationship between ABA and reactive oxygen species (ROS) that contribute to enhanced levels of lipid peroxidation has been described almost entirely in leaves and seeds where it has been found that ABA positively enhance ROS production to mediate abiotic and biotic stress responses (Yu et al., 2019), creating a positive loop between ABA signal, ROS burst and antioxidant production. However, the interaction between ABA and ROS is poorly understood in fruits, where ROS production has been mostly associated to the latest stages of fruit ripening and to fruit decay (Xu et al., 2013; H., Gao et al., 2016) and there is only evidence of ROS burst at the ripening onset in grapes and tomatoes (Kumar et al., 2013). The present experiment shows that ABA promotes higher oxidative stress during fruit development of sweet cherries on-tree by increased levels of lipid peroxidation.

Even though ABA application in cherry fruits had no effect on the fruit biomass, which increased over time with no differences between treatments, ABA promoted anthocyanin biosynthesis in cherry fruits immediately after application and anthocyanin contents remained higher in ABA-treated fruits than in control fruits all over the experiment (Figure 2). These results agree with the ripening function of ABA in non-climacteric fruits. Indeed, other research studies have already reported the incentive effects of ABA to enhance anthocyanin biosynthesis in several fruits, including sweet cherries during on-tree development (Shen *et al.*, 2017). Moreover, 4h after ABA application, total chlorophylls contents were 20% lower in ABA-treated fruits than in controls (Figure 2). In leaves, chlorophyll degradation during the late stages of senescence has been related to ABA signaling, since the Arabidopsis NAC-LIKE, ACTIVATED BY AP3/PI (NAP) transcription factor leads to higher chlorophyll degradation by enhancing the expression of *ABSCISIC ALDEHYDE OXIDASE3* (*AAO3*) that promotes ABA accumulation on senescent leaves (Yang *et al.*, 2014).

Furthermore, three abscisic acid (ABA)-responsive element (ABRE)-binding transcription factors, ABF2 (AREB1), ABF3, and ABF4 (AREB2) directly bind to and activate the *NYE1* promoter, which codes for a nonyellowing chloroplastic protein that prevents chlorophyll degradation (Ren *et al.*, 2007; Gao *et al.*, 2016). Likewise, the ABSCISIC ACID RESPONSE ELEMENT-BINDING FACTORs (LcABF1/2) have been proven to bind the promoter region of chlorophyll degradation-related genes (PAO and SGR), while LcABF2/3 bind the promoter region of LcMYB1 related to anthocyanin biosynthesis during litchi (*Litchi chinensis*) ripening (Hu *et al.*, 2019). Taken together, it seems that ABA could also be promoting transient chlorophyll degradation of cherry fruits with indistinguishable degradation of chlorophyll *a* or *b*, since the proportion only changed over-time, but no statistical differences were found between treatments (Figure 2).

3.2. Tocopherol accumulation in ABA-treated fruits

Previous analysis of sweet cherry fruits have reported low contents of this antioxidant molecule in sweet cherries (Bastos *et al.*, 2015; Mirto *et al.*, 2018) compared to other fruits that accumulate significant higher levels like avocados or mangoes (Lu *et al.*, 2009; Ornelas-Paz *et al.*, 2007). Immediately after 4h since the application of ABA to cherry fruits, α- and γ-tocopherol contents increased by 40% and 60%, respectively, compared to the control (Figure 3). Besides, both tocopherol forms remained about 20% higher in ABA-treated cherry fruits than in the control at the end of the experiment, showing that ABA treatment significantly increases endogenous contents of tocopherols in sweet cherries and therefore constitute a good target to enhance vitamin E contents and study its regulatory role in sweet cherries. There is evidence that tocopherols accumulate in plastoglobules and lipid membranes of chloroplasts and chromoplasts of leaves and fruits like tomatoes and yellow peppers because enzymes that participate in tocopherol biosynthesis are located in these plastid regions (Ytterberg *et al.*, 2006). However, it is important to note that unlike tomatoes and yellow peppers, sweet cherries do not transform chloroplasts into

chromoplasts as no carotenoids are detected at advanced stages on the ripening of *P. avium* L. var. Prime Giant (Tijero *et al.*, 2016) and to date, no other plastids have been described for sweet cherries when fully ripen. Therefore, accumulation and biosynthesis of tocopherols in sweet cherries may take place in plastoglobules released from chloroplasts following chloroplast dismantling, as it has been evidenced for senescing chloroplasts that exude lipid-rich bodies from gerontoplasts to the cytosol (van Doorn and Prisa, 2014; Liu, 2016). Nevertheless, at present this information still remains elusive for sweet cherry fruits and needs further investigation.

3.3. Phytol recycling is an active pathway for tocopherol biosynthesis in sweet cherries

The recently completed phytol recycling pathway has opened a new frame to understand events leading to vitamin E biosynthesis in plants, since chlorophyll degradation is an underpinning factor that could boost tocopherol production (Valentin *et al.*, 2006; vom Dorp *et al.*, 2015; Almeida *et al.*, 2016). In this sense, several fruits undergo fruit de-greening as part of the ripening process, which could be a primary source of phytol for tocopherol biosynthesis. For sweet cherries, chlorophyll degradation occurs prior to anthocyanin accumulation in fruit ripening and, as shown in the present study, ABA promotes this process (Figure 2). Expression analysis by RT-qPCR showed that *PacNCED* expression increased overtime, related to its function as the gene encoding for the enzyme catalyzing ABA *de novo* biosynthesis, but was unaffected by ABA treatment, which explains that ABA contents were unaffected after 5d of treatment, since *PacNCED* expression was not promoted in ABA-treated fruits (Suppl. Figure 1).

In contrast, expression of *PacVTE6* and *PacVTE5* sharply increased (with an up to 2-fold increase) at 4h and 1d after ABA treatment, to later decrease and reach similar values to control-treated fruits (Figure 4A). Moreover, ABA treatments in sweet cherries also promoted the expression of *PacHPPD* (Figure 4A), encoding the enzyme responsible for the conversion of *p*-hydroxyphenyl pyruvate to homogentisic acid that constitutes the

chromanol head of tocopherols accountable of their antioxidant activity (Figure 4B). Therefore, higher endogenous contents of ABA in fruits not only promoted the activation of the phytol recycling pathway through higher expression of *PacVTE5* and *PacVTE6*, but also enhanced the expression of *PacHPPD* (Figure 4A and 4B), therefore triggering tocopherol biosynthesis in ABA-treated cherry fruits as shown by higher contents of α- and γ-tocopherol (Figure 3). Previous studies had already shown the involvement of the phytol recycling pathway in climacteric fruits like tomatoes, where a down-regulation of *SlVTE5* reduced up to 90% total tocopherol content in mature fruits (Almeida *et al.*, 2016). Moreover, olive varieties with higher tocopherol content in their fruits also exhibited higher expression of *VTE5* during on-tree ripening (Georgiadou *et al.*, 2015). Cys-elements of tocopherol-related genes have been found for *HPT* and *HPPD*, so that both ABA and ethylene activated the transcription of these genes (Singh *et al.*, 2011; Gálvez-Valdivieso *et al.*, 2015). However, to our knowledge this is the first time the specific activation of the phytol recycling pathway through ABA signaling in plants is reported presenting evidence that ABA is indeed promoting tocopherol biosynthesis in sweet cherries. This new role for ABA could have important applications in the agronomy production of sweet cherries and other vegetable and fruit commodities where tocopherols do not accumulate at high amounts and their implementation could provide beneficial health effects.

4. Conclusion

It is concluded that ABA stimulates vitamin E accumulation in "Prime Giant" sweet cherries during ripening and that phytol recycling from chlorophyll degradation is an active pathway for vitamin E biosynthesis, where transcription of *PacVTE6* appears to be rate limiting and consequently, a potential target for vitamin E fortification in cherry fruits

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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FIGURE LEGENDS

Fig. 1. Endogenous contents of the phytohormone abscisic acid (ABA) and lipid hydroperoxides (LOOH) in sweet cherry fruits after exogenous application with 10^{-5}M ABA. Measurements were performed at 0h (just before treatments), 4h, 1d, 5d, 11d and 19d of treatments. Data are the mean ± SE of n = 8. Statistical comparisons were performed by two-way ANOVAs followed by a posthoc Duncan test. Results of statistics are shown in the inlets. Asterisks indicate significant differences at P < 0,05) in the posthoc test. NS, not significant.

Fig. 2. Biomass, total anthocyanins, total chlorophylls and ratio chlorophyll *a*/*b* after exogenous application with 10^{-5}M ABA. Measurements were performed at 0h (just before treatments), 4h, 1d, 5d, 11d and 19d of treatments. Data are the mean ± SE of n = 8. Statistical comparisons were performed by two-way ANOVAs followed by a posthoc Duncan test. Results of statistics are shown in the inlets. Asterisks indicate significant differences at P < 0,05) in the posthoc test. NS, not significant.

Fig. 3. Endogenous contents of α- and γ-tocopherol in sweet cherry fruits after exogenous application with 10^{-5}M ABA. Measurements were performed at 0h (just before treatments), 4h, 1d, 5d, 11d and 19d of treatments. Data are the mean ± SE of n = 8. Statistical comparisons were performed by two-way ANOVAs followed by a posthoc Duncan test. Results of statistics are shown in the inlets. Asterisks indicate significant differences at P < 0,05) in the posthoc test. NS, not significant.

Fig. 4. (A) Expression analysis of *PacHPPD*, *PacHPT*, *PacVTE5* and *PacVTE6* in sweet cherry fruits after exogenous application with 10^{-5}M ABA. Measurements were performed at 0h (just before treatments), 4h, 1d, 5d, 11d and 19d of treatments. Data are the mean ± SE of n = 8. Statistical comparisons were performed by two-way ANOVAs followed by a posthoc Duncan test. Results of statistics are shown in the inlets. Asterisks indicate significant differences at P < 0,05) in the posthoc test. NS, not significant. **(B)** Vitamin E biosynthesis pathway in fruits with a detail of the phytol recycling pathway used to provide phytol for tocopherol biosynthesis. HPPD and HPT are marked in blue, while VTE5 and VTE6 are marked in green. DMPBQ, dimethylphytylbenzoquinol; GGDR, genarylgenaryl reductase; HPPD,

hydroxyphenylpyruvate dioxygenase; HPT, homogentisate phytyl transferase; MEP, methylerythritol phosphate; MPBQ, methylphytylbenzoquinol; MPBQ-MT, MPBQ methyltransferase; PDP, phytyl diphosphate; PK, phytyl kinase; PPH, pheophytin pheophorbide hydrolyase; PPK, phytyl phosphate kinase; TAT, tyrosine aminotransferase; TC, tocopherol cyclase; TMT, tocopherol methyltransferase.

Figure 1

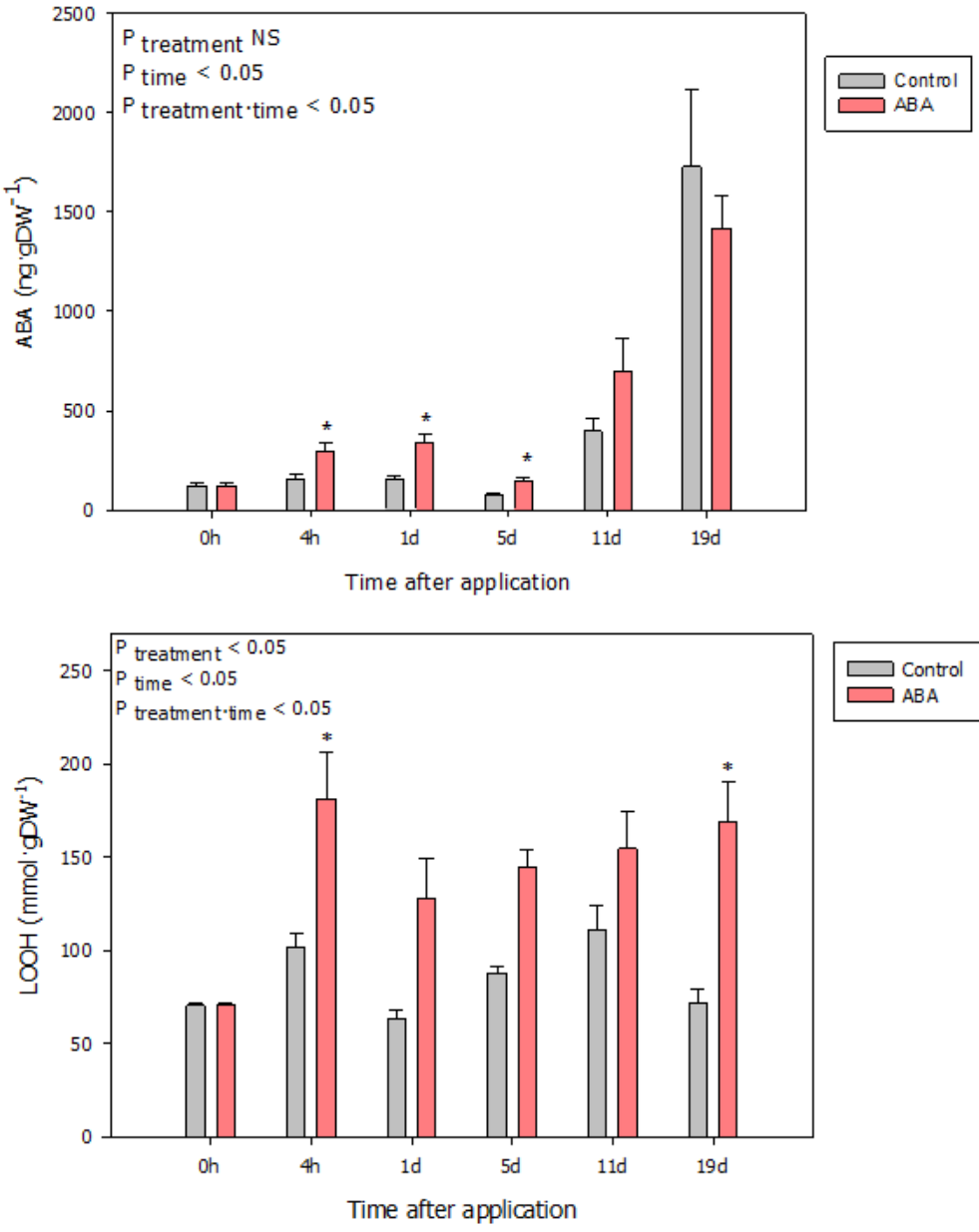

P treatment NS
P time < 0.05
P treatment·time < 0.05
Control
ABA
ABA (ng·gDW-1)
2500
2000
1500
1000
500
0
0h
4h
1d
5d
11d
19d
Time after application
P treatment < 0.05
P time < 0.05
P treatment·time < 0.05
Control
ABA
LOOH (mmol·gDW-1)
250
200
150
100
50
0
0h
4h
1d
5d
11d
19d
Time after application

Figure 2:

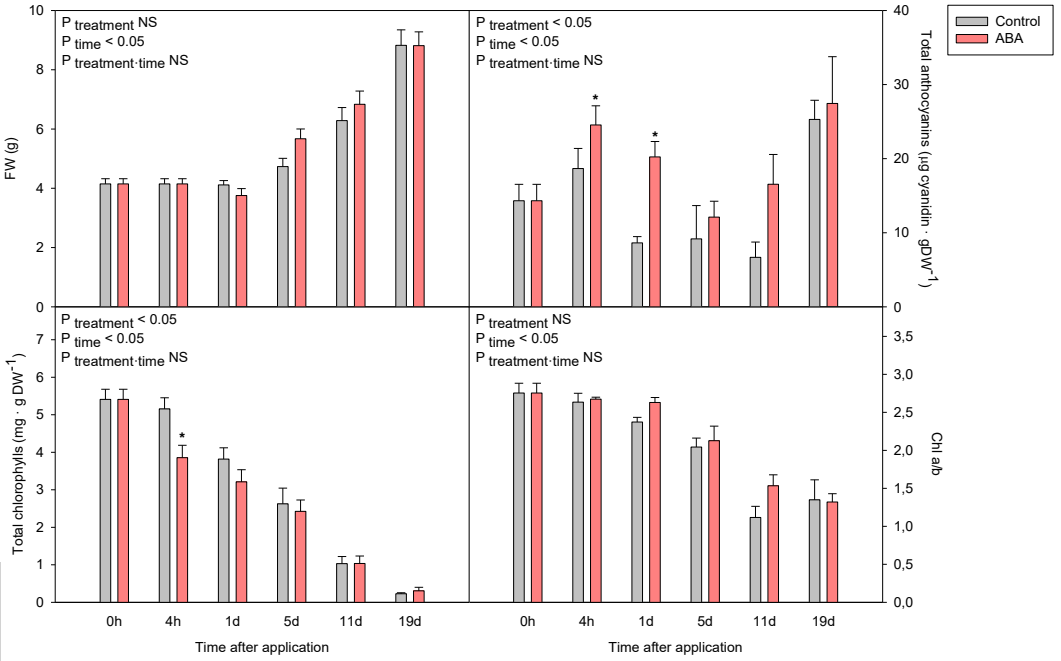

P treatment NS
P time < 0.05
P treatment·time NS
P treatment < 0.05
P time < 0.05
P treatment·time NS
P treatment < 0.05
P time < 0.05
P treatment·time NS
P treatment NS
P time < 0.05
P treatment·time NS
Control
ABA
FW (g)
Total anthocyanins (µg cyanidin · gDW-1)
Total chlorophylls (mg · g DW-1)
Chl a/b
0h
4h
1d
5d
11d
19d
Time after application
Time after application

Figure 3:

