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	Maren Müller	

Al príncipe Juan i la Conxi,

a les niñites Helena i Laura,

a la iaia i l'avi, ...

... i, sobretot, als meus bitxirulos

ÍNDEX

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Les paraules no són el meu fort, així que intentaré demostrar-vos amb fets com n'estic d'agraïda amb cadascun de vosaltres per formar part de la meva història.

And there were ugly times it's true
Some from me and some from you
And devils have a way of digging down
But a friend is not a friend for life
Unless your life is worth the strife
So hold on we'll be alright

I'm much obliged

So thank you my dear friends
and that's goodnight
All night loud (The Cat Empire)

ABREVIATURES

$^1O_2$	Singlet d'oxigen
2iP	2-Isopentenil adenina
α-Toc	α-Tocoferol
ABA	Àcid abscísic
ACC	Àcid 1-aminociclopropà-1-carboxílic
AOS	*Allene oxide* sintasa
APX	Ascorbat peroxidasa
Asc	Ascorbat
CAT	Catalasa
Car	Carotenoides
DHA	Dehidroascorbat
DHAR	Dehidroascorbat reductasa
DNA	Àcid desoxiribonucleic
ET	Etilè
Fd	Ferredoxina
F_v/F_m	Eficiència màxima del fotosistema II
GAs	Gibberel·lines
GR	Glutatió reductasa
GSH	Glutatió
GSSG	Glutatió oxidat
H_2O_2	Peròxid d'hidrogen
IPA	Isopentenil adenosina
JA	Àcid jasmònic
LMA	Massa foliar per àrea
$LOO^·$	Radicals lipídics peroxil
LOOH	Hidroperòxids
LOX	Lipoxigenasa
MDA	Àcid malondialdehid
MDHA	Monodehidroascorbat
MDHAR	Monodehidroascorbat reductasa
NADH	Nicotinamida adenina dinucleòtid
NADPH	Nicotinamida adenina dinucleòtid fosfat
NPQ	Extinció no fotoquímica
$O_2^{·-}$	Radical superòxid
$OH^{·-}$	Radical hidroxil
OPDA	Àcid oxofitodienoic
OR	OPDA reductasa
PQ	Plastoquinona
PSII	Fotosistema II
PUFA	Àcid gras poliinsaturat
ROS	Espècies reactives de l'oxigen
SA	Àcid salicílic
SAG	Gens associats a la senescència
SOD	Superòxid dismutasa
UQ	Ubiquinona
Z	Zeatina

Introducció general

INTRODUCCIÓ GENERAL

1. Estrès oxidatiu en plantes

Els processos metabòlics en plantes, com ara la fotosíntesi, la fotorespiració i la respiració, comporten l'inevitable producció d'espècies reactives de l'oxigen (ROS, de l'anglès *reactive oxygen species*) en cloroplasts, peroxisomes i mitocondris. En determinades concentracions poden actuar com a molècules implicades en la senyalització cel·lular, però a causa de la seva elevada reactivitat, un augment de les ROS provoca l'oxidació de components cel·lulars alterant-ne la seva funció biològica i provocant dany oxidatiu a la planta. Per tal d'evitar-ho, les plantes han desenvolupat diversos mecanismes antioxidants. L'equilibri entre la producció de les ROS i la seva eliminació mitjançant els antioxidants serà el que determinarà l'estat redox de la planta. Mantenir l'homeòstasi redox és vital per evitar un possible dany i a la vegada regular els seus nivells de manera que la seva funció en senyalització sigui eficaç. Per contra, aquest equilibri es pot veure pertorbat per diversos estressos, tant biòtics com abiòtics, comportant un increment dels nivells de ROS i, per tant, provocant un estrès oxidatiu.

1.1 Espècies reactives de l'oxigen

La transferència d'energia o d'electrons a l'oxigen provoca la formació de singlet d'oxigen ($^1O_2$) o el radical superòxid ($O_2^{\cdot-}$) i posteriorment del peròxid d'hidrogen (H_2O_2). Si aquests dos últims s'acumulen en presència de metalls de transició, poden generar el radical hidroxil ($OH^{\cdot-}$) (reaccions de Haber-Weiss i de Fenton), una de les ROS més reactives capaç d'atacar ràpidament qualsevol tipus de component cel·lular (Møller *et al.*, 2007) (**Figura 1**). En plantes, les formes de ROS més abundants són el $^1O_2$, $O_2^{\cdot-}$, H_2O_2 i $OH^{\cdot-}$, que són contínuament produïts com a subproductes de vàries vies metabòliques (Apel & Hirt, 2004).

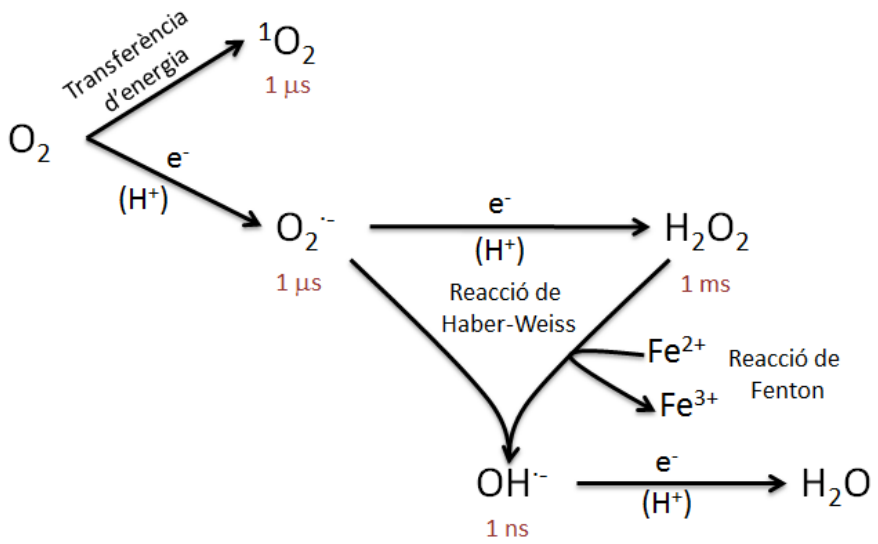

Figura 1: Formació de les ROS més comunes en plantes mitjançant la transferència d'energia o electrons a l'oxigen (O_2) fins a la formació d'aigua (H_2O). $O_2^{\cdot-}$, anió superòxid; H_2O_2, peròxid d'hidrogen; $^1O_2$, singlet d'oxigen; $OH^{\cdot}$, radical hidroxil. En vermell: vida mitjana de cadascuna de les ROS en sistemes biològics.