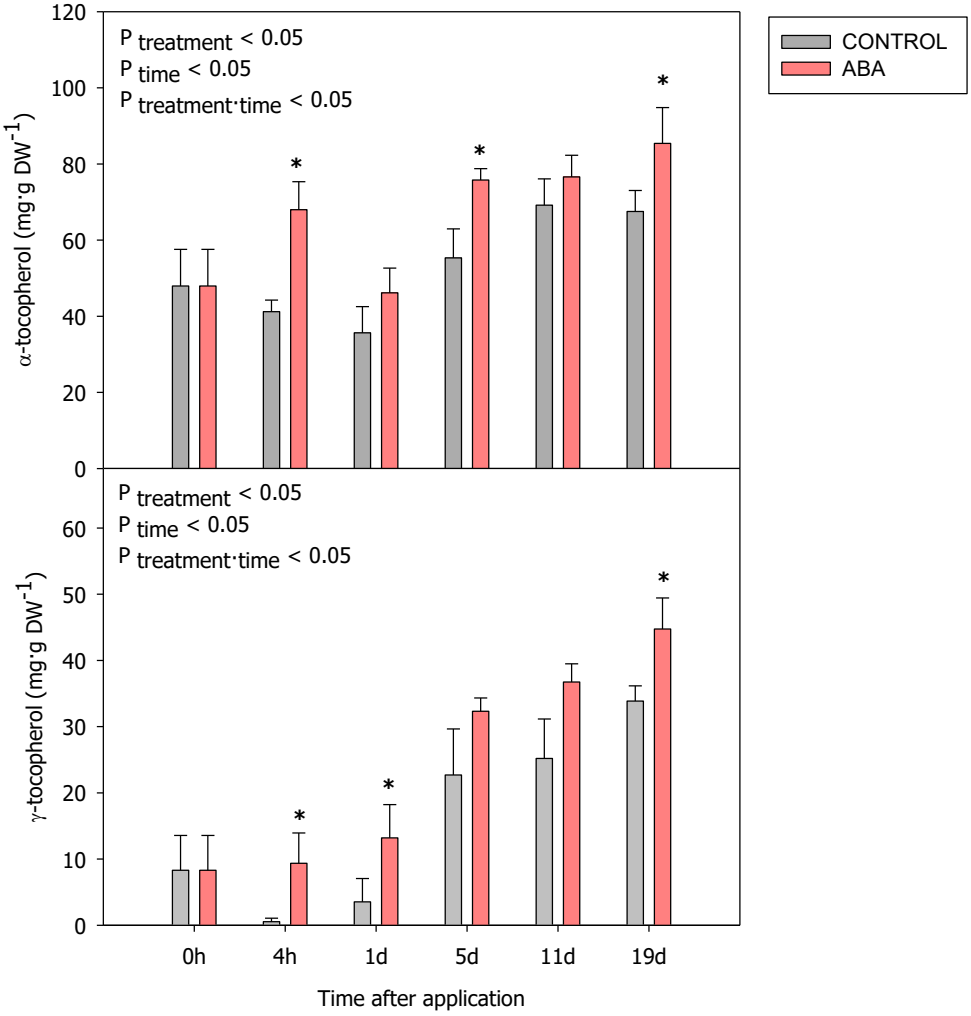
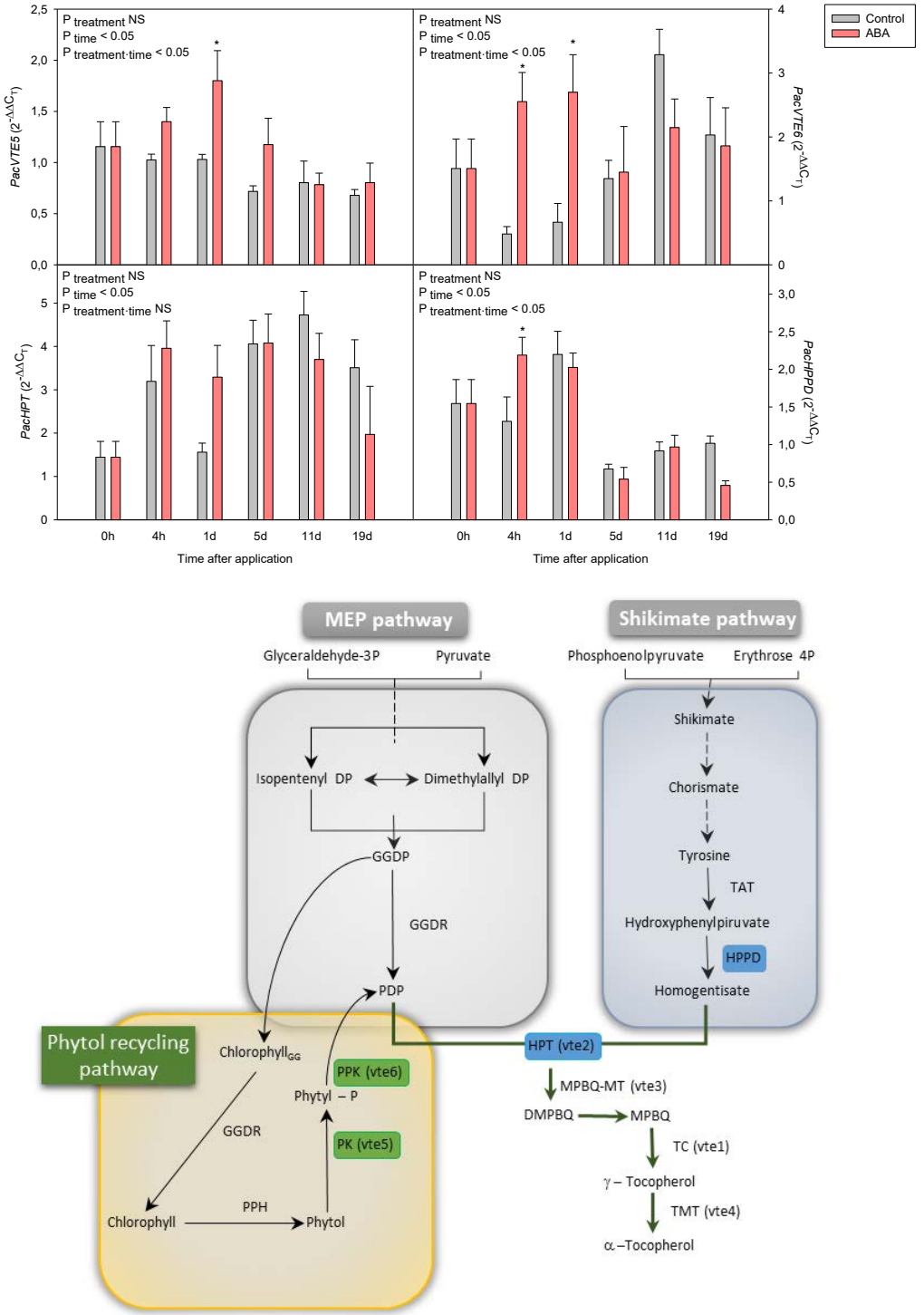

P treatment < 0.05
P time < 0.05
P treatment·time < 0.05
CONTROL
ABA
α-tocopherol (mg·g DW-1)
γ-tocopherol (mg·g DW-1)
0h
4h
1d
5d
11d
19d
Time after application

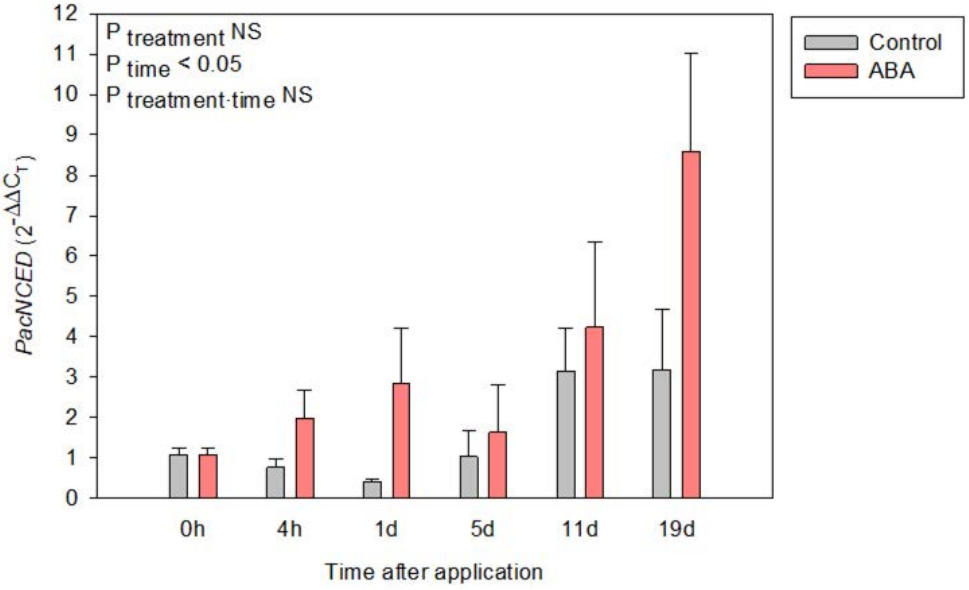
Figure 4:


P treatment NS
P time < 0.05
P treatment·time < 0.05
P treatment·time NS
Control
ABA
PacVTE5 (2-ΔΔCT)
PacVTE6 (2-ΔΔCT)
PacHPT (2-ΔΔCT)
PacHPPD (2-ΔΔCT)
0h
4h
1d
5d
11d
19d
Time after application
MEP pathway
Shikimate pathway
Glyceraldehyde-3P
Pyruvate
Phosphoenolpyruvate
Erythrose 4P
Isopentenyl DP
Dimethylallyl DP
GGDP
GGDR
PDP
Shikimate
Chorismate
Tyrosine
TAT
Hydroxyphenylpiruvate
HPPD
Homogentisate
Phytol recycling pathway
Chlorophyll GG
PPK (vte6)
Phytyl – P
PK (vte5)
PPH
Chlorophyll
Phytol
HPT (vte2)
MPBQ-MT (vte3)
DMPBQ
MPBQ
TC (vte1)
γ – Tocopherol
TMT (vte4)
α –Tocopherol

Suppl. Table 1. RT-qPCR primer pair sequences, amplicon length and Tm for each gene analysed in *P. avium* L. Var. Prime Giant

Oligo Name	Sequence (5' to 3')	Amplicon length (base pairs)	Tm (°C)	Gene accession numbers for primer design
ACT_F	GGGTATGCCCTACCTCATGC	167	60	AB665559.1
ACT_R	GAAAGAATAACCGCGCTCCG		60	
NCED_F	GATGCACGACTTCGCTATCA	150	60	FJ560909.1
NCED_R	TGATCATCTCCTGCAGCTTG		60	
HPPD_F	ATTCAAGACGCTGAGGGAGA	159	60	XM_004303840.2
HPPD_R	ACCAAAATCCCCAACTCCTC		60	XM_008231349.1
HPT _F	TATTTTTCAAGGGGGTGCTG	163	60	XM_008225533.1
HPT _R	GATGCCAGTCCCAACTGAAT		60	XM_011464033.1
VTE5_F	GGTCTCCTTGGCAATGATGT	164	60	At5g04490 NM_001346814.1
VTE5_R	CCCAAATTTTCTTCCCATGA		60	XM_004307103.2 XM_008220718.2
VTE6_F	CAGTGCTGCTGGTTGTGTTT	132	60	At1g78620 XM_004244244.3
VTE6_R	TTGCCAAAGCCGAGAATATC		60	XM_016793330.1

Suppl. Figure 1. Expression analysis of *PacNCED* in sweet cherry fruits after exogenous application with 10-5M ABA. Measurements were performed at 0h (just before treatments), 4h, 1d, 5d, 11d and 19d of treatments. Data are the mean ± SE of n = 8. Statistical comparisons were performed by two-way ANOVAs followed by a posthoc Duncan test. Results of statistics are shown in the inlets. Asterisks indicate significant differences at P < 0,05) in the posthoc test. NS, not significant.



DISCUSSION

DISCUSSION

Plants are constantly subjected to environmental and developmental cues that can compromise their growth and life performance. In this context, light becomes an important factor accountable for unbalanced cellular redox state that contributes to activate physiological and metabolic changes to fine-tune acclimation and developmental processes in plants (Li, *et al*., 2009; Foyer, 2018). Indeed, since chloroplasts are responsible of light harvesting and make use of light energy to perform photosynthesis through redox reactions taking place in thylakoid membrane complexes, these organelles are largely affected by light excess as a result from external and internal pressures (Dietz, *et al*., 2016). ROS produced within chloroplasts due to high energy quanta in photosystems have a direct impact in cellular redox state that promotes photo-oxidative stress in plants (Triantaphylidès, *et al*., 2008). To avoid cellular damage and mediate oxidative signalling from chloroplasts to the nucleus, antioxidant systems must modulate production of ROS and peroxyl radicals derived from photo-oxidative processes (Foyer & Noctor, 2005). Because chloroplasts control the overall plant performance by metabolic adjustments, plants have evolved multiple antioxidant mechanisms and strategies to prevent photo-oxidative damage (Mittler, 2002). Moreover, common mechanisms might exist in different organs subjected to light excess, but the outcomes derived from this process may differ since composition and physiological status of chloroplasts is different relying upon organ developmental stage (Pogson, *et al*., 2015). In this framework, it is important to understand from a wide perspective, physiological events leading to excessive light in chloroplasts that cause photoinhibition and cellular redox changes in different plant organs, to depict internal signals that regulate plant stress acclimation and organ development, which could have important applications in ecology and agronomy.

1. Photoinhibition in leaves, flowers and fruits: developmental stage matters

Leaves, flowers and fruits have very different functions in plants. While leaves are responsible for most part of plant photosynthesis that contributes to the production of photoassimilates for vegetative growth and reproduction (O'Leary, 1981; Evans, 1989), flowers and fruits are specialised organs involved in plant reproduction, the former bearing all reproductive structures (Lohmann & Weigel, 2002) and the later contributing to seed formation and protection along with plant propagation through seed dispersal (Gapper, *et al*., 2013). Even though the structures and functions of these organs do not seem comparable, during leaf, flower and fruit development there are several stages where these organs might share common physiological mechanisms since there share a similar cellular composition. For instance, flowers and fruits at initial stages of development possess functional chloroplasts with resembling properties to that of mature leaves, which contribute to carbon fixation and organ development (Blanke & Lenz, 1989; Lytovchenko, *et al*., 2011; Vaillant-Gaveau, *et al*., 2011; Brazel & Ó'Maoileídigh, 2019). As proposed in **Annex II**, chloroplasts have important implications in organ development and, therefore, studying events that lead to photoinhibition or chloroplast dismantling not only during leaf development, but also during flower and fruit development is essential to understand the physiological relevance of chloroplasts in acclimation and organ development, with potential biotechnological applications.

It is important to highlight that chloroplasts are not only the site to perform photosynthesis, but also the place where many metabolic reactions occur. Indeed, the chloroplast stroma harbours enzymes of the MEP (Rodríguez-Concepción & Boronat, 2002) and the shikimate (Rippert, *et al*., 2009) pathways, which are related to the biosynthesis of basic molecules like amino acids, as well as the biosynthesis of complex molecules involved in plant growth like ABA or gibberellins (GAs) and protective antioxidant

compounds like carotenoids or tocopherols, among others. Therefore, slight changes in the chloroplast status can have important consequences for the global performance of plants, while transformation of these chloroplasts may trigger events of programmed cell death. Photoinhibition in chloroplasts due to excessive light leads to a highly-oxidized environment that can induce photodamage and photobleaching in chloroplasts, ultimately leading to chloroplast dismantling, and indeed, photoinhibition is one of the first mechanisms for the plant to sense environmental and developmental cues (Foyer & Noctor, 2005). As it has been previously stated for other abiotic stresses like water deficiency (Björkman & Powles, 1984; Valladares & Pearcy, 1997), high temperatures (Havaux, 1992; Streb, *et al*., 2003) or nutrient deficiency (Simancas, *et al*., 2016), it was shown in **chapter 1** that cold temperatures during Mediterranean winter trigger PSII inactivation of mastic trees living in a natural environment, as indicated by reduced F_v/F_m values below the standard photoinhibition threshold of 0.75 when mean temperatures were below 15ºC from November to March. *P. lentiscus* showed special sensitivity to winter photoinhibition when minimum temperatures where under 0°C in December and February, where F_v/F_m values reached their minimum levels. Nevertheless, inactivation of PSII was found to be transient in this species, since mastic trees restored initial F_v/F_m values when temperatures warmed up by the end of April. Likewise, susceptibility of mastic trees to cold temperatures was also observed in the altitudinal gradient performed in February, as the highest population at 740m a.s.l. was also the most photoinhibited. Photoinhibition in overwintering plants has been described for plants from the boreal and northern-temperate region, where overwintering confers advantages in terms of carbon gain and energy costs at the expenses of overexcitation in the photosynthetic apparatus due to metabolic disturbances occurring during winter (Tanja, *et al*., 2003; Morales, *et al*., 2016; Míguez, *et al*, 2017). Photochilling stress also leads to reduced photosynthetic capacity in evergreen species, including *P. lentiscus*, where previous studies have shown lower

photosynthetic rates during winter and in altitude (Flexas, *et al*., 2001). Nevertheless, even if growth might be constrained, photoinhibition in these plants may also be considered as an effective strategy to avoid further photodamage, since PSII repair cycle is more dynamic than that of PSI, which is more affected by cold temperatures that could have a greater impact (Tikkanen, *et al*., 2014b). In this regard, transient photoinibition due to cold temperatures during winter must be seen as an investment for plants to preserve their ecological niche during winter preventing be conditioned by external factors that could compromise leaf renewal during spring, as it is the case for sclerophyllous plants. These reduced values in F_v/F_m of mastic trees were also accompanied by slight reductions in the chlorophyll content over the winter and at increasing altitude. Moreover, higher chlorophyll a/b ratio was also observed when temperatures were reduced, suggesting that photoinhibition during cold periods were likely to be inducing chlorophyll photobleaching. However, as shown for other species such as pines (*Pynus sylvestris*) or snow gum (*Eucalyptus pauciflora*) chlorophyll loss during winter photoinhibition does not compromise leaf recovery at spring and chlorophyll values can return to pre-stress levels (Ottander, *et al*., 1995; Gilmore & Ball, 2000). The dynamics of photobleaching and chlorophyll recovery might indeed contribute to acclimation and withstand adverse conditions during winter, while the capacity to overcome this situation with a rapid turnover of the chlorophyll content when conditions are restored favours leaf development.

In general, flower and fruit development entail a massive loss on chlorophyll content and there is great accumulation of other pigments like vacuolar anthocyanins or carotenoids in the conversion from chloroplasts to chromoplasts. These pigments give flower corollas and ripe fruits spectacular bright colours that contribute to the attraction of pollinators and animals that assist in seed dispersal (Grotewold, 2006). In **chapter 2**, it was shown that chlorophylls begin to decrease prior to flower opening and achieve their lowest values at anthesis, when only a small fraction of

chlorophylls remained. Nevertheless, F_v/F_m values in flower tepals were high until chlorophylls were drastically reduced at anthesis, showing that chlorophylls were efficiently harvesting light at initial bud development. Moreover, from changes in the pigment content and the chlorophyll a/b ratio it may be inferred that there was severe degradation of the antenna just before PSII photoinhibition, followed by a dramatic reduction in both antenna and reaction centers. There is little evidence in the literature about maximum efficiencies of PSII in tepals or petals of ornamental flowers, but results obtained for lily tepals were consistent with other studies of *Lilium longiflorum* (Juneau, *et al*., 2002) and *Petunia hybrida* (Weiss, *et al*., 1988), where there was a total destruction of PSII and only some activity remained at PSI. These studies show that bud flowers can harvest light and may have a relevant contribution to an initial carbohydrate demand, as it has been shown for flowers of fruity trees (Brazel & Ó'Maoileídigh, 2019). Even though most ornamental flowers like *Lilium* 'Litouwen' are sterile, the process of flower opening entails *de novo* formation and maturation of reproductive structures, which needs a high demand of sucrose efflux from different parts of the plant, at least until structures as the ovaries can be autocatalytic (Podd & Van Staden, 2002). Source to sink relationships are essential in this process, since the initial bud may partially sustain carbohydrate demands, but when there is a higher requirement of carbohydrate resources programmed cell death occurs, so that there is chloroplast disassembly by autophagy to allow nutrient recycling and resource allocation to reproductive structures (van Doorn & Woltering, 2008; Rogers, 2013). It is important to highlight that chloroplasts of 'Litouwen' flowers in **chapter 2** did not convert to chromoplasts, since there was no carotenoid accumulation, and were most likely degraded or converted to elaioplasts serving as lipid reservoirs. Indeed, chlorophyll degradation due to chloroplast disassembling constitutes another factor that contributes to greater photoinhibition and an increased production of ROS due to PSII inactivation, therefore leading to a positive feedback of chloroplast dismantling (Hörtensteiner, 2006).

Phytohormones play a major role in the control of chlorophyll breakdown and nutrient remobilization, since ethylene (in ethylene-sensitive flowers) and ABA (in ethylene-insensitive flowers) promote chlorophyll degradation during flower development (Arrom & Munné-Bosch, 2012b; Scariot, *et al*., 2014), while other hormones like GAs or cytokinins rescue the chlorophyll contents (Saks & Van Staden, 1993; Danaee, *et al*., 2011). In **chapter 2**, treatment of flower buds with Promalin®, a compound based on GAs and benzyladenine led to increased chloroplast retention since F_v/F_m values and chlorophylls remained higher for longer periods, which delayed the process of flower opening. Therefore, phytohormones seem to play an important role controlling the timing of flower opening by the control of the chloroplast status that is dependent on photoinhibition and chlorophyll degradation, and in doing so, establish a certain regulation of the point of no-return proposed by van Doorn & Woltering (2008) that lead to flower senescence.

Likewise, in **chapter 3**, fruits of *Prunus avium* also displayed a massive reduction of chlorophyll contents at the ripening onset, which were further degraded upon the exogenous application of ABA. In this case, ABA plays a fundamental role for fruit development, since sweet cherries are non-climacteric fruits and ABA triggers most metabolic changes taking place during fruit ripening (Shen, *et al*., 2014; Tijero, *et al*., 2016). In this case, there was neither a chloroplast-to-chromoplast transition since no carotenoids were accumulated over ripening. Chlorophyll breakdown is a well-documented process in fruits, since this process is relevant not only during ripening (Jacob-Wilk, *et al*., 1999; Luo, *et al*., 2013; Guyer, *et al*., 2014) but also in postharvest, where fruit re-greening may lead to important economic losses. In **chapter 3**, both chlorophyll *a* and *b* were degraded during sweet cherries ripening, which implied the complete dismantling of chloroplasts that presumably gave rise to phytol moieties that were further employed for tocopherol production. Several years ago, the Arabidopsis chlorophyllases CLH1 and CLH2 were identified (Tsuchiya, *et al*., 1999). However, further characterisation of the activity of these enzymes showed

that localization of these enzymes did not allow the proposed activity of chlorophyll breakdown during senescence (Schenk, *et al*., 2007). In contrast, removal of the central Mg^{2+} cation of chlorophylls in leaves seems to be mediated by the stay green (SGR) protein that leads to the formation of pheophytin a (Shimoda, *et al*., 2016), which by the catalytic action of pheophytin pheophorbide hydrolase (PPH) gives rise to phytol formation (Lira, *et al*., 2016). Nevertheless, this pathway does not seem to be active in fruits, since fruits of *SlPPH*-knockdown tomato plants still could produce phytol from chlorophyll degradation (Lira, *et al*., 2016). In green leaves, a new alternative pathway has also been described to control chlorophyll turnover, in particular by the enzymatic activity of a chlorophyll dephytylase (CLD1) responsible of chlorophyll hydrolysis (Lin, *et al*., 2016). However, there is no information on the potential role of this enzyme to mediate chlorophyll degradation in fruits. Conversion of chlorophyll b to chlorophyll a is mediated in plant leaves by the chlorophyll b reductases from the family non-yellow coloring (NYC), which has been shown to be under control of ABA during senescence (Gao, *et al.*, 2016). However, no information exists on transcriptional activation of these genes during fruit ripening. In **chapter 3** new evidence is provided that, indeed, ABA regulates phytol recycling from chlorophyll breakdown in non-climacteric fruits such as sweet cherries. This is undoubtedly of high interest for future research so that it paves the way to better understanding how ABA influences chlorophyll degradation in fruits during ripening at the molecular level, not only in climacteric but also in non-climacteric fruits.

Altogether, it is evidenced here, with the analysis of leaves, flowers and fruits subjected to environmental or developmental constraints, that chloroplast maintenance is an important feature of plant development and survival, either because it allows leaf recovery after photoinhibitory periods, as it was the case for mastic trees, or because it controls the timing of flower opening and ripening, as it was the case for lily flowers and sweet cherries, respectively.

2. Photoprotective mechanisms involved in plant acclimation and organ development

Avoid and overcome photoinhibition are the two main strategies in plants to balance the amount of harvested light and the energy being used for carbon fixation (Demmig-Adams & Adams, 2018). Nevertheless, underpinning specific strategies in plant photoprotection to overcome certain environmental constraints or to counteract excessive ROS formation in events of chloroplast degradation or transformation, may be essential to determine the susceptibility to external and internal cues that potentially limit plant growth and organ development.

From a vast array of phototoprotective mechanisms, light shielding and thermal dissipation are two of the main strategies to avoid excess energy quanta in the reaction center of photosystems by plants (Pintó-Marijuan & Munné-Bosch, 2014). Screening compounds like anthocyanins located at vacuoles of epidermal cells or mesophyll significantly reduce and modify light arriving to chloroplasts by filtering light of the visible spectra (Brouillard & Dubois, 1977; Ferreira Da Silva, *et al*., 2012; Rusistoni, *et al*., 2013). Because not all plants can produce the whole array of anthocyanin molecules and their chemical structure is conditioned by vacuolar pH, there has been extensive debate on the characteristics that make anthocyanins to be considered as a photoprotective strategy (Gould, 2004). Anthocyanins are partially responsible of the red autumn colours of deciduous plants and in this sense, it has been proposed that photoperiod may induce anthocyanin accumulation when the day-length begin to shorten and there is a shift on the red/far-red light, so that there is not uncontrolled chloroplast dismantling during senescence prior to winter (Feild, *et al*., 2001). In evergreen species, anthocyanins have been shown to accumulate during cold temperatures of the winter periods and also at pre-hardening (Hughes, 2011). In **chapter 1**, anthocyanins showed a dramatic accumulation in *P. lentiscus* plants triggered by cold temperatures, since these antioxidants

began to accumulate when temperatures dropped below 15°C. This increased accumulation of anthocyanins was indeed parallel to that of photoinhibition showing a high correlation. The fact that increased anthocyanin content results in lower F_v/F_m values in *P. lentiscus* plants and others, may be related to a lowered incident light arriving at photosystems, which leads to reduced PSII efficiency that prevents from enhanced ROS production during winter periods under high light and restricts further photoinhibition of PSI (Huner, *et al*., 1993). Moreover, in **chapter 1**, increased accumulation of anthocyanins during winter was recovered after the cold period, an aspect that was also observed in the altitudinal gradient study, where the population at the highest elevation was also the one having the highest anthocyanin content. In this sense, anthocyanin accumulation has been described to be promoted by cold temperatures, while warmer temperatures seem to inhibit their production (Stiles, *et al*., 2007). Likewise, it is important that anthocyanin accumulation restores after winter when there are more favourable conditions, since increased contents of these pigments could be affecting net photosynthesis when they are not preventing the photosynthetic apparatus from excessive light (Gould, 2004). Therefore, the study in mastic trees highlights the role of anthocyanin accumulation when plants are exposed to cold temperatures so that high-energy quanta do not inactivate PSII, which is an additional evidence that anthocyanins have photoprotective functions in plants. Nevertheless, in mastic trees (**chapter 1**), anthocyanins seemed to work in cooperation with other mechanisms located in close vicinity to PSII. For instance, higher DPS of the xanthophyll cycle was found in *P. lentiscus* plants both during winter photoinhibition and at increasing altitude, which agrees with the proposed role of xanthophyll conversion to dissipate energy excess (Bilger & Björkman, 1990). Xanthophylls located at photosynthetic antennae can perform a dual function in photoprotection as they can both dissipate energy by conversion of violaxanthin to zeaxanthin or lutein to lutein epoxide, or they can also prevent ^{3}Chl* formation at the LHCs in events of intense

photoinhibition by directly quenching ^{1}Chl after energy transfer (Mozzo, *et al*., 2008). While the violaxanthin cycle is accountable for the major component of NPQ (qE) in plants that is usually reversible within minutes, lutein preferentially contributes to prevent ^{3}Chl* formation due to the fact that its location in the antenna is closer to chlorophylls than that of violaxanthin (Mozzo, *et al*., 2008; Ruban, *et al*., 2012; Demmig-Adams & Adams, 2018). In other evergreen plants, NPQ has been related to higher photoprotection during winter periods and especially during recovery with warmer temperatures (Verhoeven, *et al*., 1996). In fact, Adams *et al*. (2001) proposed that phosphorylation of D1 protein along with increased pH gradient within thylakoid membranes regulate increased NPQ levels during winters, irrespective if plants could extend their growth during this period. NPQ is considered to protect chloroplasts from excessive light by preventing the lifetime of ^{1}Chl, as well as preventing over-acidification of the chloroplast stroma (Niyogi, 1999). Mutants with impaired NPQ (*npq*), due to mutations in the gene encoding for violaxanthin de-epoxidase, were able to acclimate to chilling stress when other antioxidant mechanisms were present, but were particularly susceptible to excessive light (Havaux & Kloppstech, 2001). A more dramatic phenotype was found when *npq* mutations were combined with a tocopherol deficiency, which led to marked photoinhibition in PSII and highlighted the photoprotective relevance of tocopherols and the compensatory effects of different photoprotective strategies. (Havaux, *et al*., 2005). Tocopherols, together with β–carotene, are the main antioxidants protecting PSII from $^{1}O_2$ formation (Triantaphylidès, *et al*., 2008). In mastic trees (**chapter 1**), during winter phtotoinhibition, β- carotene production was not enhanced. In contrast, tocopherol levels arose with lower temperatures and was especially relevant in an altitudinal gradient, where *P. lentiscus* showed up to 50% higher α-tocopherol content at the highest altitude of 740m a.s.l. than in the other two populations. Indeed, even though single mutations of tocopherols like *vte1* or *vte2* only lead to mid sensitivity to high light (Porfirova *et al*., 1999), α-tocopherol has been specifically linked to

plant photoprotection and acclimation to abiotic stresses (Havaux *et al.*, 2005). The production of this lipophilic antioxidant during events leading to high light and PSII inactivation is rather well documented in the literature, so that increased tocopherol production entails an adaptive advantage to sustain physiological functions in events of abiotic stress (Munné-Bosch, 2005). Moreover, the production of α-tocopherol has also been related to the altitudinal distribution and the susceptibility to stress in the plant *S. longifolia*, which is adapted to live at high altitudes and where higher levels of tocopherols were also found at the highest population (Munné-Bosch, *et al*., 2016).

To allow plant acclimation to abiotic stresses it is important an intense communication (retrograde signalling) between chloroplasts and the nuclei, since chloroplast genome mostly encodes for proteins related to the photosynthetic machinery, while the nuclei harbours stress-responsive genes (Kaul, *et al*., 2000). Growing evidence shows that ROS derived from chloroplasts during photoinhibitory processes activate intracellular signalling, either by specific cell receptors or through redox sensitive compounds, to induce nuclear gene expression (Dietz, *et al*., 2016; Foyer, 2018). During abiotic stresses, retrograde signalling requires a fine-tuned production of ROS emaning from the thylakoid structures so that their production does not surpass the antioxidant capacity and leads to uncontrolled photo-oxidative damage (Foyer, 2018). In this sense, tocopherol production in *P. lentiscus* may be controlling the extent of $^1O_2$ produced from PSII firstly to avoid excessive photodamage and secondly to mediate acclimation by retrograde signalling (Muñoz & Munné-Bosch, 2019). In fact, this process of retrograde control is not only limited to the capacity of tocopherols to limit ROS production, but recently it has also been found that tocopherol contents might affect the production of miRNA and regulate their function by modulating the accumulation of 3′-phosphoadenosine 5′-phosphate (PAP), which protects miRNA from being degraded (Fang, *et al*., 2019).

Considering all results from the study of *P. lentiscus* and winter photoinhibition, it is shown the great complexity in the mechanisms conferring photoprotection. Therefore, leaves subjected to events of PSII inactivation require the activation and coordination of multiple photoprotective strategies that cover from light avoidance to specific quenching and scavenging of ROS. The production of molecules assisting in plant photoprotection show a higher specificity for their activation, since their function is transitory and is usually time-dependent to photoinhibition events. The fact that photoprotective mechanisms are only activated when environmental conditions compromise chloroplast integrity reinforce the idea proposed in the previous section that chloroplasts have a fundamental role during stress acclimation and maintenance of this organelle is essential so that the plant can return to normal physiological conditions when there are more favourable conditions for its growth. In order to control chloroplast maintenance, antioxidant compounds have a primordial role regulating redox balance and allowing communication between chloroplasts and the nucleus, so that stress-related signals are activated.

Results in leaves contrast with those obtained for flowers and fruits, mainly because there is no recovery of chloroplasts after photoinhibition in these organs. This feature makes that accumulation of photoprotective molecules in flowers and fruits mostly related to their antioxidant function *per se* rather than the potential to promote photoprotection of reaction centers. Nevertheless, during development of these structures, photoprotection is also relevant especially when chlorophylls begin to degrade, so that there is no uncontrolled production of ROS that could lead to premature photodamage of organ structures and compromise their development. For both flowers (**chapter 2**) and fruits (**chapter 3**) chlorophyll loss at initial stages of development was accompanied by a reduction in the content of carotenoids, which did not accumulate in chromoplasts structures and were shown to promote *de novo* biosynthesis of ABA, as evidenced by higher contents of this phytohormone during fruit development, as well as shown

by higher expression of *PacNCED* of control-treated fruits at the moment of ripening onset. In contrast, tocopherol production was enhanced both at flower opening and at fruit maturation, coincident with higher chlorophyll breakdown.

The newly completed pathway of phytol recycling for the production of phytol-DP for tocopherol biosynthesis from chlorophyll degradation, has also shed light on the vitamin E function in photosynthetic organisms, since it has connected the relevant role of tocochromanols in protecting from oxidative stress to events leading to chlorophyll breakdown and the significance for chloroplast integrity (Muñoz & Munné-Bosch, 2019). In both the flower and fruit models (**chapter 2** & **3**) tocopherol accumulation started at crucial stages of organ development, where chloroplasts are more sensitive because they are reducing the amount of chlorophyll that can lead to enhanced photo-oxidative stress (Hörtensteiner, 2006). Therefore, tocopherol production at these stages seems to assist in photoprotection to the initial stages of chlorophyll degradation to avoid uncontrolled oxidative burst. Tocopherol production in fruits has been described to be controlled by light, through the PHYTOCHROME-INTERACTING FACTOR 3 (SlPIF3) that regulates transcriptional repression of *GERANYLGERANYL DIPHOSPHATE REDUCTASE (SlGGDR)* and that is involved in tocopherol biosynthesis (Gramegna, *et al*., 2019). Cys-elements of tocopherol-related genes have also been identified for *p-hydroxyphenylpyruvate dioxygenase* (*MiHPPD*), where ethylene seems to play a role controlling its transcription in the ripening of mangoes (Singh, *et al*., 2011). Nevertheless, no elicitors have been described for enzymes related to the phytol recycling pathway. Since it was essential to understand how tocopherol production was regulated in organs where there is massive chlorophyll breakdown, in **chapter 3** ABA was evaluated as a promoter phytohormone for the tocopherol biosynthesis from chlorophyll recycling in sweet cherry fruits, based that ABA is the main phytohormone leading the ripening process and that a strong correlation was previously found between ABA contents and

tocopherol accumulation in *Prunus avium* (Tijero, *et al*., 2016). Exogenous application of ABA prior to the ripening onset of cherry fruits led to transient increases in ABA contents in the fruit and a higher accumulation of anthocyanins (Shen, *et al*., 2014; Oh, *et al*., 2018), as it was described in several other experiments. More interestingly, exogenous ABA triggered both the production of α- and γ-tocopherol while chlorophyll degradation was also immediately enhanced. Moreover, ABA specifically induced the transcription of the phytol kinase (*PacVTE5*) and the phytyl phosphate kinase (*PacVTE6*). These results indicate that exogenous ABA promoted the accumulation of tocopherols in cherry fruits by the activation of the phytol recycling pathway from the chlorophyll breakdown, which could be also the mechanism of natural accumulation of tocopherol contents in sweet cherries. This is of paramount importance since sweet cherries do not naturally accumulate tocopherols in large amounts and could help to design new strategies to increment vitamin E contents in plants, which may have beneficial health properties for human consumption (Khadangi & Azzi, 2019). Moreover, a similar pathway could be happening also in lily flowers, whose development is known to be regulated by ABA (Arrom & Munné-Bosch, 2012b), and in so, biofortification of tocopherol in *Lilium* varieties dependent on this phytohormone could promote increased vase-life. There is evidence that tocopherol accumulation takes place in plastoglobules and lipid membranes of chloroplasts and chromoplasts of leaves and fruits (Arango & Heise, 1998; Ytterberg, *et al*., 2006). However, it is important to note that no chromoplasts were being differenciated in sweet cherries, therefore tocopherol production promoted by ABA is likely to be taking place in plastoglobules released from chloroplasts following chloroplast dismantling, as it has been evidenced for senescing chloroplasts that exude lipid-rich bodies from gerontoplasts to the cytosol (Liu, 2016), although this requires further validation in further experiments.

Considering all photo- and antioxidant protective molecules accumulated under abiotic stress and developmental processes, it is shown

that plants activate photoprotective mechanisms to try to preserve the integrity of chloroplasts, as it was seen in mastic trees where different photoprotective strategies were activated in coordination so that plants can overcome environmental constraints, while degradation of these chloroplasts also requires higher photoprotection in developmental processes, as it is shown in flower opening and fruit ripening onset where the production of antioxidants was coincident to the point of chlorophyll degradation, which indeed promotes the accumulation of potent antioxidant molecules like tocopherols.