El cloroplast és l'orgànul més susceptible de patir estrès oxidatiu a causa dels elevats nivells d'oxigen als que està exposat, originats de la fotòlisi de l'aigua en el fotosistema II (PSII), combinat amb la cadena de transport d'electrons durant la fotosíntesi.

En el primer pas de la fotosíntesi, l'energia de la llum absorbida pels pigments fotosintètics en el PSII és utilitzada per dur a terme la hidròlisi de l'aigua, reacció que genera oxigen lliure com a producte residual i, a més, cedeix electrons a les clorofil·les dels centres de reacció. Aquests electrons seran transferits a través del PSII fins la plastoquinona (PQ), que al seu temps, els cedirà al citocrom *b*$_6$*f*. El citocrom és el complex que connecta els dos fotosistemes, oxidant el plastoquinol (plastoquinona reduïda) i reduint la plastocianina, que s'oxidarà en el PSI. La ferredoxina (Fd) és una proteïna oxidoreductasa que actua com a acceptor d'electrons del PSI en el costat estromàtic de la membrana tilacoidal. La funció de la Fd és connectar el PSI amb l'enzim final, la Fd-NADP$^+$ oxidoreductasa, que redueix el NADP$^+$ a l'acceptor final d'electrons, el NADPH, que serà utilitzat per la

fixació de CO_2 en el Cicle de Calvin (Haehnel, 1984; Nelson & Yocum, 2006; Foyer *et al.*, 2012).

El PSI és el major generador de ROS en les membranes tilacoidals, ja que és on es troben els acceptors d'electrons. En condicions adverses, com per exemple l'excés de llum, el flux d'electrons pot ser redistribuït, i passar de la Fd a la PQ en el transport cíclic d'electrons. Quan aquests acceptors no són suficients, una part del flux d'electrons és desviat de la Fd a l'oxigen, reduint-lo a $O_2^{\cdot-}$ via la reacció de Mehler. Aquest serà eliminat a través del cicle de l'aigua-aigua, en el qual hi estan implicats diversos antioxidants del cloroplast com la superòxid dismutasa (SOD) i l'ascorbat (apartat 1.2.). En resum, els electrons generats del consum d'una molècula d'aigua en el PSII redueixen l'oxigen tornant de nou a formar-se aigua en el PSI (**Taula 1**)(Asada, 1999).

Taula 1: Reaccions del cicle de l'aigua-aigua.

1	$2\ H_2O \rightarrow 4e^- + 4H^+ + O_2$	Fotòlisi de l'aigua en el PSII
2	$2\ O_2 + 2e^- \rightarrow 2\ O_2^{\cdot-}$	Reacció de Mehler
3	$2\ O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$	SOD
4	H_2O_2 + 2 ascorbat $\rightarrow$ 2 H_2O + 2 MDHA	APX
5	2 MDHA + $2e^- + 2H^+ \rightarrow$ 2 ascorbat	MDHAR
TOTAL	**$2\ H_2O + O_2^* \rightarrow O_2 + 2\ H_2O^*$**	

En condicions adverses, quan la captació de llum absorbida pels pigments fotosintètics excedeix la taxa del seu consum en el PSII, s'origina la formació del triplet de clorofil·la excitat en el PSII ($^3P_{680}$), capaç de generar $^1O_2$ al reaccionar amb l'oxigen (Fischer *et al.*, 2013). Els principals substrats oxidats pel $^1O_2$ són els àcids grassos poliinsaturats (PUFAs, de l'anglès *polyunsaturated fatty acids*) de les membranes (Triantaphylidès *et al.*, 2008). La peroxidació dels PUFAs per contacte amb $^1O_2$ genera una reacció en cadena en la qual es formen hidroperòxids lipídics i diversos aldehids, com per exemple l'àcid malondialdehid (MDA), capaços de

formar conjugats amb el DNA i les proteïnes inhibint-ne la seva funció biològica (Farmer & Mueller, 2013). Com a conseqüència, la peroxidació lipídica disminueix la fluïdesa de la membrana, augmentant-ne la seva permeabilitat i causant danys secundaris a les proteïnes de membrana (Møller *et al.*, 2007).

Les ROS generades en el cloroplast estan implicades en el procés de fotoinhibició. La fotoinhibició es dóna quan un excés de llum provoca la inhibició de l'activitat del PSII mitjançant la inactivació dels seus components com el complex generador d'oxigen i la degradació de la proteïna D1 (implicada en mantenir units els components del PSII) (Hakala *et al.*, 2005; Ohnishi *et al.*, 2005), ocasionant una reducció de la taxa fotosintètica. Les plantes disposen d'un sistema de reparació que consisteix en reciclar ràpidament la proteïna D1, per tal de prevenir el dany fotooxidatiu (Aro *et al.*, 1993; Nath *et al.*, 2013). No obstant, quan la taxa de dany en el PSII supera la taxa de reparació, per exemple en condicions d'estrès ambiental, es dóna la fotoinhibició (Murata *et al.*, 2007; Takahashi & Murata, 2008). El dany causat per la llum en el PSII provoca un augment de la producció de ROS que incrementen la fotoinhibició. Tot i que les ROS poden atacar els components del PSII (Miyao, 1994), aquest efecte està causat principalment per la inhibició dels mecanismes de reparació del PSII, impedint la síntesi *de novo* de la proteïna D1 (Nishiyama *et al.*, 2004, 2011).

Si bé la producció de ROS en el cloroplast és molt important qualitativament, ja que pot afectar processos vitals de la cèl·lula, quantitativament els peroxisomes són la font intracel·lular majoritària de ROS en forma de H_2O_2. Durant la fotorespiració, part del carboni que es perd com a conseqüència de l'activitat oxigenasa de la Rubisco és reciclat mitjançant el cicle del glicolat. En aquest, la reacció d'oxidació del glicolat a glioxilat a través de l'enzim glicolat oxidasa produeix grans quantitats de H_2O_2 (del Rio *et al.*, 2006; Foyer *et al.*, 2009).

Com a conseqüència de la sèrie de reaccions redox que es donen en la cadena de transport d'electrons durant la respiració, els mitocondris són una altra font important de ROS. Un desequilibri redox entre la cadena de transport d'electrons i l'ATP sintasa comporta la formació del $O_2^{\cdot-}$ en els complexes I i III de la membrana mitocondrial interna (Blokhina & Fagerstedt, 2010).

Les ROS també poden generar-se a l'apoplast, a través de les NADH i NADPH oxidases i peroxidases ubicades a la membrana plasmàtica, generalment implicades en l'esclat oxidatiu com a resposta en mecanismes de defensa (Torres & Dangl, 2005).