3. Factors affecting the extent of photo-oxidative stress in leaves, flowers and fruits

Production of ROS has long been related to redox unbalance and higher photo-oxidative stress in plants (Foyer & Noctor, 2000; Gill & Nuteja, 2010; Muñoz & Munné-Bosch, 2018). In nature, the oxidative status of chloroplasts is easily disturbed by environmental factors such as high light or temperatures, leading to altered redox status of plant cells (Foyer & Noctor, 2000). In that line, sensing redox status of chloroplasts is essential to trigger genetic responses so that plants are able to acclimate to changing conditions (Plannschmidt *et al*., 2001). One of the main components of the ROS signalling transduction is the activation of redox susceptible molecules that have longer viability than ROS themselves (Foyer & Noctor, 2015). For a long time, photo-oxidative stress was believed to compromise plant survival due to irreversible damage of cellular components (Schraudner, *et al*., 1997; Rawyler, *et al*., 2002). At present there is new evidence conferring important signalling roles to compounds derived from the ROS action, as it is the case of β-cyclocitral from β-carotene oxidation (D'Alessandro & Havaux, 2019) or compounds derived from lipid peroxidation such as LOOH or oxylipins (Weber, *et al*., 2004; Kopriva, 2013). While LOOH are primary products of lipid peroxidation with relative instability due to rapid conversion, oxylipins, including jasmonates and MDA, are secondary products with

longer viability in plant cells and in this sense, secondary peroxidation products are potential molecules to mediate the retrograde signalling pathway (Morales & Munné-Bosch, 2019). Low molecular antioxidant systems like tocopherols specifically regulate the extent of lipid peroxidation by (i) reducing ROS levels and (ii) preventing the formation of lipid peroxyl radicals that could further contribute to lipid peroxidation chain reactions (Schneider, 2005).

In **chapter 1**, lipid peroxidation was particularly relevant when sex and year of shoot development were considered, being MDA the one with a particular role in plant acclimation. In that matter, sexual dimorphism of dioecious plants has been related to differences in reproductive costs, which involves morphological and physiological differences between male and female plants (Juvany & Munné-Bosch, 2015; Tonnabel, *et al*., 2017). Indeed, some studies have evidenced that female plants show higher sensitivity to adverse conditions due to highest investment and resource allocation (Han, *et al*., 2013; Simancas, *et al*., 2016). With this regard, female and male plants of mastic trees were evaluated at three points of the seasonal study where chloroplast integrity could be compromised, including February (when it was the coldest month), May (coincident with the flowering period for both males and females) and August (when it was the highest fruiting period for females). The analysis at these time points showed that females had a higher sensitivity than males, including months where they were not investing in higher reproductive efforts. Sensitivity was not shown by decreased F_v/F_m ratios but with higher contents of MDA. Even though contents of β-carotene per chlorophyll unit were lower for female plants in the fruiting period, so that plants could be more susceptible to environmental constraints, increased MDA levels did not compromise in any case leaf survival. Moreover, females also showed higher chlorophyll contents and lowest chlorophyll a/b ratio during fruiting, which could be associated to higher photosynthetic rates to meet carbon demands for fruit development (Jonasson, *et al*., 1997; Seyedi, , *et al*., 2019).

When leaves from shoots developed from different years were analysed in *P. lentiscus* plants (**chapter 1**), a significative interaction between MDA production and leaf phenology was found. While mastic trees do not suffer leaf abscission prior to the winter periods, their duration on-tree is of around one year (Jonasson, *et al*., 1997; Munné-Bosch & Peñuelas, 2003). During the experiment of winter photoinhibition (**chapter 1**), leaves did not show higher increases on the total amount of lipid hydropoeroxides that indeed, were reduced during cold periods. On the contrary, MDA levels progressively increased during the cold season and even though detoxificating molecules like tocopherols could have prevented their formation, MDA levels only slightly decreased when warmer temperatures were restored to later increase in the flowering period, achieving maximum values prior to leaf abscision. Nevertheless, formation of new shoots allowed the system to restore at the antioxidant and peroxidation level. It is important to highlight that even though tocopherols could not prevent MDA formation, their contents increased parallel to MDA and what it is more, leaves did not abscise until new shoots were fully mature. This scenario is consistent with the idea that MDA levels may be involved in retrograde siganling to facilitate chloroplast dismantling and nutrient remobilisation taking place at the latest stages of development prior to senescence (García-Plazaola & Becerril, 2001; Munné-Bosch & Peñuelas, 2003), an aspect that requires further investigation.

Similarly, in flowers and fruits (**chapter 2** & **3**) a progressive increase in lipid peroxidation was found over the course of organ development as evidenced by increases in LOOH and MDA. It is important to note that even though there was a higher oxidative environment, both organs had a normal progress of their development and their senescence did not occur until many days later since the beginning of oxidative stress. Indeed, flowers and fruits follow programmed cell death that is dependent not only in ROS production, but also in the source/sink relationship and metabolic and molecular networks (Rogers, 2013). Moreover, it is important to highlight that at the

extent of lipid peroxidation during flower opening in **chapter 2** conditioned the timing and progress of the process. Increased levels of both LOOH and MDA were detected at opening but important reductions of MDA were found in flowers treated with Promalin® at anthesis, which resulted in a delay on the timing of flower opening. Such reductions of MDA were preceded by increased levels of LOOH and jasmonic acid (JA) contents in Promalin®-treated flowers at the begining of the experiment, which therefore suggest a positive regulatory role of both LOOH and JA derived from lipid peroxidation. JA is a phytohormone that primary mediates plant immune responses (Nomura, *et al.*, 2012). Nevertheless, the signaling network of JA is also involved in acclimation to high light and JA is produced in these conditions by the enzimatic catabolism of lipid membranes (Alsharafa, *et al*., 2014; Tikkanen, *et al*., 2014a). Moreover, antioxidant compounds like tocopherols have been identified as compounds that can mediate the production of JA by preventing the extent of lipid peroxidation and that under some circumstances a trade-off between JA production and tocopherol accumulation occurs (Munné-Bosch, *et al*., 2007). Results obtained in **chapter 2** also showed a role for JA in controlling the timing of flower opening in response to GAs and cytokinins application, which led to lower levels of MDA. This suggests that a signalling network exists between phytohormones and the extent of photo-oxidative stress in plant cells. This idea is further supported by increased levels of lipid hydroperoxides in cherry fruits as a response to exogenous application of ABA in **chapter 3**. Interplay between phytohormones and ROS is of paramount importance in chloroplast signalling to regulate growth and development, as it has been evidenced by the experiments that have been performed here in leaves, flowers and fruits. Other experiments also support the idea that ROS may act as secondary messengers in hormonal signalling, even though the specific mechanisms that facilitate ROS or redox regulation of plant growth and development has not been elucidated (Xia, *et al*., 2015). Likewise, the nature of retrograde signalling by secondary lipid peroxidation products like

MDA is still elusive, even though has been proven to induce genetic transcription (Weber, 2004) and therefore might be an important component of the chloroplast retrograde signalling. In so, further research should focus on both the interaction between phytohotmones and ROS, as well as the relevance of secondary lipid peroxidation products in acclimation and organ development at the molecular level.

4. Ecophysiological and biotechnological applications of the responses to photo-oxidative stress

Geographic distribution of plant species is conditioned by multiple factors, including climate, topography, soil, and also other biological factors like plant competition and physiological traits that allow plants to establish in specific niches (Fenollosa & Munné-Bosch, 2019; Willy & Van Burskirk, 2019). In **chapter 1** *P. lentiscus* where particularly sensitive to cold conditions not only during winter but also in altitude, which evidence that specific environmental conditions trigger a physiological response that can potentially constraint its distribution. Ecophysiological studies that include specific physiological parameters with higher sensitivity to specific abiotic stresses may constitute a powerful tool to create distribution models and better understand competitiveness of a plant species.

Moreover, from the analysis of both flowers and fruits it has been evidenced that the regulation of chloroplast integrity by the interplay of phytohormones and photo-oxidative stress is essential for the development of new commercial products. For instance, Promalin®, whose commercialisation was originally intended to promote fruit set in apples and pear trees, has been proven to promote a number of other developmental processes and it is currently widely used in agriculture and horticulture. Understanding the specific responses of secondary peroxidation signals could also promote the production of new commercial products with applications in the agronomy sector but to do so, it is important to identify specific redox processes taking place during organ development. From the

experiment of lily flowers (**chapter 2**) it was essential to identify that chloroplast integrity retention strongly influences the timing of flower opening and new biotechnological applications could be drawn to this direction, since the horticultural sector might be interested in changing the timing of flower opening, not only to ornamental flowers, but also for food production since off-season products offer better economical profits (Kuchler & Arnade, 2016). Moreover, understanding which environments could naturally regulate these processes could be essential in a future trend of agriculture that demands food production with the employment of less chemical products.

On the other hand, the rising demand of food with added healthy properties require a better understanding of the processes that lead to better quality products in fruits and vegetables, so that it can meet consumer's expectations. In this line, production of food with higher antioxidant properties will satisfy the necessity required by customers, as well as the possibility to extend the shelf-life of this commodities due to better physiological properties. However, to do so it is particularly relevant to understand metabolic pathways and possible elicitors that directly regulate the production of antioxidants in plants, so that other than genetic modifications, specific products or techniques can be applied for their implementation. One example could be considered the **chapter 3** where the identification of the promotion of phytol recycling by the phytohormone that oversees its development could have important biotechnological applications.

CONCLUSIONS

CONCLUSIONS

- Mastic trees display strong photoinhibition at cold temperatures during overwintering and at high elevations that does not compromise shoot survival, at least in part because of the activation of photoprotective mechanisms of light shielding through anthocyanins, along with higher DPS and increased tocopherol content, particularly that of α-tocopherol, that might help provide an adequate oxidative balance in acclimation.
- Females of mastic trees show higher sensitivity to stressful environmental conditions as indicated by enhanced production of MDA, even though no reductions of F_v/F_m were registered, which emphasizes the high adaptation of female plants to withstand photo-oxidative stress. On the other hand, the analysis of natural populations of *P. lentiscus* plants puts in relevance the importance of shoot renewal on evergreen long-lived plants as a mechanism to fully restore the system.
- The extent of photoinhibition, the capacity of photo- and antioxidant protection and the production of primary and secondary products of lipid peroxidation is finely controlled in a time-dependent manner during tepal de-greening and flower opening in lilies.
- Enhanced photoinhibition and photo-oxidative stress occurs after the start of tepal de-greening and just prior to anthesis. In that manner, Promalin® may exert a delaying effect on flower opening through a crosstalk between hormones and lipid peroxidation products in lilies.
- During ripening of sweet cherries “Prime Giant”, ABA stimulates vitamin E accumulation through phytol recycling from chlorophyll degradation, which is shown to be an active pathway for vitamin E biosynthesis. In this pathway, transcription of *PacVTE6* appears to be rate limiting and consequently, a potential target for vitamin E fortification in cherry fruits.
- The study of diverse plant organs in different species highlights the relevance of keeping or dismantling chloroplasts in processes of acclimation and development through the activation of photoprotective strategies and specific hormonal pathways.

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ANNEX I

Annex I

Vitamin E in Plants: Biosynthesis, Transport, and Function

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Opinion

Vitamin E in Plants: Biosynthesis, Transport, and Function

Paula Muñoz[1] and Sergi Munné-Bosch[1,*]

Vitamin E, which includes both tocopherols and tocotrienols, comprises lipid-soluble antioxidants that modulate lipid peroxidation. Recently, significant advances have been made in our understanding of vitamin E biosynthesis, transport, and function. The phytyl moiety from chlorophyll degradation is used for tocopherol biosynthesis. An α-tocopherol-binding protein (TBP) has been identified in tomato (SlTBP) serving in intraorganellar vitamin E transport in plants. Moreover, α-tocopherol not only scavenges free radicals through flip-flop movements in the lipid bilayer, but may also contribute to fine-tuning the transmission of specific signals outside chloroplasts. Vitamin E, and α-tocopherol in particular, appear to be essential for plant development and help to provide the most suitable response to a number of environmental stresses.

Vitamin E in Plants: Tocopherols and Tocotrienols

Almost a century has passed since the discovery of a dietary factor essential for reproduction in mice [1]. This **vitamin** (Glossary) was named '**tocopherol**' from the Greek words τόκος (*tókos*, birth), and φέρειν (*phérein*, to bear or carry) meaning 'to carry a pregnancy', with the ending '-ol' related to its chemical alcohol structure. Although the eight different molecular forms of **vitamin E**, four tocopherols and four **tocotrienols**, are potent **antioxidants**, the human body tends to accumulate α-tocopherol due to the activity of the liver **α-tocopherol transfer protein (α-TTP)**, which enriches plasma with α-tocopherol [2]. Besides α-TTP, which resides only in the liver, a system of **tocopherol-binding proteins (TBPs)** leads to the localization of tocopherols in various human tissues where they are required [3]. α-TTP has not been identified in the plant kingdom, but recently a TBP has been identified in tomato [4]. Living under relatively high-oxygen conditions has made plants and animals evolve complex, but also fascinating, mechanisms of antioxidant protection [5,6], including the production (plants) or intake (animals) of vitamin E [7,8]. Living in a high-oxygen environment can result in inactivation of electron transport processes (e.g., **photoinhibition** of photosynthesis in plants, dysfunction of mitochondrial electron transport in both plants and animals) if production of **reactive oxygen species (ROS)** is not counterbalanced adequately. Sustained production of ROS can be harmful to cells because they can damage proteins, lipids, and nucleic acids; but when they accumulate transiently, they participate in **retrograde signaling**, which in turn is essential in mediating both developmental and acclimation processes [9,10].

All of the eight lipid-soluble antioxidants that constitute vitamin E owe their chemical structure to a polar chromanol head linked to a hydrophobic prenyl tail (tocopherols with a completely saturated prenyl tail and tocotrienols with three unsaturated carbons, at 3′, 7′, and 11′). Both tocopherols and tocotrienols can be divided into α, β, γ, and δ forms, depending on the number and position of the methyl groups on their chromanol head. Slight differences in these chemical structures strongly influence their antioxidant activity in various systems [11] and therefore their presence and accumulation in the plant kingdom [7,12]. Although α-tocopherol is universally distributed in the plant kingdom and is the predominant vitamin E form in photosynthetic tissues, γ-tocopherol and tocotrienols predominate in the seeds of several dicots and monocots, respectively [7,12,13]. Due to their capacity to (physically) quench and (chemically) scavenge singlet oxygen ($^1O_2$) and regulate the extent of lipid peroxidation in chloroplasts, some vitamin E forms (α-tocopherol in particular) have been shown to play an antioxidant role not only in leaves [14] but also in some fruits [15] and flowers [16,17]. Significant advances have been made over the past 5 years in the biophysics [18], biochemistry [19–21], and understanding of the function [22,23] of vitamin E in plants. Here, we aim to discuss the biological significance of these recent findings in relation to other key achievements made in this field previously, while providing new avenues for future research.

Highlights

α-Tocopherol is a lipophilic antioxidant synthesized from the methyl-erythritol and shikimate pathways in chloroplasts that modulate the extent of lipid peroxidation in plants.

Increased α-tocopherol content, which is both biochemically and functionally related to chlorophyll loss, improves photoprotection.

The recent discovery of an α-tocopherol-binding protein sheds light on intraorganellar prenyllipid transport in plants.

High α-tocopherol content in some nonphotosynthetic tissues poses intriguing questions about the biosynthesis, transport, and function of vitamin E in plants.

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Vitamin E Biosynthesis: Novel Insights

Vitamin E biosynthesis occurs at the inner envelope of plastids via a combination of two main pathways (Figure 1). The shikimate pathway gives rise to the chromanol ring from homogentisate (HGA). Meanwhile, the methylerytrithol phosphate (MEP) pathway provides the prenyl tail from geranylgeranyl diphosphate (GGDP) and phytyl diphosphate (phytyl-DP) for the synthesis of tocotrienol and tocopherol, respectively. An additional pathway for phytyl-DP production from chlorophyll degradation, also known as the phytol recycling pathway (Figure 2), was discovered almost 15 years ago after the identification and characterization of the arabidopsis (*Arabidopsis thaliana*) *vitamin E pathway gene 5-1* (*vte5-1*) [24]. Seeds and leaves of *vte5-1* mutants showed 80% and 65% reductions in total tocopherol content, respectively, compared with the wild type. *vte5-1* mutants were deficient in the phytol kinase, named VTE5, responsible for phytol phosphorylation in the first of a two-step reaction leading to phytyl-DP formation [24]. This alternative metabolic route, allowing phytol recycling (Figure 2), was recently completed in arabidopsis with the characterization of the phytyl-phosphate kinase VTE6, which catalyzes the second-step phytol phosphorylation [19]. This phytol recycling pathway has also been reported in tomato (*Lycopersicon esculentum*) plants, where vte5-silenced plants (*SlVTE5*) showed lower tocopherol content than the wild type in both leaves and fruits [20]. It is interesting to consider that chlorophyll degradation occurs not only in plant responses to environmental **stresses** but also during several developmental processes, such as leaf senescence or seed maturation, as well as during the ripening of several fruits and the senescence of some flowers [15,17]. Several studies have shown a strong correlation between chlorophyll loss and tocopherol production during these physiological processes [15,17,25,26]. Chlorophyll synthase and geranylgeranyl diphosphate reductase (GGDR) are also involved in vitamin E biosynthesis, mediating the production of chlorophyll linked to a phytyl moiety ($chlorophyll_{phy}$), from which phytol is released during chlorophyll breakdown [27]. The identity of the enzymes involved in chlorophyll dephytylation is less clear and while pheophytinase has been proposed as the enzyme that catalyzes chlorophyll breakdown in senescent leaves, other hydrolases such as CLD1 may allow phytol remobilization during fruit ripening and seed maturation [28,29].

Tocotrienols differ from tocopherols not only in their chemical structure but also in that they have a unique distribution in particular tissues (e.g., seeds [12], latex [30]) and plant species (e.g., some clades of the palm family [12]), although in several other examples they are found together with tocopherols, particularly in seeds [12,31,32]. Their uneven distribution in the plant kingdom and the fact that tocotrienols do not naturally substitute tocopherols suggest that, while tocopherols are essential compounds that are distributed throughout the entire plant kingdom universally, tocotrienols function as secondary metabolites. Although the differential production of tocopherol and tocotrienol forms is complex and strongly specific to both tissue and species, the biosynthetic pathway for tocotrienol production has mostly been characterized [33]. However, emerging studies on this topic exemplify its complexity and the importance of plant diversity and uniqueness in determining tocotrienol/tocopherol production. A complete characterization of vitamin E biosynthesis in rice (*Oryza sativum*) has recently allowed the identification of an additional geranylgeranyl reductase (OsGGR2 [21]) that is not present in arabidopsis or *Synechocystis* sp., which have only one *GGR* gene each. Since geranylgeranyl reductase is responsible for the formation either of phytyl-DP from GGDP or of chlorophyll from geranylgeranyl chlorophyll (Figure 2), rice callus with inactivated OSGGR1 and OsGGR2 produces tocotrienols but not tocopherols. This offers strong potential as a resource for the production of pure tocotrienol with nutraceutical applications [21]. Furthermore, a better understanding of tocotrienol biosynthesis will have important implications in ecology. The differential production of tocopherols and tocotrienols, which is mainly regulated by the activity of HGA geranylgeranyl transferase and HGA phytyl transferase, respectively [33,34], has been suggested to be modulated through jasmonic acid-mediated upregulation of the vitamin E biosynthesis flux towards tocopherol instead of tocotrienol accumulation during seed germination in dwarf palms [35].