1.2. Antioxidants

Per tal de mantenir l'homeòstasi redox de la cèl·lula, les plantes disposen d'antioxidants encarregats d'eliminar les ROS evitant un possible dany oxidatiu (**Figura 2**). Generalment, aquests components poden estar dividits en: antioxidants lipòfils, basats en l'habilitat de tamponar els radicals lliures de certes molècules com els carotenoides (xantofil·les i β-carotè) i els tocoferols; en antioxidants hidròfils com el glutatió i l'ascorbat, així com els antioxidants enzimàtics, basats en les reaccions químiques catalitzades per enzims com la SOD, l'ascorbat peroxidasa (APX), la glutatió reductasa (GR) i la catalasa (CAT) a més, algunes espècies poden acumular flavonoides (entre ells els antocians) en fulles, els quals tenen cert potencial antioxidant.

Els carotenoides protegeixen l'aparell fotosintètic de la toxicitat del $^1O_2$, i poden eliminar-lo tant físicament com química (Triantaphylidès & Havaux, 2009; Ramel *et al.*, 2013). En l'eliminació física es produeix una transferència d'energia, mentre que en l'eliminació química es generen productes oxidats. El principal mecanisme per prevenir la formació de ROS en el PSII és la dissipació d'energia en forma de calor mitjançant el cicle de

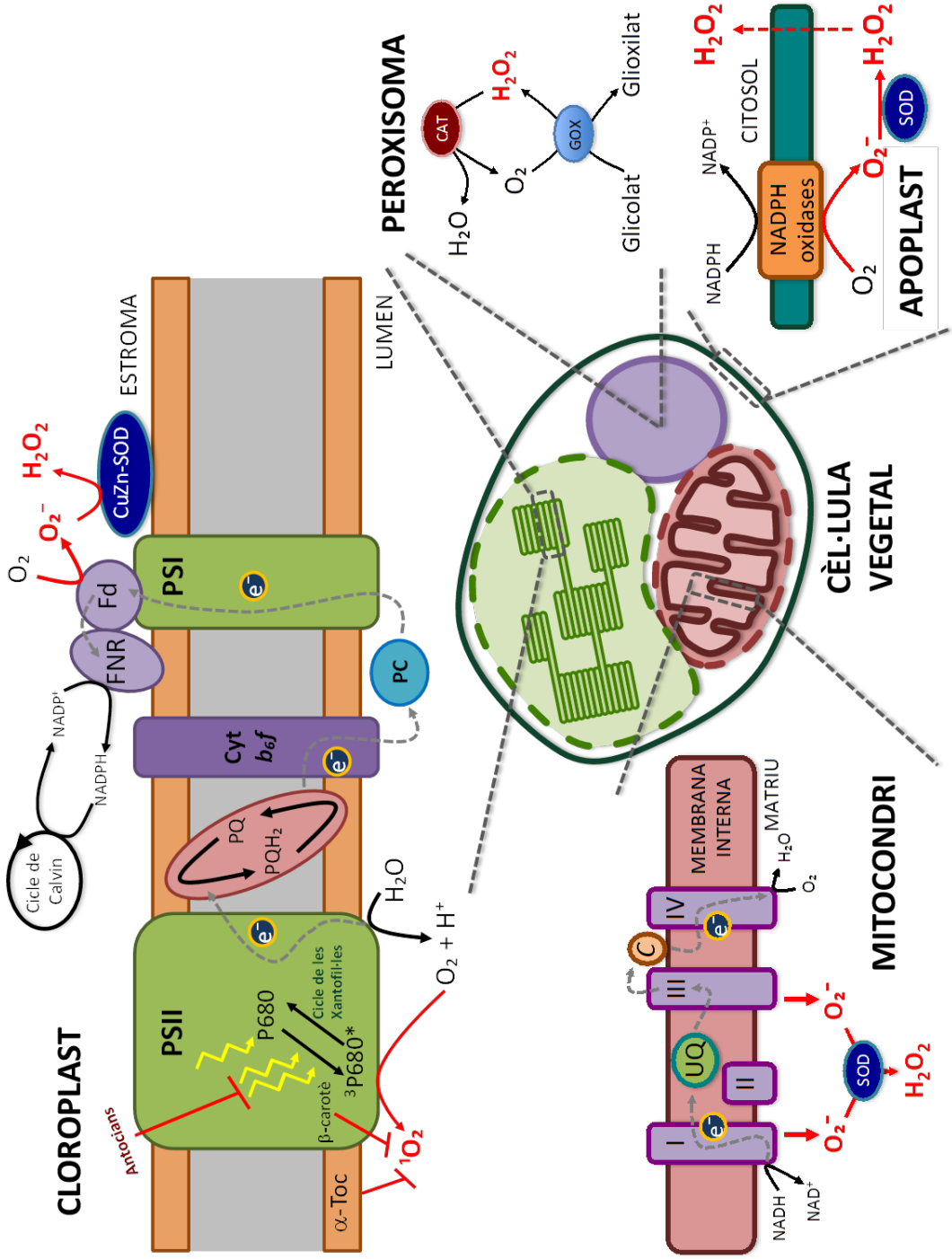

Figura 2: Formació de les espècies reactives de l'oxigen i principals mecanismes antioxidants en les cèl·lules vegetals. En el cloroplast: α-Toc, α-tocoferol; Cytb_6f, citocrom b_6f; P680, clorofil·la; Fd, ferredoxina; FNR, Fd-$NADP^+$ oxidoreductasa; PSI, fotosistema I; PSII, fotosistema II; PC, plastocianina; PQH_2, plastoquinol; PQ, plastoquinona; SOD, superòxid dismutasa; $^3P680^*$, triplet de clorofil·la excitat. En el mitocondri: I,II,III,IV, fan referència als 4 complexes de la cadena de transport d'electrons implicats en la respiració en els mitocondris; *C*, citocrom c ; UQ, ubiquinona. En peroxisomes: CAT, catalasa; GOX, glicolat oxidasa. ROS: $O_2^{\cdot-}$, anió superòxid; H_2O_2, peròxid d'hidrogen; $OH^{\cdot}$, radical hidroxil; $^1O_2$, singlet d'oxigen.

les xantofil·les. Les xantofil·les són pigments situats en els complexos antena del PSII capaços d'eliminar el triplet de clorofil·la excitat (Jahns & Holzwarth, 2012). En el cicle de les xantofil·les, la zeaxantina es forma a partir de la violaxantina, via l'intermediari anteraxantina, a través de l'enzim violaxantina de-epoxidasa, en condicions d'intensitat lumínica elevada. Un cop disminueix la intensitat de la llum, es reverteix el procés (Demmig-Adams & Adams, 1992, 1996; Horton *et al.*, 1996). En cas que, tot i l'acció d'aquest sistema, s'arribi a formar $^1O_2$, aquest pot ser eliminat per altres carotenoides com el β-carotè. El β-carotè està situat en els centres de reacció del PSII i és capaç d'eliminar el $^1O_2$ ja sigui mitjançant la transferència d'energia o per reaccions químiques en les que es generen productes d'oxidació com el β-carotè endoperòxid (Telfer, 2005; Ramel *et al.*, 2012a).

Els tocoferols són un grup d'antioxidants lipòfils units a les membranes (Wang & Quinn, 2000) que són sintetitzats únicament per organismes fotosintètics. Pertanyen al grup dels compostos de la vitamina E, on l'α-tocoferol n'és la forma predominant en fulles. Els tocoferols tenen dues activitats antioxidants principals: detoxificar el $^1O_2$ i aturar la reacció en cadena de la peroxidació lipídica. L'eliminació del $^1O_2$ es pot donar mitjançant un mecanisme físic de transferència de càrregues o químicament en el qual el $^1O_2$ oxida l'α-tocoferol a α-tocoferol quinona mitjançant el trencament irreversible de l'anell cromanol (Munné-Bosch & Alegre, 2002a; Trebst, 2003).

La seva propietat antioxidant de protegir les membranes tilacoidals de la propagació de la peroxidació lipídica rau en la capacitat del seu anell cromanol heterocíclic de donar l'hidrogen fenòlic als radicals lipídics generant un radical tocoferoxil (Kamal-Eldin & Appelqvist, 1996). Aquest últim pot reciclar-se de nou a α-tocoferol mitjançant el cicle de l'ascorbat-glutatió (Munné-Bosch & Alegre, 2002a; Falk & Munné-Bosch, 2010).

Les plastoquinones també estan implicades en l'eliminació de ROS en la cadena de transport d'electrons (Mubarakshina & Ivanov, 2010). Recentment s'ha descobert que el plastoquinol elimina el $^1O_2$ més eficientment que el tocoferol, però es troba en unes concentracions molt més baixes (Nowicka & Kruk, 2012), cosa que sembla indicar que el tocoferol n'és l'eliminador principal.

L'eliminació del $O_2^{\cdot-}$ en el PSI està catalitzada per l'enzim SOD, generant oxigen i H_2O_2. En els cloroplasts, la SOD es troba ancorada a la membrana tilacoidal en la isoforma CuZn-SOD, prop del PSI. La seva localització és clau per a una major eficàcia de la reacció, ja que el $O_2^{\cdot-}$ no pot difondre a través de les membranes a causa de la seva càrrega negativa (Asada, 1999).

El H_2O_2 resultant de la reacció catalitzada per la SOD és eliminat per l'APX, primer enzim implicat en el cicle de l'ascorbat-glutatió (**Figura 3**). En aquest primer pas del cicle, l'APX utilitza l'ascorbat com a donador d'electrons, reduint el H_2O_2 a aigua i generant la seva forma oxidada, el monodehidroascorbat. Aquest pot regenerar-se per la monodehidro-ascorbat reductasa utilitzant el NADPH com a equivalent de reducció. El monodehidroascorbat es pot dismutar espontàniament en dehidroascorbat, una forma oxidada de l'ascorbat, que a través de l'oxidació del glutatió i

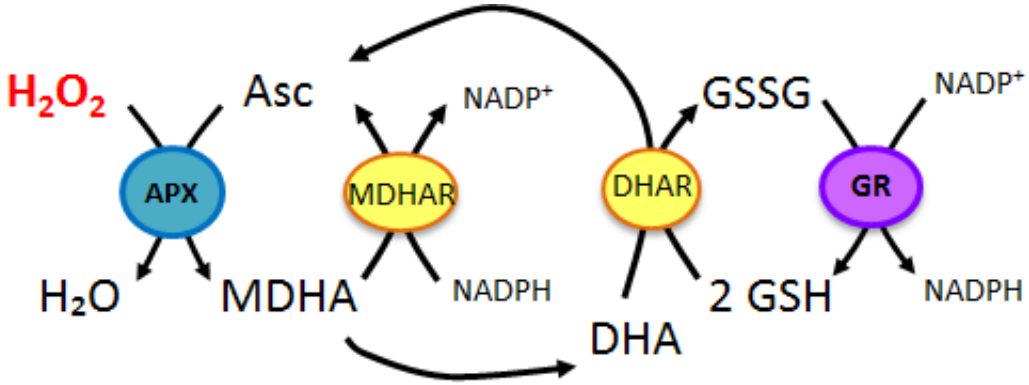

Figura 3: Cicles de l'ascorbat-glutatió, un dels principals sistemes antioxidants de les cèl·lules vegetals encarregat d'eliminar el peròxid d'hidrogen. Es troba tant als cloroplasts com als mitocondris, peroxisomes i citosol. Asc, ascorbat; APX, ascorbat peroxidasa; DHA, dehidroascorbat; DHAR, dehidroascorbat reductasa; GSH, glutatió; GSSG, glutatió oxidat; GR, glutatió reductasa; MDHA, monodehidroascorbat; MDHAR, monodehidroascorbat reductasa; H_2O_2, peròxid d'hidrogen.

l'enzim dehidroascobat reductasa, pot regenerar-se a ascorbat. La GR és l'enzim encarregat de mantenir quantitats suficients de glutatió en estat reduït (Foyer & Noctor, 2011). Aquest sistema d'eliminació de ROS es troba tant als cloroplasts com als mitocondris i peroxisomes (Caverzan *et al.*, 2012). En el peroxisoma però, la CAT és l'antioxidant majoritari encarregat de la detoxificació del H_2O_2, capaç de dismutar-lo directament en aigua i oxigen (Apel & Hirt, 2004; Mittler *et al.*, 2004).