Vitamin E Transport: Evidence and Biological Implications

α-Tocopherol is synthesized by the subsequent action of enzymes located in both the inner membrane of the chloroplast envelope and plastoglobuli (Figure 3). The latter location is where

Glossary

α-Tocopherol transfer protein (α-TTP): human liver protein that enriches the plasma with α-tocopherol.
Antioxidant: a molecule with a low reduction potential that can donate electrons or hydrogen atoms thereby preventing the oxidation of other molecules.
Photoinhibition: reduction of photosystem efficiency caused by excess excitation energy in the electron transport chain.
Photo-oxidative stress: physiological condition related to increased production of ROS under excess light in chloroplasts.
Photoprotection: mechanisms to avoid, reduce, or tolerate excess excitation energy in chloroplasts.
Reactive oxygen species (ROS): produced from aerobic metabolism, including superoxide anions (O_2^{-}), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^{-}), and singlet oxygen ($^1O_2$).
Retrograde signaling: chloroplast- or mitochondria-to-nucleus signaling.
Stress: physiological condition by which a stress factor (see below) limits growth at the cellular, tissue/organ, or organism level.
Stress factor: any condition, either biotic or abiotic, that limits growth at the cellular, tissue/organ, or organism level.
Tocopherol: molecular form of vitamin E comprising a chromanol ring and a saturated prenyl tail.
Tocopherol-binding protein (TBP): protein that transports tocopherol and other prenyllipid molecules, favoring the localization of tocopherols at various chloroplast compartments.
Tocotrienol: molecular form of vitamin E comprising a chromanol ring and a prenyl tail with three unsaturations at 3′, 7′, and 11′.
Vitamin: any of various fat-soluble or water-soluble organic substances essential in minute amounts for correct growth and development that cannot be synthesized in the organism for which they are 'vital'.
Vitamin E: lipophilic antioxidant essential for human and animal development but synthesized only by photosynthetic organisms (with the exception of the malaria parasite *Plasmodium falciparum*, the latter harboring a remnant chloroplast from its algal ancestor).

some enzymes in the biosynthesis pathway, such as VTE1, and NAD(P)H-dependent quinone oxidoreductase (NDC1), which is involved in the recycling of tocopherol quinone, are anchored [36]. While some key enzymes involved in the biosynthesis of vitamin E precursors, such as tyrosine aminotransferase (TAT) and 4-hydroxyphenylpyruvate dioxygenase (HPPD) (Figure 1), can be allocated either in the cytoplasm or in plastids of plant cells [37], all of the enzymes catalyzing the final steps of tocopherol and tocotrienol production are found in plastids. Consequently, plastids have classically been considered the unique subcellular localization of vitamin E in plant cells.

However, the recent discovery of SITBP in tomato plants, found not only in source and sink leaves but also in fruits [4], may challenge our previous views on the limited subcellular location of vitamin E in plants. Earlier studies had already suggested that tocopherols may be located not only in leaf chloroplasts and seed plastids, but also in seed lipid bodies [12,38] and in the vacuoles and nuclei of

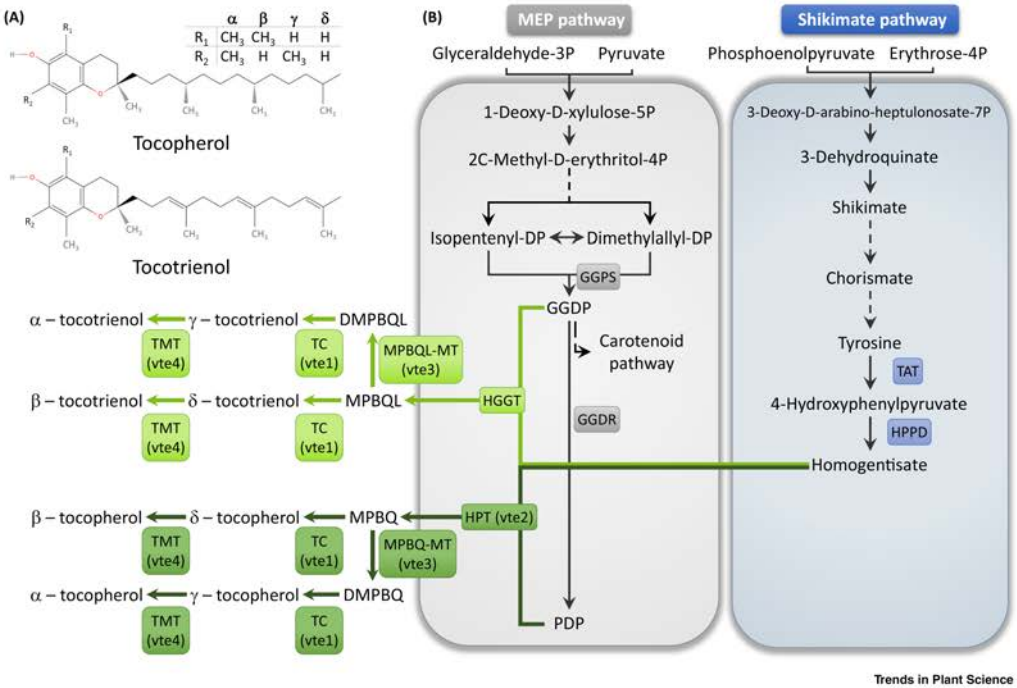

Figure 1. Vitamin E Chemical Structure and Biosynthesis in Plants.

(A) Vitamin E chemical structure. A chromanol head and a prenyl tail constitute the chemical structure of tocopherols and tocotrienols. While tocopherols have a saturated tail, tocochromonals have three unsaturations (orange lines), at 3′, 7′, and 11′. α, β, γ, and δ homologs differ in the number and position of methyl groups in R1 and R2. (B) Biosynthesis of tocopherols and tocotrienols in plants. Tocopherols and tocotrienols are formed from the combination of the methylerythritol phosphate and shikimate pathways. Abbreviations: DMGGBQ, dimethylgeranylgeranylbenzoquinol; DMPBQ, dimethylphytylbenzoquinol; GGDP, geranylgeranyl diphosphate; GGDR, geranylgeranyl diphosphate reductase; GGPS, geranylgeranyl diphosphate synthase; HGGT, homogentisate geranylgeranyl transferase; HPPD, hydroxyphenylpyruvate dioxygenase; HPT, homogentisate phytyl transferase; MEP, methylerythritol phosphate; MGGBQ, methylgeranylgeranylbenzoquinol; MPBQ, methylphytylbenzoquinol; MPBQ-MT, MPBQ methyltransferase; PDP, phytyl diphosphate; TAT, tyrosine aminotransferase; TC, tocopherol cyclase; TMT, tocopherol methyltransferase.

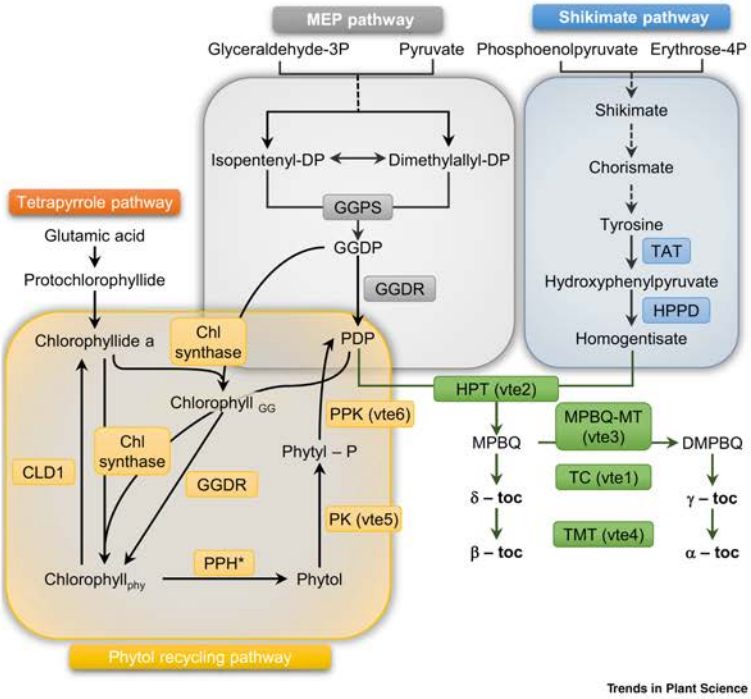

Figure 2. Linking Tocopherol Biosynthesis with Chlorophyll Degradation in Plants.
The phytol recycling pathway is essential to provide phytol for tocopherol biosynthesis when the methylerythritol phosphate (MEP) pathway may be limiting. The asterisk indicates that pheophytin pheophorbide hydrolase (PPH) is an essential component of the chlorophyll breakdown machinery of senescent leaves leading to tocopherol production by the subsequent action of phytyl kinase (PK) and phytyl phosphate kinase (PPK). Other hydrolases may, however, contribute to chlorophyll dephytylation during fruit ripening and seed maturation. Abbreviations: CLD1, chlorophyll dephytylase 1; DMPBQ, dimethylphytylbenzoquinol; GGDR, geranylgeranyl diphosphate reductase; GGPS, geranylgeranyl diphosphate synthase; HPPD, hydroxyphenylpyruvate dioxygenase; HPT, homogentisate phytyl transferase; MEP, methylerythritol phosphate; MPBQ, methylphytylbenzoquinol; MPBQ-MT, MPBQ methyltransferase; PDP, phytyl diphosphate; TAT, tyrosine aminotransferase; TC, tocopherol cyclase; TMT, tocopherol methyltransferase.

mesophyll cells [39]. Moreover, although tocopherols have been mostly related to photosynthetic tissues, accumulation of both tocopherols and tocotrienols exists in several nonphotosynthetic tissues. From these nonphotosynthetic tissues, seeds accumulate the largest amounts of vitamin E, including a diverse composition in their forms [35,40], while fruits [15,20,41], flowers [16,17], and tubers [42,43] store α- and, to a lesser extent, γ-tocopherol. Some vitamin E-related genes have been identified in these nonphotosynthetic organs, especially in seeds where almost the full biosynthetic pathway has been described. However, there are still some questions regarding how either vitamin E forms or their precursors can be synthesized in nonchloroplastic tissues and several attempts at vitamin E biofortification in these tissues have failed because of physiological constraints [29,43]. Although the tomato SITBP protein targets chloroplasts and TBPs serve to conduct tocopherols and other prenyllipids between the plastid compartments in plants [4], more research is needed to study other

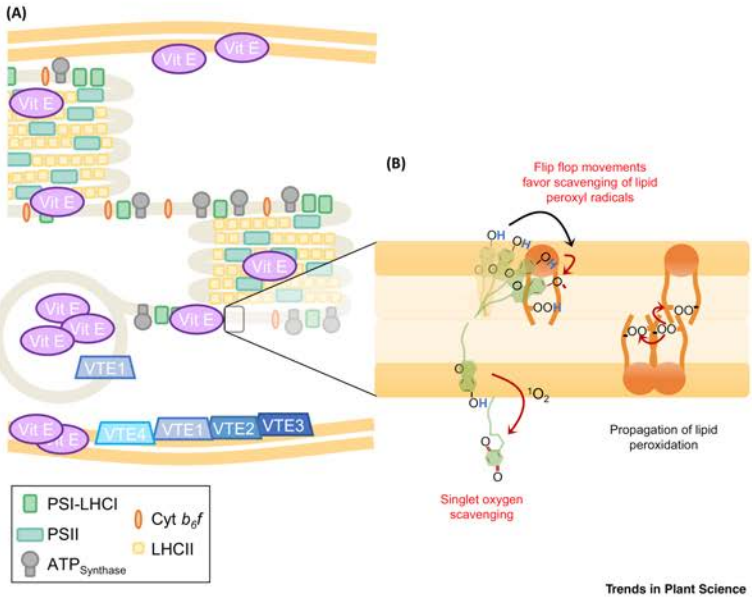

Figure 3. Subcellular Location and Antioxidant Role of Vitamin E in Plants.
(A) Vitamin E is synthesized in the chloroplasts. Although several enzymes involved in the synthesis of tocopherols have been localized at the inner membrane of the chloroplast envelope, others are found in plastoglobuli. (B) Antioxidant role of tocopherols in the lipid bilayer. Flip-flops of tocopherols (green structures) are intimately related to the trapping of radicals (-OO$^{\cdot}$) near the lipid–water interface, allowing the lipid peroxyl radicals to be efficiently removed. This process is essential to avoid the propagation of lipid peroxidation by the peroxyl neighborhood. Tocopherols can also efficiently scavenge reactive oxygen species (ROS) like singlet oxygen ($^1O_2$). Abbreviations: Cyt b_6f, cytochrome b_6f complex; LHCII, light-harvesting complex of photosystem II; PSI-LHCI, photosystem I–light-harvesting complex of photosystem I; PSII, photosystem II; Vit E, vitamin E; VTE1, VITAMIN E 1 or tocopherol cyclase; VTE2, VITAMIN E 2 or homogentisate phytyltransferase; VTE3, VITAMIN E 3 or methylphytylbenzoquinol methyltransferase; VTE4, VITAMIN E 4 or tocopherol methyltransferase.

TBPs that may serve in interorganellar transport. In this respect, it will be interesting to examine the possibility of vitamin E in the interface between chloroplasts and endoplasmic reticulum (ER) membranes, thereby affecting interorganellar lipid metabolism [4], as it has been shown that tocopherols could be accessed by enzymes in the ER using transorganellar complementation studies [44]. Interestingly, using a GFP fusion protein expression system in mammalian cells, it was observed that some TBPs translocate from cytosol to nuclei in an α-tocopherol-dependent fashion [45]; thus, putative interorganellar vitamin E trafficking systems should be studied in plants.

SITBP, which shows an overall similarity of 24.8% with human α-TTP and harbors an α-tocopherol-binding pocket [4,46], binds not only to α-tocopherol but also to other isoprenoid prenyllipids, such as phylloquinone [4]. This suggests that SITBP is involved in broad lipid-trafficking regulation in tomato plants and that vitamin E transport in plants seems to be less specific than in mammals. However, important differences in the structure and function of TBPs may have evolved in the plant kingdom over millions of years; thus, further research is needed to unravel the complexity of these proteins. To date the only described example of a plant TBP has been shown in tomato plants. SITBP cannot be considered completely homologous to either a mammalian TBP or TTP in the strict sense,

but it is a member of the plant PATELLIN gene family. The PATELLIN protein family members AtPATL1 and AtPATL2 are known to bind to phosphoinositides and to interact with kinases participating in vesicle-trafficking events [47] and membrane regeneration [48], respectively. Moreover, phylogenetic analysis has revealed that SITBP belongs, as occurs with AtPATL1 and AtPATL2, to the SEC14 protein family, which is broadly distributed from yeast to humans and participates in phosphatidylinositol 4,5-bisphosphate (PIP_2)-mediated lipid transport [49]. Although SITBP is characterized by the presence of a chloroplast signal peptide [4], this discovery paves the way for further research concerning the transport of tocopherols by other, still-unidentified TBPs outside chloroplasts (Box 1).

Vitamin E in Membranes: Biophysical Chemistry, Antioxidation, and Beyond

The amphipathic molecular structure of tocopherols and tocotrienols make it suitable for them to fuse in lipid bilayers at the water–lipid interface, where they play an important role in maintaining membrane integrity and stability. One of the major functions of vitamin E in cyanobacteria, plants, and animals that is well recognized is its protective effect against membrane lipid peroxidation through the donation of a proton from the hydroxyl of its chromanol head (Figure 3). This reaction can prevent the propagation of lipid peroxidation, since lipid peroxyl radicals can rapidly oxidize other fatty acids [57]. α-Tocopherol (chemically) scavenges lipid peroxyl radicals thus stopping the propagation of lipid peroxidation and leading to the formation of α-tocopheroxyl radicals, which are recycled back to α-tocopherol through the ascorbate–glutathione cycle [58]. Several models have been suggested for the mechanisms that allow peroxyl radical scavenging in membranes [59–61]; however, new evidence points to a flip-flop mechanism in the lipid bilayer as the most probable [18]. Movement of vitamin E molecules within membranes is an effective means of scavenging peroxyl radicals by making hydroxyl groups more accessible to peroxidized phospholipid tails (Figure 3). α-Tocopherol molecules stay inside the lipid bilayer (e.g., in the lipid matrix of thylakoids or the inner chloroplast envelope) with their hydroxyl groups in contact with the bilayer surface [18]. Interestingly, when lipid peroxidation is initiated in the water phase in liposomes prepared from plant lipid membranes,

Box 1. Transport of Vitamin E in Plants

The recent discovery of SITBP [4] has changed our view on tocopherol transport in plants, showing intraorganellar vitamin E transport within chloroplasts. Transport of vitamin E outside chloroplasts has not yet been proved, but it is tempting to speculate that vitamin E might also move outside chloroplasts and long distances through the phloem, playing a role in nonphotosynthetic sink tissues.

Vitamin E Transport within Chloroplasts

Continuous exchange of vitamin E and other prenyllipids occurs between the inner chloroplast envelope (where several vitamin E biosynthesis enzymes occur), grana (where α-tocopherol acts as an antioxidant and protects the photosynthetic apparatus), and plastoglobuli (where α-tocopherol accumulates as a reservoir). Prenyllipids such as plastoquinol and α-tocopherol accumulate in the plant in response to a number of environmental stresses leading to excess light in chloroplasts, partly accumulating in plastoglobuli [50], which are considered to be a reservoir of vitamin E in close contact with thylakoid membranes and the inner envelope of chloroplasts [51]. TBPs may facilitate rapid exchange of prenyllipids between intraorganellar membranes in chloroplasts.

Vitamin E Transport Outside Chloroplasts

As occurs with other lipids and lipophilic compounds, continuous exchange of vitamin E is likely to occur between the chloroplast envelope and the ER [45]. Since SITBP contains a peptide signal to chloroplasts [4], other, still-unidentified TBPs might play such a role to facilitate interorganellar vitamin E transport out of chloroplasts, which remains to be proved experimentally.