Els flavonoides són una família de metabòlits secundaris en plantes que poden actuar com a antioxidants gràcies a la seva capacitat per cedir electrons o àtoms d'hidrogen. Molts aspectes d'aquesta suposada funció segueixen sent incerts i objecte d'investigació, com per exemple la seva rellevància en mig de tota la maquinària antioxidant del cloroplast. Així doncs, la funció antioxidant dels flavonoides encara és un tema de debat (Hernández *et al.*, 2009). Els antocians són flavonoides solubles causants de les coloracions vermelles en fulles que han estat proposats com a mecanisme de protecció de l'excés de llum en l'aparell fotosintètic. Poden acumular-se a l'epidermis de les fulles, a la capa del mesòfil en palissada, actuant com a pantalles protectores de llum, prevenint la sobreexcitació de la maquinària fotosintètica mitjançant la reducció de la quantitat de llum absorbida pel mesòfil del cloroplast (Feild *et al.*, 2001; Hoch *et al.*, 2001). Cal tenir en compte però, que el resultat pot variar entre espècies o depenent de les condicions de camp. Per exemple, en les fulles amb coloració vermella s'observen símptomes d'aclimatació a l'ombra en relació a les fulles verdes (fet consistent amb la seva funció fotoprotectora). Tot i així, les espècies que presenten fulles vermelles durant l'hivern i les que no conviuen en els mateixos hàbitats, sota les mateixes condicions de radiació, i presenten capacitats fotosintètiques similars, sense indicis d'un avantatge per les fulles vermelles (Hughes, 2011). També s'ha proposat la idea d'una possible coevolució entre les fulles vermelles i els insectes (Schaefer & Wilkinson, 2004). Aquest fet sembla indicar que els factors pels quals unes

espècies tornen les fulles vermelles durant l'hivern i altres no, segueixen sense estar clars.

1.3. Dany *vs.* senyalització

Inicialment, les ROS es consideraven productes tòxics derivats del metabolisme aeròbic a causa de la seva acumulació en condicions d'estrès ambiental sever, en el qual un augment de l'estrès oxidatiu podria causar danys irreversibles als components cel·lulars com el DNA, lípids, proteïnes i sucres (Van Breusegem & Dat, 2006; Asada, 2006). Tanmateix, en els últims anys s'ha demostrat que les plantes produeixen activament i en quantitats controlades les ROS com a molècules de senyalització per controlar processos com ara el creixement, el cicle cel·lular, la mort cel·lular programada, les respostes a estrès abiòtic, la defensa contra patògens i el desenvolupament (Mittler *et al.*, 2004, 2011; Miller *et al.*, 2010; Swanson & Gilroy, 2010; de Pinto *et al.*, 2012).

Per assegurar la correcta execució de les funcions de senyalització de les ROS i prevenir la toxicitat, és necessari un bon equilibri entre la producció de ROS i la seva eliminació mitjançant els antioxidants.

Condicions ambientals adverses com l'excés de llum, les temperatures extremes, la salinitat, el dèficit hídric, etc. originen un estrès en la planta que implica un increment de les quantitats de ROS (Cruz de Carvalho, 2008; Suzuki *et al.*, 2012). Una limitació de la fixació de CO_2 a causa de les condicions d'estrès condueix a una disminució en la reducció de carboni per la Rubisco en el cicle de Calvin. En conseqüència, no es consumiran equivalents de reducció (NADPH) provocant una manca d'acceptors d'electrons en la fotosíntesi ($NADP^+$) i, per tant, la cadena de transport d'electrons s'atura. Quan la taxa d'absorció d'energia de la llum pels pigments fotosintètics excedeix la taxa del seu consum en els cloroplasts, es produeix un augment de ROS (Takahashi & Murata, 2008). Altes concentracions de ROS poden provocar toxicitat, mentre que nivells relativament baixos poden ser utilitzats com a senyals d'aclimatació. S'ha

observat que l'estrès oxidatiu pot induir l'expressió de gens implicats en funcions antioxidants utilitzant el H_2O_2 com a senyal (Neill *et al.*, 2002; Gechev *et al.*, 2002; Navabpour *et al.*, 2003; Kravchik & Bernstein, 2013). Una altra de les funcions en senyalització de les ROS és la inducció de la mort cel·lular programada (de Pinto *et al.*, 2012) via un augment de H_2O_2 a l'apoplast per les NAD(P)H oxidases com a defensa contra patògens; o per mitjà del $^1O_2$, que la indueix no com a resultat d'un dany oxidatiu, sinó com a conseqüència de l'activació de gens (Kim *et al.*, 2012).

La senyalització per ROS està altament integrada amb la senyalització hormonal, permetent a les plantes regular processos de desenvolupament així com respostes adaptatives a senyals ambientals (Mittler *et al.*, 2011; Bartoli *et al.*, 2013). Aquesta interacció és altament complexa ja que també hi entren en joc les relacions entre les diferents hormones. Les citocinines, auxines i gibberel·lines (GAs) són hormones capaces d'integrar les senyals ambientals amb el creixement i desenvolupament de la planta. Les ROS poden interferir en el metabolisme de les auxines, produint canvis morfològics que ajuden a evitar els efectes nocius de possibles estressos ambientals. També intervenen en la senyalització per GAs, basada en la regulació de les proteïnes DELLA, proteïnes inhibidores del creixement i de les ROS, que també intervenen en la intercomunicació entre GAs i altres hormones com el JA i l'ABA. L'àcid salicílic (SA), l'àcid jasmònic (JA), l'etilè (ET) i l'àcid abscísic (ABA) són les hormones típicament conegudes com les implicades en respostes a l'estrès. El SA sol estar involucrat en respostes contra atacs de patògens. Un increment de ROS per les NAD(P)H oxidases en resposta a un estrès està relacionat amb l'acció del SA. L'ET indueix la generació de ROS, i al mateix temps, el H_2O_2 estimula l'expressió de proteïnes sensibles a l'ET i d'enzims implicats en la seva biosíntesi (Vandenabeele *et al.*, 2003). La relació entre les ROS i les hormones també pot donar-se a través d'altres components implicats en l'estat redox de la cèl·lula, com els antioxidants. Entre els gens de resposta al JA s'inclouen antioxidants i proteïnes associades amb defensa així com gens que

codifiquen per enzims implicats en el cicle de l'ascorbat-glutatió. L'ascorbat i el glutatió juguen un paper molt important en la senyalització hormonal a causa d'estar implicats en la regulació dels nivells de ABA i GAs. Alguns processos no només estan regulats per la interacció de les ROS amb una sola hormona sinó per vàries d'elles. Per exemple, el glutatió és un modulador de les vies de senyalització entre SA i JA (Noctor *et al.*, 2012), i en la mort cel·lular programada induïda pel $^1O_2$ hi estan implicades les vies de senyalització d'ET, SA i JA (Bartoli *et al.*, 2013; Fischer *et al.*, 2013). L'augment de H_2O_2 a les cèl·lules de guarda causat pel tancament d'estomes participa en la interacció entre l'ABA i l'ET en aquest procés (Cho *et al.*, 2009). També s'ha observat que la regulació redox de l'equilibri entre les vies de senyalització del ABA i les GAs podria influenciar processos com la germinació o la dormició, i la inducció floral.