Vitamin E Transport Outside Mesophyll Cells

Vitamin E, and α-tocopherol in particular, has been located outside mesophyll cells, including vascular tissue, flowers, fruits, nodules, and other nonphotosynthetic tissues [17,46,52,53]. It is possible that plastids from nonphotosynthetic tissues harbor vitamin E biosynthesis-related genes and can synthesize tocopherols, but it may also be speculated that still-unknown TBPs allow long-distance transport of α-tocopherol in plants through the vascular tissue. This might explain earlier studies showing that tocopherols play a role in the regulation of flowering [54], which need to be urgently revisited. The translocation of TBPs by the phloem from sources to sinks (Figure I) might facilitate antioxidant protection and signaling in nonphotosynthetic tissues, a possibility that requires further investigation. Although recent findings of lipids and lipid-binding proteins in phloem exudates suggest that a long-distance lipid signaling exists in plants [55], vitamin E transport through the vascular tissue in plants remains to be proved experimentally. Interestingly, however, previous studies have shown that vitamin E is essential for phloem loading under low-temperature acclimation in arabidopsis [56].

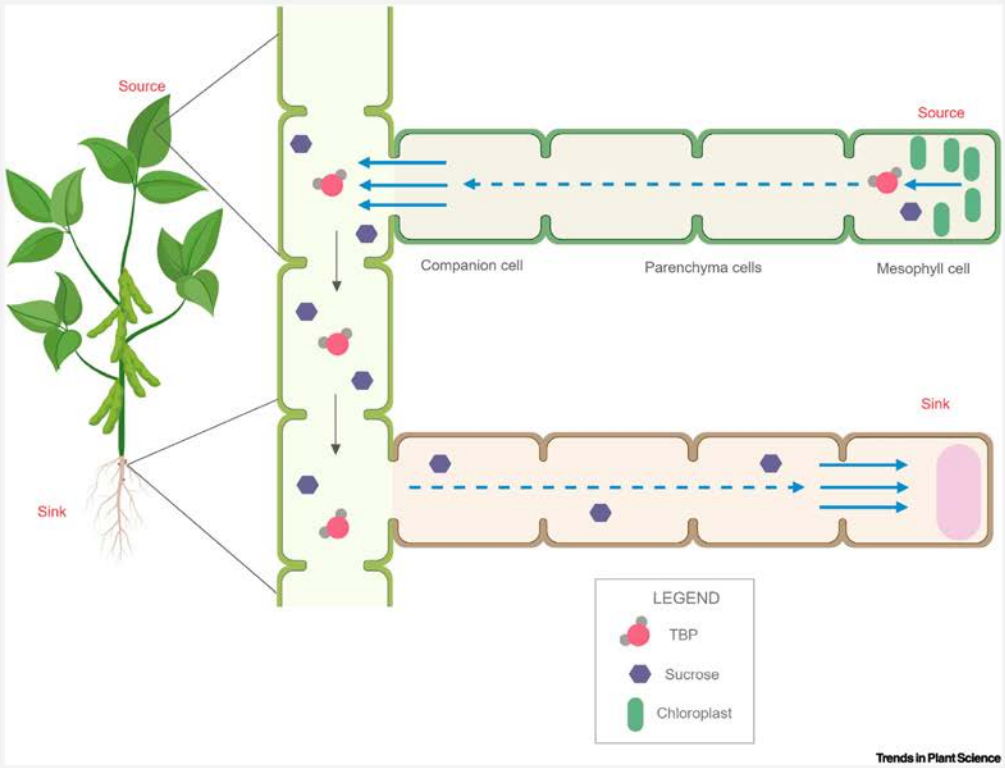

Figure I. Hypothetical Model Illustrating Still-Unproved Long-Distance Transport of Tocopherols though the Phloem in Plants.

This figure was created with the help of a template taken from the Biorender account at https://app.biorender.io/cell_press. Abbreviation: TBP; tocopherol-binding protein.

tocopherols are more effective than prenylquinols (e.g., plastoquinol-9, ubiquinol-10, α-tocopherol-quinol). By contrast, if the peroxidation is initiated in the hydrophobic interior of liposome membranes, long-chain prenyllipids, such as plastoquinol-9 and plastochromanol-8, are considerably more active than tocopherols in the inhibition of lipid peroxidation [62]. Rapid flip-flop movements are essential here and determine the antioxidant capacity of vitamin E in environments with high oxidation rates, and membrane composition, fluidity, and temperature, which are all interconnected, greatly influence the antioxidant activity of tocopherols [18,63,64].

Vitamin E not only protects plant membranes from the propagation of lipid peroxidation, but also modulates the accumulation of ROS. The most unstable ROS, first produced during photoinhibition and believed to be the driver of the process, is singlet oxygen ($^1O_2$ [65]). Formation of $^1O_2$ occurs in photosystem (PS)II and PSI when energy is not efficiently transferred from chlorophylls to downstream acceptors because they are already reduced [65,66]. Consequently, triple-state chlorophylls (^{3}Chl) are produced from photoactivation, because electron spins rephase into a lower-energy excited state [67]. As a result, the reaction between ^{3}Chl and triplet oxygen ($^3O_2$) can increase $^1O_2$ levels. Interaction of ^{3}Chl with $^3O_2$ can be avoided by cyclic electron transport, an increased pH gradient across the

thylakoid membrane, and nonphotochemical quenching (NPQ), which are all interconnected [68], or by direct quenching of ^{3}Chl by β-carotene at light-harvesting antenna complexes (LHCs) [69]. Besides carotenoids, the production of $^1O_2$ can also be neutralized by vitamin E [70]. α-Tocopherol has been shown to be very efficient in the physical (quenching) and chemical (scavenging) neutralization of $^1O_2$ in biological membranes [63]. While the highly efficient quenching of $^1O_2$ by tocopherols finally leads to the destruction of only one tocopherol molecule by approximately 120 quenched $^1O_2$ [71], the scavenging of $^1O_2$ by tocopherols is a 1:1 reaction (Figure 3). This latter reaction leads to the formation of tocopherol quinones, which can be recycled back by the subsequent activities of NAD(P)H-dependent quinone oxidoreductase (NDC1), an unknown dehydratase in the presence of ascorbate and glutathione, and tocopherol cyclase (VTE1) [36,72]. Consequently, vitamin E, and most particularly α-tocopherol, has been shown to play an essential role in **photoprotection** when leaves are subject to **photo-oxidative stress**, which may be caused either by intense light or by a number of environmental **stress factors** leading to excess excitation energy in chloroplasts, including water deficit, salinity, extreme temperatures, or metal toxicity [14,73]. Besides $^1O_2$, tocopherols could also help to eliminate hydroxyl radicals produced by the Fenton and Haber–Weiss reactions in chloroplasts when the ascorbate–glutathione cycle does not operate correctly or is overwhelmed by metal accumulation [74–77]. Both the prevention of the propagation of lipid peroxidation and the elimination of ROS help to maintain the correct fluidity of thylakoid membranes in stress situations. Tocopherol increases in response to a number of environmental stimuli will also help to physically stabilize thylakoid membranes, particularly when plants are exposed to high light and extreme temperatures [22,78].

Cellular signaling in plants is a complex phenomenon governed by several interconnected pathways. Heat-induced changes in membrane fluidity activate phospholipases and kinases on the plasma membrane within minutes, including phospholipase D and phosphatidylinositol phosphate kinase. Phosphatidic acid and PIP_2 then accumulate rapidly, with the heat-induced PIP_2 localized to the plasma membrane, nuclear envelope, nucleolus, and punctate cytoplasmic structures [79]. Additionally, PIP_2 is hydrolyzed by membrane-bound phospholipase C and releases diacylglycerol and inositol 1,4,5-trisphosphate, which increases rapidly within 3 min of heat shock in arabidopsis [80]. Interestingly, differentially expressed genes in leaves and ripe fruits from SlTBP-knockdown tomato lines indicate an alteration not only of photosynthesis and lipid, pigment, and isoprenoid metabolism but also of redox homeostasis and inositol/phosphoinositide metabolism [4]. This is particularly interesting because it indicates that, although SlTBP is preferentially located in plastids, this protein may help tocopherols play a role in retrograde signaling, their deficiency not only affecting lipid metabolism in the chloroplast envelope–ER interface but altering intracellular signaling processes operating in the plasma membrane [4]. Although it is still not fully known how signals propagate from the chloroplast to the plasma membrane, it has been shown that plants deficient in tocopherols undergo profound gene reprogramming under heat stress [23] and in low temperatures [81], as well as under water deficit, salinity [82], and mild phosphate starvation [83]. Retrograde signaling in plants is characterized by chloroplast-to-nucleus communication through signals emanating from chloroplasts or regulated by chloroplast-related effectors. In this regard, it has been shown that tocopherol-deficient plants respond to environmental stress by altering not only lipid metabolism and the endogenous content of both enzymatic and nonenzymatic lipid peroxidation products [81–84], but also endogenous phytohormone content and signaling, mainly related to jasmonates and ethylene [82,83]. Heat stress in tocopherol-deficient arabidopsis has been shown to alter the endogenous content of 3′-phosphoadenosine 5′-phosphate, an inhibitor of exoribonucleases, thus protecting miRNAs from degradation [23]. This sheds new light on the role of vitamin E in stress sensing and intracellular signaling, showing that gene reprogramming under tocopherol deficiency may be associated, at least in part, with the regulation of miRNA biogenesis.

The influence of vitamin E in stress sensing and signaling may be associated with its role in photoprotection and in the modulation of lipid peroxidation in thylakoid membranes (Figure 4). The action of vitamin E in stress sensing and signaling may be seen in the context of the overall mechanisms operating in chloroplast-to-nucleus retrograde signaling, with other effectors (e.g., ROS [84–87], carotenoids [88–90]) playing key regulatory roles in cooperation with vitamin E. Therefore, because

tocopherol can limit the production of $^1O_2$, it can affect transcription and phosphorylation of proteins like EXECUTER1 (EX1) or protein signaling cascades such as the one formed by OXI1, PTI1, PTI4, and MAPK3/6, which plays a key role in signal transduction to the nucleus. The study of double and triple mutants and overexpression lines with altered tocopherol and carotenoid composition will

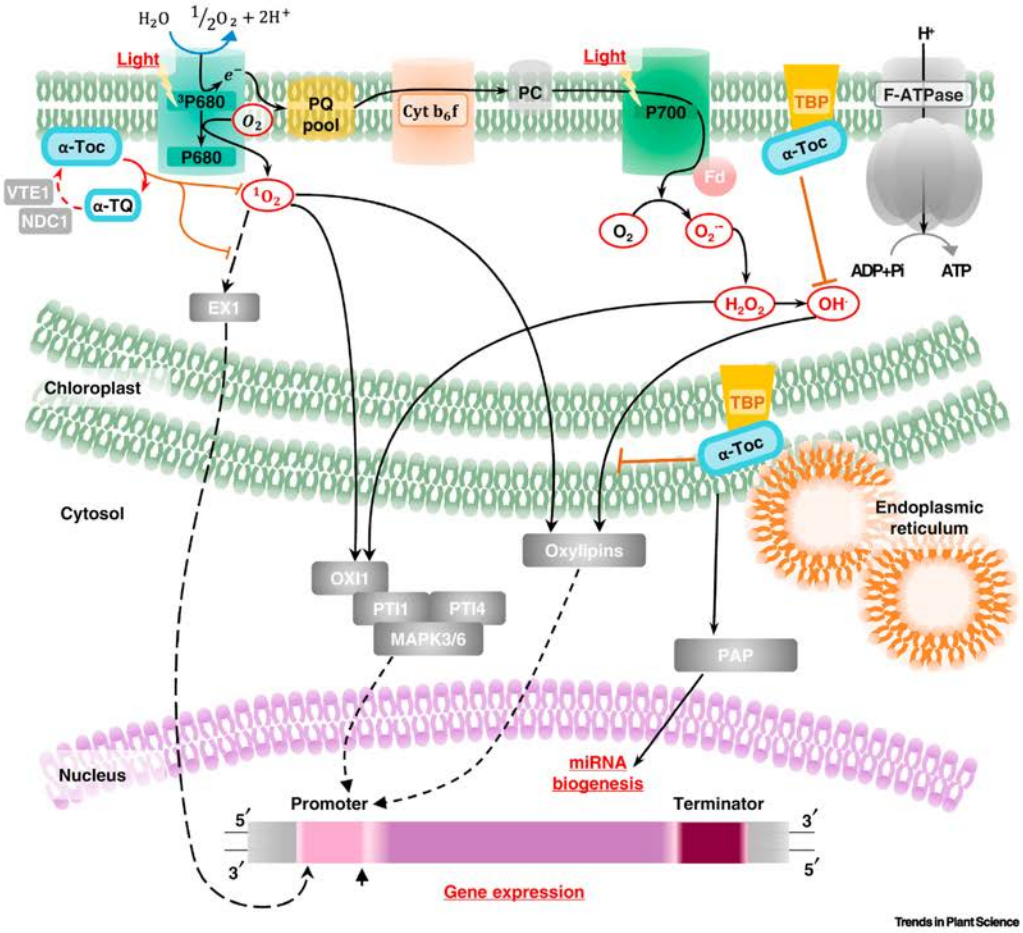

Figure 4. Proposed Model to Explain the Possible Role of α-Tocopherol in Intracellular Signaling in Plants.
Vitamin E might act as a chloroplast sentinel for stress sensing and signaling and altered vitamin E content in the chloroplast may help to transmit retrograde signals outside chloroplasts in stressful situations that result in miRNA biogenesis and gene reprogramming in the nucleus. 3′-Phosphoadenosine 5′-phosphate and oxylipins have been shown to be essential components to transmit the signal from the chloroplast to the nucleus. Positive and negative regulatory actions are indicated by arrows and lines with bars, respectively. Broken arrows/lines are indicative of still not fully characterized pathways. Abbreviations: EX1, EXECUTER1; H_2O_2, hydrogen peroxide; MAPK3/6, mitogen-activated protein kinase 3/6; NDC1, NAD(P)H-dependent quinone oxidoreductase; $^1O_2$, singlet oxygen; $O_2^{\cdot-}$, superoxide anion; $OH^{\cdot}$, hydroxyl radical; OXI1, OXIDATIVE SIGNAL INDUCIBLE1; PAP, 3′-phosphoadenosine 5′-phosphate; PTI, protein kinase Pto-interacting; α-Toc., α-tocopherol; α-TQ, α-tocopherol quinone; TBP, tocopherol-binding protein; VTE1, tocopherol cyclase.

undoubtedly help us better understand the underlying mechanisms governing tocopherol-mediated retrograde signaling in plants in the near future [91,92]. The advent of gene editing in plants using the clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (Cas9)-based genome editing tool [93] will help to confirm previous studies using mutant and transgenic lines with altered vitamin E content and delineate in the near future specific responses of different tocopherol forms in both photosynthetic and nonphotosynthetic tissues.

Concluding Remarks and Future Perspectives

The recent discovery of SITBP completely upturns our understanding of the transport and function of vitamin E in plants. SITBP shows 24% homology with liver α-TTP, a Sec14 domain lipid exchanger, which in humans picks up α-tocopherol from endosomal membranes and transports it to the plasma membrane, where α-tocopherol is released and replaced by PIP_2, which is then transported back to the endosome [2]. Although SITBP contains a transit signal to plastids and has not been found outside chloroplasts, it has been shown to influence inositol/phosphoinositide metabolism in the plant plasma membranes of tomato leaves and ripe fruits, thus posing intriguing questions for future research (see Outstanding Questions). It will undoubtedly be a great challenge for the near future to identify and characterize new TBPs in plants that help us elucidate whether tocopherols can be transported outside chloroplasts and how tocopherols crosstalk with various retrograde signals to fine-tune the most adequate response to every particular developmental and stress situation.

Outstanding Questions

Why do some plant species accumulate more tocotrienols than tocopherols in specific tissues?

Can vitamin E be transported outside chloroplasts and additionally for long distances through the vascular tissue?

Which are the retrograde signals derived from stress sensing by vitamin E specifically produced for each type of stress factor? What occurs under multiple stresses?

Will gene editing help us improve in the near future our current understanding of the biosynthesis, transport, and function of vitamin E in plants?

Acknowledgments

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ANNEX II

Annex II

Photo-oxidative stress during leaf, flower and fruit development

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Photo-Oxidative Stress during Leaf, Flower and Fruit Development[1][OPEN]

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Chloroplasts are essential for plant growth and development through their roles in photosynthesis and signaling. Reactive oxygen species (ROS) and antioxidants play crucial roles in regulating developmental processes, from organ initiation to senescence, not only in leaves but also in some types of flowers and fruits. Flowers and fruits bearing chloroplasts during their early stages of development share some similarities with leaves but also important differences in terms of photosynthesis-derived ROS, the duration and extent of photooxidative stress, and redox signaling. Here, we discuss recent advances in our understanding of the common mechanisms underlying redox signaling in leaves, flowers, and fruits, focusing on key spatiotemporal processes that determine specific responses in each organ. Chloroplasts play a central role in cellular processes during organ development, with photooxidative stress a key effector of redox signaling during organ development in leaves as well as in some types of flowers and fruits.

The growth and development of organs are characterized by several well-defined and interconnected key stages. Organ initiation, marked by pluripotent meristematic cells that divide and differentiate to become a new organ, is followed by organ growth, which involves further cell proliferation through reiterative mitotic cycles and subsequent cell expansion. Maturity defines the stage when cells no longer expand and the organ reaches a fully competent state. Lastly, senescence is the final developmental stage of a plant organ, usually leading to programmed cell death (Beemster et al., 2005; Anastasiou and Lenhard, 2007; Thomas, 2013). Since different organs share this characteristic set of developmental events, it is possible that similar underlying regulatory mechanisms might be involved. However, although leaves and petals have common evolutionary origins (Friedman et al., 2004), leaves, flowers, and fruits have completely different functions in plant development. Leaves transform light energy into chemical energy to provide photoassimilates, while petals enable pollination and, therefore, sexual reproduction and fruits, in turn, facilitate seed dispersal.

Several types of petals and fruits contain functional chloroplasts at early stages of development. However, the spatiotemporal dynamics of plastid differentiation differs between leaves, flowers, and fruits. In general, chloroplasts of mesophyll cells remain active during most of leaf ontogeny (until they become gerontoplasts in the last senescing stage), although loss of the cytochrome b_6f complex, electron flow, and proton conductivity may start earlier than chlorophyll degradation (Schöttler et al., 2017). By contrast, chloroplasts rapidly differentiate into chromoplasts in flower corollas, even before anthesis (Ščepánková and Hudák, 2004; Gan and Fischer, 2007; Arrom and Munné-Bosch, 2012) or during the ripening of the fruit exocarp (Lytovchenko et al., 2011; Lado et al., 2015). Chloroplast differentiation into gerontoplast typically occurs in senescing leaves (either yellow/orange or anthocyanin-rich red leaves), while chloroplast differentiation into chromoplast occurs in the flowers of some species (e.g. tepals from lilies [*Lilium* spp.] that turn from green to yellow or whitish) and several types of fruits (e.g. citrus fruits such as lemons [*Citrus limon*] and oranges [*Citrus sinensis*]). Indeed,

ADVANCES

- Chloroplast-related retrograde signaling is essential for organ development.
- Photooxidative stress is strongly dependent on environmental conditions, but also on the type of organ and its developmental stage.
- Redox signaling plays a key role in plastid differentiation in leaves, flowers, and fruits.
- Photooxidative stress may trigger redox signaling during organ development with important spatiotemporal differences between leaves, flowers, and fruits.

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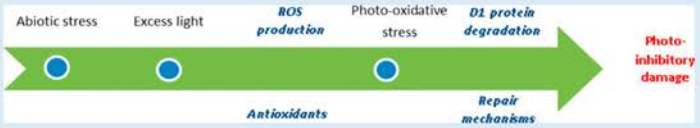
flower and fruit development is incredibly diverse, with some of the color changes involved not always detected by our retinas. For instance, experiments with petunia (*Petunia hybrida*) flowers have shown that expanded pink corollas contain photosynthetically active chloroplasts with high chlorophyll content (compared with the very early green stages), thus suggesting that chloroplasts also can play specific roles in flower development in anthocyanin-rich reddish petals, at least in some species (Katz and Weiss, 1998).

As aerobic organisms, plants cannot avoid ROS formation. In leaves, ROS production occurs mainly in chloroplasts, peroxisomes, mitochondria, and the apoplast due to the redox-related processes occurring in these organelles (Takahashi and Badger, 2011). Excessive levels of light in chloroplasts, caused by drought, salinity, extreme temperatures, high levels of light, or a combination of these factors, lead to photoinhibition and photooxidative stress, thus potentially causing photoinhibitory damage to the photosynthetic apparatus (Box 1). Although it is generally assumed that mitochondria, peroxisomes, and the apoplast are the main sources of ROS in flowers and fruits (Qin et al., 2009b; Rogers and Munné-Bosch, 2016), flower corollas and fruit exocarps in several species also accumulate excessive levels of light during the early developmental stages, generally until organ maturation is reached. Thus, photoinhibition and photooxidative stress also can occur in these organs (Arrom and Munné-Bosch, 2010; Hengari et al., 2014; Naschitz et al., 2015; Gang et al., 2016). ROS are known to be toxic, but recent studies have indicated that ROS are involved in signaling during growth, development, and responses to biotic/abiotic stress (Bell et al., 2009; Xia et al., 2015). ROS signaling is tightly regulated, their production and elimination being controlled by antioxidants that determine the lifetime and specificity of the ROS signal (Foyer and Noctor, 2005a; Mittler et al., 2011). ROS with different natures generate distinct signals. For example, singlet oxygen ($^1O_2$) is a very reactive species that lasts only a few microseconds, while hydrogen peroxide (H_2O_2) is more stable, with a longer lifespan and a greater diffusion distance, which makes it a more likely molecule to trigger a transduction signal to the nucleus. Moreover, site formation and detoxification systems ultimately determine the specificity of the oxidative signaling processes (Foyer and Noctor, 2005b; Munné-Bosch et al., 2013; Mignolet-Spruyt et al., 2016). There are several questions regarding the role of ROS in plant organ development. For example, what types of ROS are produced in the chloroplasts of flowers and fruits compared with those in leaves? Can chloroplasts play a role in redox signaling during the growth and development of flowers and fruits? To what extent do chromoplasts contribute to cellular redox signaling during flower and fruit development?

Although the biology of ROS production and elimination in chloroplasts and its implications for redox signaling have been reviewed in the last 5 years (Munné-Bosch et al., 2013; Dietz et al., 2016), a comparative analysis of the role of photooxidative stress and chloroplast-derived redox

BOX 1. Basic Concepts Related to Photooxidative Stress in Plants

- **Antioxidant**: Enzyme or low molecular weight compound that prevents the oxidation of essential cellular components (e.g. lipids, proteins, nucleic acids).
- **Excess light**: amount of light not used for photosynthesis that may be dissipated to prevent damage in chloroplasts.
- **Photoinhibition**: inhibition of photosynthesis at the photosystem II level (reduction in photosystem II quantum yield).
- **Photoinhibitory damage**: injuries caused by excess light in chloroplasts; it can be reversible or not (depending on the severity and duration of excess light).
- **Photooxidative stress**: oxidative stress caused by excess light in chloroplasts.
- **Photooxidative damage**: photoinhibitory damage associated with oxidative stress.
- **Oxidative stress**: imbalance between reactive oxygen production and its elimination.
- **Reactive oxygen species (ROS)**: unstable compound derived from molecular oxygen.
- **Redox signalling**: cell signalling based on reduction/oxidation processes.
- **ROS quenching**: Physical elimination of reactive oxygen species.
- **ROS scavenging**: Chemical elimination of reactive oxygen species.



signals in leaves, flowers, and fruits is still lacking. Here, we aim to go beyond our previous comparative analysis of ROS production and elimination in leaves and flowers (Rogers and Munné-Bosch, 2016) by providing a new conceptual framework for chloroplasts as central players in redox signaling in leaf, flower, and fruit development. This review will discuss recent advances in our understanding of photooxidative stress and redox signaling in leaves, flowers, and fruits, focusing on key spatiotemporal processes that determine specific responses in each organ. This might have important applications in agri-food biotechnology for the commercialization of leafy vegetables, flowers, and fruits.

CHLOROPLASTS IN LEAF, FLOWER, AND FRUIT DEVELOPMENT

Chloroplasts play a central role in cellular energy and redox processes. One of the most important events in the history of life was the origin of oxygenic photosynthesis. Life would not have been possible without the changes in atmospheric composition produced 2 billion years ago by ancient photosynthetic prokaryotes similar to cyanobacteria. The subsequent symbiosis of these photosynthetic organisms with eukaryotic cells gave rise to chloroplasts, powerhouse organelles that are now present in leaves and other photosynthetic tissues (Allen, 2015). In leaves, chloroplasts perform photosynthesis and are essential for C, N, and S reduction and assimilation (Kopriva and Rennenberg, 2004) as well as for the biosynthesis of amino acids, fatty acids, chlorophylls, and carotenoids, the latter essential for both the photosynthetic and antioxidant machinery (Joyard et al., 2009). Moreover, chloroplasts contribute to the production of phytohormones, such as abscisic acid, GA, auxins, salicylates, jasmonates, and melatonin, and essential antioxidants, such as vitamin E (Joyard et al., 2009). Chloroplasts also play a crucial role in redox processes and retrograde signaling, which are translated into metabolic changes through genetic regulation and control of the cellular redox state (Pfannschmidt, 2003; Asada, 2006; Dietz et al., 2016). However, during leaf senescence induced by aging and/or environmental stress, leaf chloroplasts change from a source to a sink due to a lower photosynthetic rate elicited by the disassembly of the photosynthetic apparatus and chlorophyll breakdown, which is indicated by low maximum photochemical efficiency of PSII in the dark-adapted state (Juvany et al., 2013). The complete dismantling of chloroplasts during leaf senescence generates nonphotosynthetic gerontoplasts (Fig. 1A). This process involves increased ROS production and a massive degradation of macromolecules by proteolysis and autophagy, remobilizing nutrients from leaves to other plant organs, mainly flowers and fruits (Gregersen et al., 2008; Guiboileau et al., 2013).

The corollas of many flowering plants and the exocarp of most fruits exhibit green colors at very early developmental stages. This green color is related to the presence of chlorophyll in the chloroplasts, which have been shown to be photosynthetically active in several types of flowers and fruits such as petunias, lilies, orchids, apples (*Malus domestica*), strawberries (*Fragaria* spp.), and tomatoes (*Solanum lycopersicum*; Weiss et al., 1990; Blanke, 2002; Juneau et al., 2002; He and Woon, 2008; Cocaliadis et al., 2014; Naschitz et al., 2015). The yellow and orange colors acquired by flowers and fruits are due to carotenoids, a well-known group of lipophilic pigments that accumulate in chromoplasts (Fig. 1, B and C). In chromoplasts, carotenoids accumulate in large quantities in a wide range of lipid-associated proteins (PAPs) that determine chromoplast morphology (Ytterberg et al., 2006). Chloroplast differentiation into chromoplast starts with chlorophyll degradation and the disassembly of grana and thylakoids, which is similar to the process that occurs during chloroplast differentiation into gerontoplasts in senescing leaves. However, for the chloroplast-to-chromoplast transition, there is not a massive loss in the plastome, and sugars are imported from the cytosol to the chromoplast to sustain biosynthetic activities. Anthocyanins contribute to the red, purple, and blue colors of flowers and fruits. In contrast to carotenoids, large amounts of anthocyanins accumulate in vacuoles, giving rise to a number of colors depending on their chemical nature and vacuolar pH (Jaakola, 2013). Whitish flowers and fruits occur when chlorophylls have already degraded and carotenoids and/or anthocyanins are not (or are no longer) present, such as in senescing lilies (Fig. 1C).

A lot of attention has been paid to the biosynthetic mechanisms underlying carotenoid accumulation in the chromoplasts of flowers (Kishimoto and Ohmiya, 2006; Chiou et al., 2010) and fruits (Fraser et al., 1994; Kato et al., 2004; Fu et al., 2016), mainly because of consumer tastes. Moreover, there have been several projects to enhance carotenoid accumulation in food products by genetic engineering to overcome typical dietary diseases associated with carotenoid deficiency in developing countries (Ye et al., 2000; Diretto et al., 2007). However, little is known about the chloroplast-to-chromoplast transition, and only some genes have been identified as regulating this process in studies using the *Orange* cauliflower (*Brassica oleracea botrytis*) mutant, which has altered chromoplast biogenesis (Paolillo et al., 2004; Lu et al., 2006), and the *high pigment* mutant lines of tomatoes, which show altered chromoplast number and size (Mustilli et al., 1999; Cookson et al., 2003). Chromoplast generation not only strongly depends on carotenoid accumulation, which is influenced by ROS production (Pan et al., 2009), but also is influenced by N availability, sugar accumulation, and phytohormones such as GAs, cytokinins, abscisic acid, and ethylene (Iglesias et al., 2001). Regreening of tissues occurs in some leaves, flowers, and fruits when gerontoplasts or chromoplasts become active chloroplasts in the increased presence of GAs and/or cytokinins and a nitrate source (Goldschmidt, 1988; Zavaleta-Mancera et al., 1999; Prebeg et al., 2008). In chloroplast regeneration, the thylakoid system is restored from the invagination of the inner membrane of chromoplasts or membrane-bound

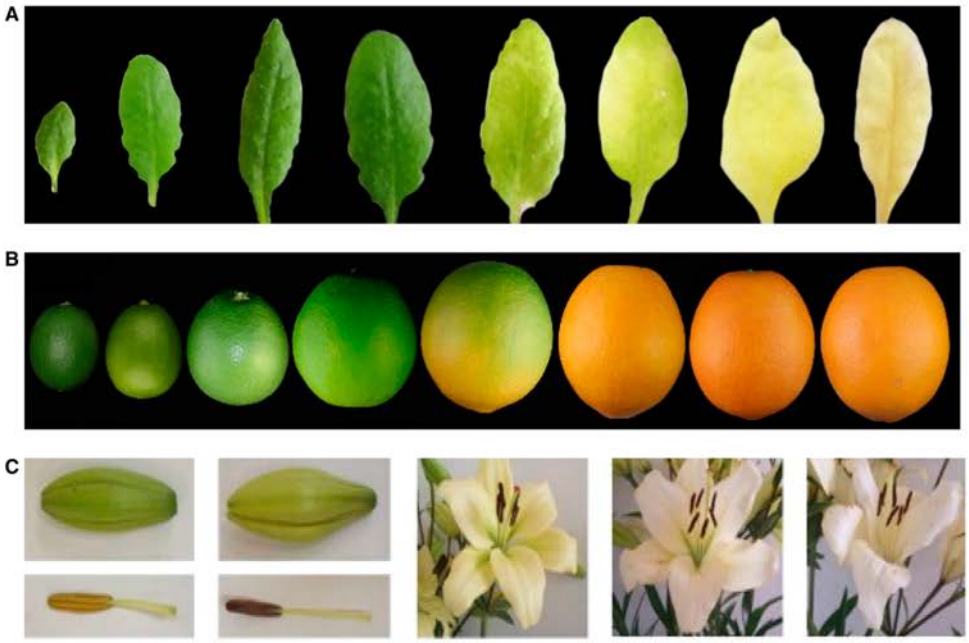


Figure 1. Comparative visualization of leaf, fruit, and flower development. A, Arabidopsis leaves experience leaf senescence during the mature stage, thus leading to the conversion of chloroplasts to gerontoplasts. B, Citrus fruits show a gradual shift from chloroplasts to chromoplasts in the exocarp, which mark the change in color from green to orange. C, Flowers also show characteristic color changes due to the conversion of chloroplasts to chromoplasts, such as tepals of lilies (cv LA Courier). In this model, however, carotenoid-accumulating chromoplasts, which can be observed at anthesis, are lost during senescence.

bodies, as described for cucumbers (*Cucumis sativus*; Prebeg et al., 2008). Although postharvest regreening of some types of flowers and fruits is regarded as negative because of the loss in quality (Hsu et al., 1989; Chen et al., 2013), there is a huge potential for chloroplast regeneration in planta at the intermediate stages of flower and fruit development in agri-food biotechnology. Delaying flower senescence and fruit ripening before harvest could enhance postharvest life and increase size and organoleptic properties by inducing higher photoassimilate production during the green stages and activating redox mechanisms to improve the antioxidant content, as has been shown for some fruits (Cocaliadis et al., 2014; Rademacher, 2015) and flowers (Arrom and Munné-Bosch, 2012; Imsabai and van Doorn, 2013). Degreening and regreening are indeed applied in some cases to increase market sales, such as for citrus fruits (Box 2).

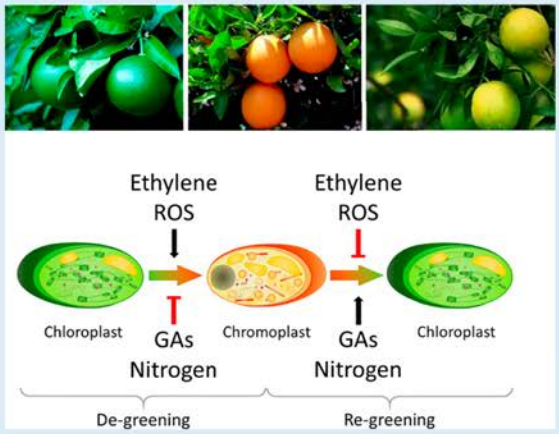
PHOTOOXIDATIVE STRESS IN LEAVES, FLOWERS, AND FRUITS

Oxygen was a key factor in promoting aerobic metabolism on Earth, supplying enough power to develop complex structures such as the eukaryotic organisms we know today. However, the establishment of aerobic metabolism inevitably led to the production of ROS. In plant cells, ROS are generated mainly in four cellular locations: chloroplasts, peroxisomes, mitochondria, and the apoplast (Apel and Hirt, 2004). In leaves, the excessive energy in chloroplasts can be dissipated as thermal energy in nonphotochemical quenching or used to produce triple-state chlorophylls in the antenna and at the reaction center of PSII. Subsequently, triple-state chlorophyll activates oxygen, leading to the production of $^1O_2$, a highly unstable ROS. Superoxide anions ($O_2^{\bullet -}$) also can be produced by the direct reduction of oxygen in PSI (Mehler reaction) due to the absence of electron acceptors like ferrodoxin or $NADP^+$. $O_2^{\bullet -}$ is then converted rapidly by superoxide dismutase (SOD) into H_2O_2, which is a more stable ROS, although hydroxyl radicals ($OH^{\bullet -}$), the most short-lived ROS, can be produced from H_2O_2 through the Fenton and Haber-Weiss reactions (Fig. 2A; Anthony et al., 2005; Asada, 2006; Pintó-Marijuan and Munné-Bosch, 2014). ROS have harmful effects on lipid peroxidation as well as on carbohydrate, protein, and DNA oxidation, leading to

BOX 2. Degreening and Regreening in Fruits

Degreening of fruits, such as oranges, is used as a postharvest treatment to accelerate marketing. Treatment of fruits at stage BBCH79 (see fig. below) with ethylene-releasing compounds accelerates chlorophyll degradation and makes it visually identical to ripen oranges. Although these fruits are not as good organoleptically as fruits that have ripened on the tree, they find a good price on the market.