Moltes de les respostes desencadenades per les ROS impliquen canvis en l'expressió de gens en el nucli i, per tant, requereixen una comunicació recíproca entre el cloroplast i el nucli. La informació sobre l'estat redox dels orgànuls proporcionada pels nivells de ROS en determinades situacions, com la fotoinhibició o la inducció de la senescència, pot portar a la inducció de gens implicats en mecanismes antioxidants, evitant així un possible dany fotooxidatiu o una incompleta remobilització de nutrients, respectivament (Pfannschmidt & Munné-Bosch, 2013). Una altra de les implicacions de les ROS en el procés de senescència és com a senyal d'inducció de la transcripció del gen *JUNGBRUNNEN1*, per mitjà del H_2O_2. JUB1 és un factor de transcripció que activarà altres gens implicats en l'eliminació de les ROS, reduint-ne els seus nivells intracel·lulars, i endarrerint el procés de senescència (Wu *et al.*, 2012).

L'acció de les ROS com a molècules de senyalització pot ser directa o indirecta. La naturalesa altament reactiva de les ROS dificulta molt una acció directa, per exemple, el $O_2^{\cdot-}$ no pot transferir passivament a través de les membranes a causa de la seva càrrega. Per contra, el $O_2^{\cdot-}$ pot ser

convertit en H_2O_2 que sí que és capaç de travessar membranes de forma passiva o a través de canals d'aigua (aquaporines). De forma indirecta les ROS tenen l'avantatge de poder intervenir en la regulació de senyals a través de l'acció hormonal, activant cascades de senyalització, ja sigui a través de Ca^{2+} o per fosforilació de proteïnes (MAP kinases) (Quan *et al.*, 2008; de Pinto *et al.*, 2012; Wrzaczek *et al.*, 2013) o mitjançant derivats de la seva oxidació, com per exemple la generació de peròxids lipídics. La peroxidació lipídica desencadenada per l'oxidació dels PUFA a la membrana per les ROS, genera missatgers anomenats oxilipines. Aquest grup inclou el JA, per tant, la regulació de gens via els missatgers derivats de la peroxidació lipídica són clau en la modulació de respostes de desenvolupament i defensa (Mueller & Berger, 2009). La peroxidació lipídica pot estar induïda no només per mecanismes no-enzimàtics (com el $^1O_2$), sinó també enzimàticament via l'activitat lipoxigenasa (LOX) (Devoto & Turner, 2003). Els gens per la LOX es troben sobre-expressats durant la inducció de la mort cel·lular programada via $^1O_2$ (Fischer *et al.*, 2013), corroborant la relació amb el JA, explicada anteriorment.

Un altre exemple és la formació de β-ciclocitral, compost derivat de l'oxidació del β-carotè pel $^1O_2$ en condicions d'alta intensitat lumínica. Aquest compost indueix canvis en l'expressió de gens associats a la defensa davant d'estressos, i la majoria d'aquests gens han estat identificats com a gens de resposta al $^1O_2$. A causa de la seva naturalesa volàtil i que es tracta d'un component soluble en lípids, podria estar implicat en la transferència d'informació fora del cloroplast (Ramel *et al.*, 2012b). Tots aquests fets demostren que la producció de ROS no és necessàriament un símptoma de dany cel·lular, sinó que pot representar una senyal per ajustar el metabolisme de la cèl·lula a les circumstàncies alterades.

2. Edat cronològica *vs*. edat fisiològica

L'edat cronològica d'una planta es defineix com el temps passat des de la germinació fins al moment actual, i pot mesurar-se en dies, setmanes, mesos o anys. No obstant, atès que les plantes estan exposades a un seguit de factors externs que poden afectar-ne les funcions vitals, una mesura més acurada de l'estat d'una planta podria ser l'edat fisiològica. L'edat fisiològica dependrà de l'edat cronològica, però també de les condicions ambientals a les que ha estat sotmesa aquesta planta al llarg de la seva vida. L'edat cronològica, a diferència de la fisiològica no ens dóna informació sobre els canvis en els components de l'aptitud biològica d'una planta, ni del seu estat de deteriorament fisiològic. Per tant, és possible que dos individus amb una mateixa edat cronològica, difereixin en la seva edat fisiològica i a l'inrevés.

2.1. Importància en fulles

La vida d'una fulla està marcada per un seguit de processos necessaris per al seu desenvolupament per tal d'arribar a assolir la seva funció com a òrgan fotosintètic. Aquests processos, des de la formació del primordi foliar fins a la senescència, estan molt lligats a l'edat cronològica de la fulla.

Es poden diferenciar tres fases en el procés de formació de la fulla (Sylvester *et al.*, 1996). La primera fase és l'organogènesi o iniciació del primordi foliar a partir de les cèl·lules perifèriques del meristema apical del brot. Aquest procés està induït per un augment del transport d'auxines. Seguidament es dóna la morfogènesi primària, que comprèn la divisió i la proliferació cel·lular. L'últim estadi correspon a la morfogènesi secundària, caracteritzada per l'expansió cel·lular. En les dues últimes etapes de morfogènesi es troben involucrades les citocinines i auxines, ja que són les hormones implicades en la divisió i l'expansió cel·lular, respectivament. Durant la primera fase d'iniciació, el primordi foliar està considerat com un òrgan heterotròfic ja que el seu aparell fotosintètic encara no s'ha desenvolupat del tot i tots els nutrients i fotoassimilats necessaris per al

desenvolupament foliar provenen d'altres òrgans. Tot i que en aquest estadi la fulla emergent sigui fotosintèticament activa, encara depèn dels fotoassimilats importats per al seu creixement (Turgeon, 1989).

La fulla jove segueix creixent fins assolir la forma i mida final, moment en el qual generalment està considerat com la transició a fulla madura. Per tant, la fulla madura és aquella que ha assolit la seva màxima expansió. En aquesta fase la fulla passa a ser fotosintèticament activa i totalment autòtrofa i es converteix en una font de carboni. La fulla madura és totalment competent per poder combatre els estímuls adversos als que pot estar sotmesa.

La senescència foliar està considerada l'últim estadi del desenvolupament d'una fulla, en el qual es produeixen canvis altament regulats a nivell molecular, cel·lular, bioquímic i fisiològic. Tot i que la inducció de la senescència està molt lligada a l'edat de la fulla així com al seu desenvolupament, també està altament lligada a les condicions ambientals.