Regreening of oranges and other fruits on the tree can be used as an effective means to prevent ripening and, when applied to late-ripe varieties, such as Lane Late (in the case of sweet oranges), can delay harvest and increase the period of oranges in the market (see fig. below).



programmed cell death during ROS overproduction (Gill and Tuteja, 2010). However, under redox homeostasis, there is a balance between ROS production and its detoxification by both enzymatic and nonenzymatic antioxidants (Foyer and Noctor, 2005a; Juvany et al., 2013). Antioxidants determine the duration and location of specific ROS, thus eliciting different signaling responses. $^1O_2$ has been described as a short-lasting ROS because it has a lifetime of approximately 4 μs and can diffuse relatively short distances (it can only travel around 200 nm, but chloroplasts are around 5–10 μm wide; Gill and Tuteja, 2010; Ogilby, 2010). Carotenoids, specifically β-carotene, tocopherols, and plastoquinone, are the main antioxidants triggering $^1O_2$ detoxification by (physical) quenching or (chemical) scavenging (Miret and Munné-Bosch, 2015). Arabidopsis (*Arabidopsis thaliana*) *fluorescent* (*flu*) mutants accumulate large amounts of chlorophyll precursors, including protochlorophyllide, in the dark and produce large amounts of $^1O_2$ when illuminated (Meskauskiene et al., 2001). Illumination of these mutants for 15 min after a period of darkness has been shown to activate a distinct set of genes as a result of $^1O_2$ production in chloroplasts, showing the relevance of lifetime and site production on ROS signaling (op den Camp et al., 2003). In contrast to $^1O_2$, H_2O_2 can be produced in different compartments in plant cells and is a more stable ROS with higher diffusion distances of around 1 μm (Vestergaard et al., 2012), thus with the potential of being able to diffuse from chloroplasts to nuclei to modulate gene expression (Exposito-Rodriguez et al., 2017). The AsA-glutathione cycle plays a major role in H_2O_2 detoxification in the water-water cycle. When H_2O_2 is produced, it is converted rapidly by ascorbate peroxidase into water and monodehydroascorbate (MDA). MDA is reduced subsequently to AsA by reduced ferredoxin or MDA reductase. Provided that MDA is not reduced directly to AsA, it spontaneously converts into AsA and dehydroascorbate,

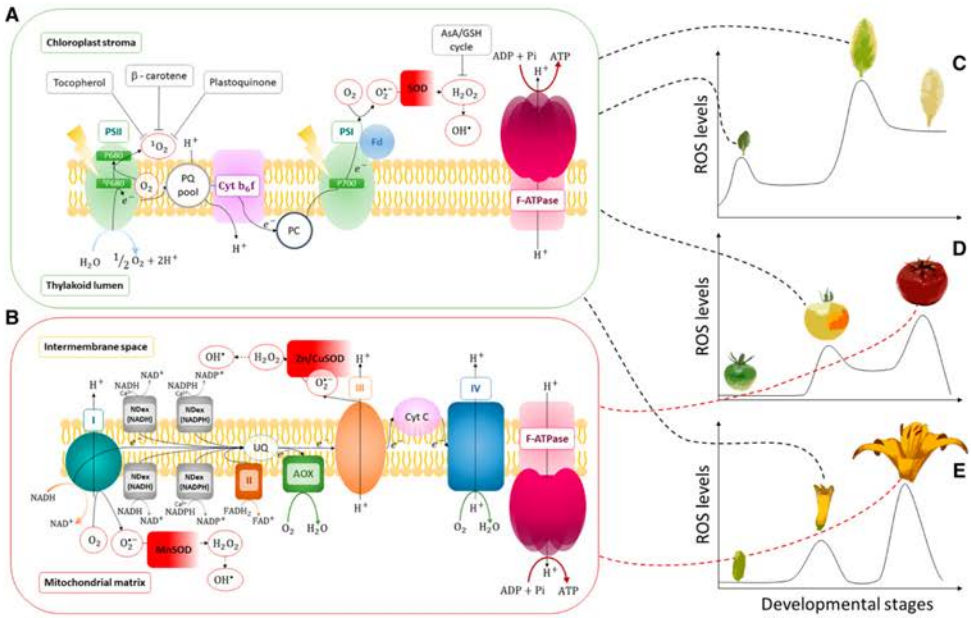


Figure 2. ROS production and temporal dynamics during organ development. A, Schematic representation of the chloroplast under excess light conditions. $^1O_2$ is produced in PSII when triple-state chlorophyll forms (^{3}P680). Antioxidants like tocopherols, β-carotene, and plastoquinone can effectively eliminate $^1O_2$. $O_2^{\bullet-}$ is produced through the direct reduction of oxygen in PSI (Mehler reaction) in the absence of electron acceptors like ferrodoxin (Fd) or $NADP^+$, and it can convert into H_2O_2 by SOD. If H_2O_2 is not efficiently eliminated by the ascorbate/glutathione (AsA/GSH) cycle, it leads to the production of $OH^{\bullet}$ in the Fenton and Haber-Weiss reactions. B, Schematic representation of a mitochondrion under oxidative stress. Complexes I and III are the main producers of $O_2^{\bullet-}$, which can be converted to H_2O_2 by MnSOD or Zn/CuSOD. C, D, and E, Simplified models for ROS production during leaf, fruit, and flower development, respectively. AOX, Alternative oxidase; Cyt b_6f, cytochrome b_6f complex; Cyt C, cytochrome *c*; NDex(NADH), external rotenone-insensitive NADH dehydrogenase; NDex(NADPH), external rotenone-insensitive NADPH dehydrogenase; NDin(NADH), internal rotenone-insensitive NADH dehydrogenase; NDin(NADPH) internal rotenone-insensitive NADPH dehydrogenase; PC, plastocyanin; PQ pool, plastoquinone pool; I, complex I (NADH:ubiquinone oxidoreductase); II, complex II (succinate dehydrogenase); III, complex III (cytochrome bc_1 complex); IV, complex IV (cytochrome *c* oxidase).

which is further reduced by dehydroascorbate reductase into reduced glutathione (GSH), which is finally recovered by glutathione reductase (Asada, 2006).

In leaves, ROS are produced not only under environmental conditions that trigger photoinhibition and photooxidative stress in chloroplasts but also during the very early and late stages of leaf development (Juvany et al., 2013). Photooxidative stress occurs in senescing leaves as well as in very young leaves when the photosynthetic machinery is still under construction, the xanthophyll cycle-dependent energy dissipation mechanisms are still not fully operational, and ROS production is increased due to the excessive energy in chloroplasts (Fig. 2C; Szymańska and Kruk, 2008; Lepeduš et al., 2011; Juvany et al., 2012). The occurrence and intensity of the double peak in ROS production during leaf development strongly depend on the specific patterns of leaf development in each plant species. For instance, species with folded leaves during the early stages of leaf development are not exposed to high levels of light and, therefore, do not suffer photooxidative stress. Leaf longevity and environmental conditions also affect the timing and intensity of the second ROS peak, which is typically associated with the start of leaf senescence (Zimmermann and Zentgraf, 2005; Juvany et al., 2013).

Petal senescence and fruit ripening share some similar morphological and biochemical processes with leaf senescence, like chloroplast disassembly and protein degradation. ROS are indeed involved in flower development and fruit ripening, with oxidative stress occurring not only in the mitochondria (Fig. 2B), due to protein carbonylation and the increased respiratory rate during ripening affecting the redox state once sugars

become a limiting factor (Qin et al., 2009a; Kan et al., 2010; Tian et al., 2013), but also in plastids during the chloroplast-to-chromoplast transition. H_2O_2 levels increase in the berry skin of grape (*Vitis vinifera* 'Pinot Noir') at the onset of ripening (veraison), when the most crucial events during berry ripening occur, including the change in color of the skin (Pilati et al., 2014). Studies on tomatoes also have revealed an elevation in H_2O_2 levels during changes in color of the skin (Jimenez et al., 2002; Kumar et al., 2016). It appears, therefore, that color break during ripening is associated with increased oxidative stress in ripening fruits that is linked to a respiratory burst in the mitochondria and enhanced ROS production during chromoplast generation, which is essential for carotenoid production (Bouvier et al., 1998). In postharvest fruits, ROS are one of the main factors causing fruit decay (Vicente et al., 2006; Pavez et al., 2013). As in leaves, fruits also show a double peak in ROS production during their development, the first peak occurring at the start of ripening and the second during overripening either at preharvest or postharvest (Fig. 2D). Flowers also show a double peak in ROS production during development, but the timing differs. H_2O_2 accumulation in daylily (*Hemerocallis* spp. hybrid) petals show a double oxidative burst (Fig. 2E), one at the time of flower bud opening, once chlorophylls have mostly been degraded, and the other at the start of flower senescence (Panavas and Rubinstein, 1998). Unfortunately, however, the effects of chloroplast-to-chromoplast differentiation and the spatiotemporal location of ROS production on flower development have not been investigated yet in detail, although it appears that light and photosynthetic electron transport promote anthocyanin synthesis, as seen in petunia, and, therefore, might be essential in triggering flower development (Weiss and Halevy, 1991).

Fruit sunburn, which is linked to photooxidative stress in several types of fruits, such as watermelons (*Citrullis vulgaris*), tomatoes, peppers (*Capsicum annuum*), cucumbers, apples, and strawberries, is still an important agronomic concern in the agri-food biotechnology industry. Three degrees of sunburn may occur: (1) photooxidative sunburn, in which fruits become photobleached by excessive levels of light (due to sudden changes in light conditions within the canopy); (2) sunburn browning, which is caused by changes in the light environment and enhanced temperatures and is associated with the severe degradation of chlorophylls and carotenoids; and (3) sunburn necrosis, which is associated with even higher temperatures that destroy tissues on the sun-exposed side of the fruit by causing a loss in cell integrity and cell leakage (Torres et al., 2006; Racsko and Schrader, 2012). Protection against sunburn includes developing sufficient leaf cover in the canopy to shade the fruit, activating the xanthophyll cycle-dependent excess energy dissipation system, and providing effective antioxidant protection through low-molecular-weight antioxidants, such as carotenoids, tocopherols, ascorbate, glutathione, and flavonoids, and by effectively using the ascorbate-glutathione cycle and other enzymatic antioxidants (Torres et al., 2006; Chen et al., 2008; Zhang et al., 2015).

REDOX SIGNALING DURING LEAF, FLOWER, AND FRUIT DEVELOPMENT

Although ROS have been traditionally viewed as harmful molecules, they are currently considered essential signaling molecules that are involved in plant responses to abiotic and biotic stress, plant growth, and development. In this context, photooxidative stress in leaves, flowers, and fruits affects redox signaling through the specific oxidation of target and signaling molecules. Lipids and proteins are excellent targets mediating cellular responses to imbalances in the redox state in chloroplasts, as fatty acids and amino acids (specifically sulfur-containing amino acids like Cys, Tyr, Trp, and His) are very susceptible to oxidative modification (Foyer and Noctor, 2005b; Han, 2017). There is limited information on the molecular mechanisms underlying ROS retrograde signaling in the chloroplasts of leaves, flowers, and fruits, although transcriptomic and proteomic approaches performed in recent years have provided important data.

Some ROS, like $^1O_2$ and OH•, have limited diffusion distances. Thus, mechanisms other than direct diffusion must exist for redox signaling from chloroplasts to the nucleus. By contrast, H_2O_2 is more stable and can diffuse directly from chloroplasts to the nuclei (Exposito-Rodriguez et al., 2017), thus influencing gene expression in the nuclei both directly and indirectly (Fig. 3). Glutathione is an important signaling molecule, acting as a buffer between ROS and Cys groups and inducing calcium (Ca^{2+}) release into the cytosol to mediate ROS retrograde signaling via MAPK pathways (Gómez et al., 2004; Ray et al., 2012). Moreover, ROS affects the production of phytohormones like ethylene, GAs, and jasmonates (Shumbe et al., 2016). Studies using mutants have been essential in determining the genetic modifications produced by ROS. The Arabidopsis *flu* mutant was described to produce $^1O_2$ in leaves when illuminated after a period of darkness because of an overproduction of protochlorophyllide in the dark (op de Camp et al., 2003). Wagner et al. (2004) found that *EX1* was responsible for programmed cell death in bleached seedlings and growth inhibition in Arabidopsis *flu* mutants, with the EX1 protein located at the grana margins of chloroplasts mediating responses to $^1O_2$ production (Wang et al., 2016). Moreover, a recent study performed in the Arabidopsis mutant *chlorina1* showed that programmed cell death induced by $^1O_2$ under high levels of light was mediated by *OXI1* via an EX1-independent pathway (Shumbe et al., 2016). In that study, *oxi1* mutants showed increased salicylic acid production and decreased expression of the genes associated with GA, ethylene, and jasmonic acid biosynthesis. Jasmonic acid regulates programmed cell death, while its precursor, 12-*oxo*-phytodienoic acid, plays

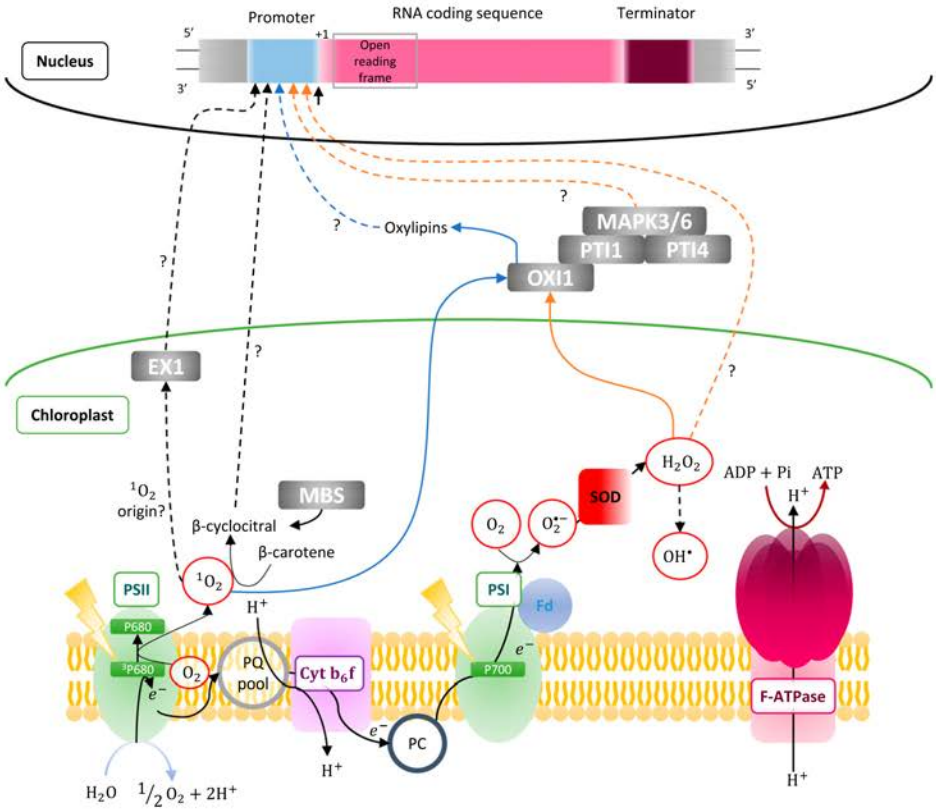


Figure 3. Retrograde signaling during photooxidative stress in chloroplasts. $^1O_2$ along with H_2O_2 have been described as signaling molecules able to modulate gene expression. EXECUTER1 (EX1) located at the grana margins has been reported to mediate responses to $^1O_2$ production, as well as OXIDATIVE SIGNAL INDUCIBLE1 (OXI1), which mediates $^1O_2$ response in an EX1-independent pathway, possibly through oxylipins. Moreover, oxidation products such as β-cyclocitral also are essential to mediate $^1O_2$ signaling. In contrast, H_2O_2 is a more stable form of ROS and direct modulation of gene expression could be possible, although this molecule also activates retrograde downstream signaling through mitogen-activated protein kinases (MAPK3/6) interacting with OXI1, protein kinase Pto-interacting1 (PTI1), and PTI4. Continuous lines show pathways and interactions already described in the literature, while discontinuous lines are unknown pathways. Cyt b_6f, Cytochrome b_6f complex; Fd, ferrodoxin; MBS, METHYLENE BLUE SENSITIVITY; PC, plastocyanin; PQ pool, plastoquinone pool.

a major role in the acclimation process during photooxidative stress. Although H_2O_2 signaling transduces through the MAPK3 and MAPK6 signaling cascades activated by the complex of *OXI1* with PTI1 and PTI4 (Rentel et al., 2004), $^1O_2$ does not appear to activate these signaling cascades, as *oxi1* mutants do not show any changes in the expression of genes encoding these proteins, indicating a different transduction pathway for *OXI1* under $^1O_2$ regulation (Shumbe et al., 2016). Although $^1O_2$ signaling has been studied only in leaves thus far, the advent of molecular genetics in new models, such as tomatoes, paves the way for studying these signaling processes in both flowers and fruits. Indeed, carotenoid oxidation products generated during $^1O_2$ scavenging, such as β-cyclocitral, also have been shown to be essential in transducing signals triggered by photooxidative stress and specific $^1O_2$ signatures (Ramel et al., 2012). Furthermore, a small zinc finger protein, MBS, is required to induce $^1O_2$-dependent gene expression (Shao et al., 2013). Interestingly, however, *mbs1* mutants supplemented with β-cyclocitral are unable to positively regulate antioxidant-related genes that are usually activated in the wild type, in which the zinc finger transcription factor MBS1 is fully active and enhances plant tolerance to high levels of light (Shumbe et al., 2017).

Lipids, rather than proteins, seem to be a target for oxidation in chromoplasts during the ripening of

OUTSTANDING QUESTIONS

- Mechanisms of retrograde signalling in flowers and fruits are still poorly understood. Do *EX1* and *OXI1* genes play a role in flower and fruit programmed cell death?
- Do leaves, flowers, and fruits follow the same mechanisms for plastid differentiation? To what extent gerontoplasts in senescing leaves regulate cell death similarly to chromoplasts in flowers and fruits?
- How do leaves, flowers, and leaves finely regulate the threshold of ROS production to strictly regulate cell growth, differentiation, and death? Have these mechanisms been evolutionary conserved?

peppers (Martí et al., 2009), indicating that jasmonates are likely to play a major role in retrograde signaling as modulators of redox signaling during fruit ripening. This is consistent with the effects of jasmonates, particularly the free jasmonic acid form, on ethylene biosynthesis and color development in other fruits, such as apples and tomatoes (Fan et al., 1998). Furthermore, the activities of SOD and components of the AsA-GSH cycle are specifically up-regulated in chromoplasts during fruit ripening, thus suggesting that these enzymes may play a role in protecting plastids and could modulate signaling molecules such as $O_2^{\bullet-}$ and H_2O_2 (Martí et al., 2009). In addition to fruits, flower corollas also are an excellent model in which to study redox signaling triggered by photooxidative stress. In this regard, it is essential to consider the spatiotemporal accumulation of plant pigments during flower development. Carotenoid accumulation during the chloroplast-to-chromoplast transition implies a distinct photoprotective capacity of the organ compared with flowers accumulating anthocyanins in the vacuoles. Red, blue, purple, and yellow flowers all have completely different absorbance spectra that determine the extent of photooxidative stress in chloroplasts and redox retrograde signaling during flower development. Although still in its infancy, research in flower corollas already has shed some light on the regulation of developmental stages by chloroplasts. For instance, in petunia corollas, which accumulate large amounts of chlorophyll until flower opening, PSI appears to be more active than PSII in electron transport, and photosynthesis saturation occurs at low light intensities (Weiss et al., 1988). This might imply that chloroplasts in petunia corollas produce large amounts of ROS, eliciting retrograde signaling with specific H_2O_2 signatures. Interestingly, components of the PSI core complex have been shown to continue accumulating in both petunia and carnation (*Dianthus caryophyllus*) corollas even after anthesis (Vainstein and Sharon, 1993), suggesting that specific redox signatures may regulate flower development that are not necessarily the same as those occurring in leaves. However, each plant species displays distinct pigmentation in flower corollas, which determines the extent of photoprotection and, therefore, the potential ROS signatures produced during flower development, an area that undoubtedly warrants further investigation.

CONCLUSION AND PROSPECTS

Molecular genetic studies have been pivotal in dissecting the key regulatory hubs in redox signaling. Flowers and fruits bearing chloroplasts during their early stages of development share some similarities with leaves but also important differences in terms of photosynthesis-derived ROS, the duration and extent of photooxidative stress, and redox signaling. Photooxidative stress may be considered a key effector of redox signaling during organ development, not only in leaves but also in some types of flowers and fruits. However, the unique patterns of pigmentation in flowers and fruits and the photosynthetic activity of fruit exocarps and flower corollas ultimately determine the specific redox signatures arising from chloroplasts. However, there are still many gaps in our knowledge on the redox mechanisms underlying flower and fruit development (see Outstanding Questions), and further research is needed to increase our understanding of flower and fruit development. This is not only essential for a better understanding of basic biology but also has important economic implications in the agri-food biotechnology sector.

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