Aquest procés s'indueix en fulles madures de manera gradual i està caracteritzada per tres fases. El procés s'inicia partint de vies de senyalització que desembocaran en un canvi d'expressió dels gens associats a la senescència (SAG). Com a conseqüència d'aquest canvi transcripcional, es produeix una etapa de remobilització de nutrients, on els components de la fulla senescents són descompostos per tal d'exportar-se i nodrir altres parts de la planta com primordis foliars, fulles emergents, òrgans reproductius o òrgans de reserva. Per tant, durant aquesta fase es donen els canvis més importants en la fulla tant a nivell molecular i estructural incloent la degradació de clorofil·les, la disminució de l'activitat fotosintètica i la pèrdua de la integritat cel·lular. La correcta regulació d'aquesta reestructuració és vital per tal d'optimitzar eficientment el procés de reciclatge de components cel·lulars. La fase final de la senescència foliar s'esdevé com a resultat de l'acumulació de factors inductors de la mort

cel·lular que comporten la pèrdua de la integritat cel·lular i, finalment, la mort. Aquesta última etapa es dóna quan el nucli i la maquinària relacionada amb els àcids nucleics es degrada, provocant la mort cel·lular i conseqüentment la pèrdua de funcionalitat de la fulla (Lim *et al.*, 2007).

Qualsevol fulla, al llarg de la seva vida passa per totes aquestes etapes del desenvolupament foliar. En aquest sentit, saber l'edat cronològica de la fulla pot ser-nos d'utilitat per determinar de manera aproximada en quina etapa del desenvolupament es troba la fulla. L'instant en el qual té lloc cadascun dels processos pels que ha de passar una fulla al llarg de la seva vida determinarà la seva longevitat foliar, que es defineix com el temps transcorregut des de l'aparició d'una fulla fins la seva mort. Per tant, la longevitat foliar pot variar tant entre espècies com dins de la mateixa planta (**Figura 4**).

Al comparar entre espècies s'observen diferents estratègies respecte al desenvolupament foliar. Les fulles d'arbres de fulla caduca tenen una fotosíntesi més elevada, assoleixen l'etapa de senescència a la tardor, havent patit abscisió abans del hivern amb una curta vida foliar. Per altra banda, les fulles de plantes perennes tot i tenir una capacitat fotosintètica més baixa, ho compensen amb una vida foliar més llarga i es mantenen durant tot l'hivern. La longevitat foliar difereix entre les diferents estratègies de vida de les plantes (Diemer & Körner, 1996; Kikuzawa & Ackerly, 1999).

El que determinarà la diferència en la longevitat foliar entre fulles dins una mateixa planta serà el moment quan es donen les varies etapes del seu desenvolupament. En aquest sentit, la senescència és un procés molt influent. Encara que la senescència foliar es dóna com un procés de mort programada i és depenent de l'edat (Jansson & Thomas, 2008; Yoshida, 2003), també pot estar induïda per senyals ambientals com ara temperatures extremes, alta radiació, exposició a ozó, manca d'aigua o nutrients i infeccions per paràsits (Munné-Bosch *et al.*, 2001; Chon *et al.*,

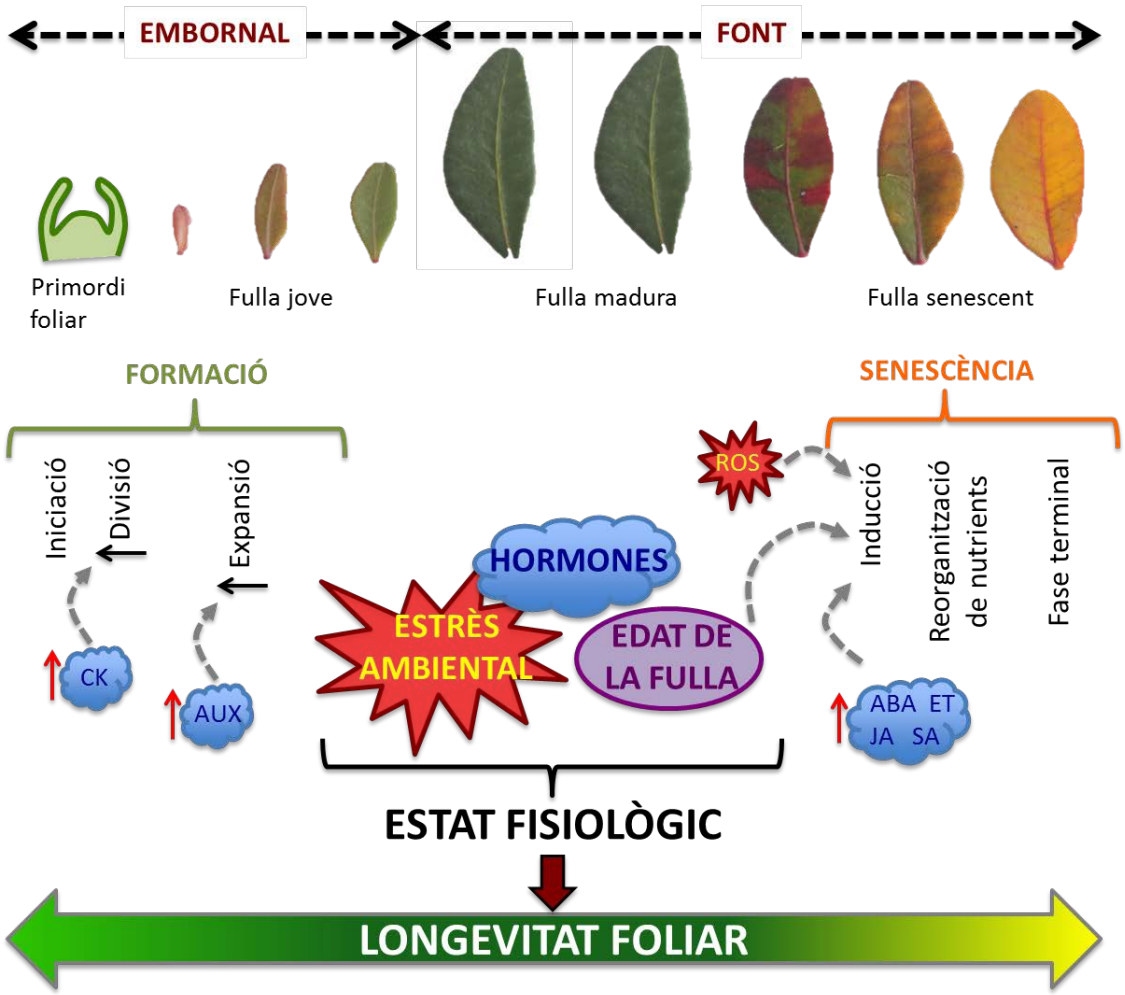

Figura 4: Esquema del desenvolupament foliar. La longevitat foliar pot estar afectada tant per factors endògens (com l'edat de la fulla o la regulació hormonal), com per factors exògens (com els estressos ambientals al que pot estar sotmesa). ABA, àcid abscísic; JA, àcid jasmònic; SA, àcid salicílic; AUX, auxines; CK, citocinines; ROS, espècies reactives del oxigen; ET, etilè.

2002; Munné-Bosch & Peñuelas, 2003; Yang *et al.*, 2003; Gielen *et al.*, 2007; Abreu & Munné-Bosch, 2008). Tot i que es tracta d'un procés deleteri, la senescència està implicada en la maximització de la fotosíntesi a nivell de planta sencera (Hikosaka *et al.*, 2010). A través de la remobilització de nutrients, la senescència contribueix a garantir la supervivència de tota la planta en condicions adverses i a optimitzar la producció de descendència (Munné-Bosch & Alegre, 2004).

Els factors exògens com els estressos ambientals, afecten a l'estat redox de la fulla provocant canvis en el seu estat fisiològic, que alhora influirà en la longevitat foliar. D'aquesta manera, per una banda l'edat cronològica d'una fulla ens pot ajudar a determinar les fases del seu desenvolupament

així com determinar el moment en el qual s'inicia la senescència foliar (ja que està regulada per l'edat de la fulla i el seu estat de desenvolupament). Per l'altra, l'edat fisiològica, que engloba tots els successos pels que ha passat una fulla al llarg de tota la seva vida, pot ser un factor determinant en la longevitat foliar.

2.2. Importància en planta sencera

Les plantes amb flor es poden diferenciar en anuals i biennals o perennes segons el tipus d'estratègies de vida que segueixen. Les plantes anuals o biennals són aquelles que completen el seu cicle de vida en una sola etapa reproductiva. Les espècies que presenten aquesta estratègia dediquen tota la seva vida en assolir la biomassa necessària per invertir tots aquests recursos acumulats en una sola etapa reproductiva, seguida per la mort de la planta sencera. Aquesta estratègia, per tant, implica la transformació de tots els meristemes indeterminats en meristemes determinats florals, perdent així la capacitat de seguir produint brots vegetatius. En el cas de les plantes anuals i biennals és fàcil poder relacionar l'edat cronològica de la planta amb el seu estat de desenvolupament. Cal tenir en compte, però, que no es tracta d'una premissa que es compleix en tots els casos, ja que també es troben sota la influència dels estressos ambientals que poden fer variar la seva longevitat (Thomas *et al.*, 2000).

Per contra, les plantes perennes són aquelles capaces de reproduir-se vàries vegades al llarg de la seva vida i mantenir una quantitat de meristemes indeterminats que seguiran formant fulles i brots, atorgant-los una gran longevitat. El rang d'anys viscuts en plantes perennes pot arribar des dels 10 anys en algunes espècies herbàcies, fins als 5000 anys com és el cas de l'espècie *Pinus longaeva* (les espècies clonals poden arribar a viure encara molts més anys, però en aquesta tesi només es tindran en compte plantes individuals) (Thomas, 2013). En canvi, els humans poden arribar a una longevitat d'uns 122 anys. Aquesta diferència tant gran en l'escala temporal entre l'espècie humana i el món vegetal, en concret les plantes

perennes, suposa un repte en quan a l'estudi de l'edat. Així i tot, la fascinació per la longevitat dels arbres ens ha portat a buscar mètodes per a determinar-ne la seva edat cronològica exacta. Un dels més coneguts és el comptatge d'anells en els troncs dels arbres (Stokes & Smiley, 1996) o en les arrels en el cas de les espècies perennes herbàcies (Arx & Dietz, 2006). El fet que l'augment de la mida en una planta sigui un efecte intrínsec del seu creixement, ens pot servir per a determinar-ne la seva edat d'una manera indirecta, amb l'excepció d'aquelles que es troben en estadis molt avançats del desenvolupament ja que la taxa de creixement tendeix a disminuir (Johnson & Abrams, 2009).

Malgrat les evidències de la gran longevitat en aquestes espècies, un dels misteris avui en dia encara pendent de resoldre és si les plantes perennes envelleixen (Munné-Bosch, 2007, 2008; Peñuelas & Munné-Bosch, 2010; Thomas, 2013). El concepte d'envelliment es defineix com l'acumulació de canvis en el desenvolupament vegetal responsable d'alteracions lentes, progressives i seqüencials que acompanya la planta al llarg de la vida (Noodén & Guiamet, 1996). Per tant, de manera innegable una planta al llarg de la seva vida va acumulant canvis en el desenvolupament responsables d'alterar gradualment el seu estat fisiològic. Aquest canvis estan causats no tant sols pel pas del temps, sinó també per les condicions ambientals a les que poden estar exposades. Al temps que la planta creix en edat i mida, càrregues fisiològiques com la demanda de nutrients i d'aigua augmenten. En arbres més vells s'ha detectat una disminució de la taxa fotosintètica (Yoder *et al.*, 1994). Amb l'augment de la mida de la planta amb el pas dels anys, la conductància hidràulica cap a les fulles per a la transpiració disminueix, causant una reducció de la conductància estomàtica que, al mateix temps, provoca la conseqüent disminució de la fotosíntesi i per tant, del creixement (Magnani *et al.*, 2000; Day *et al.*, 2001; Ryan *et al.*, 2006).

L'estat hormonal és un altre dels factors que es veuen afectats amb el pas del temps. Al comparar pins de diferents edats, Valdés *et al.* (2004) van observar una disminució del contingut d'auxines i citocinines en les fulles dels arbres més vells. Una disminució dels nivells de citocinines podria ajudar a contribuir a la reducció de la fotosíntesi, gràcies al paper clau d'aquestes hormones en la generació del cloroplast (Zubo *et al.*, 2008; Okazaki *et al.*, 2009). Un altre factor probablement implicat en la reducció de la fotosíntesi és un augment dels nivells d'ABA amb l'edat (Valdés *et al.*, 2004; Munné-Bosch & Lalueza, 2007), ja que indueix el tancament d'estomes.

Altres canvis observats amb l'edat han estat l'augment de l'estrès oxidatiu (Munné-Bosch & Alegre, 2002b; Munné-Bosch & Lalueza, 2007), la pèrdua del vigor de les gemmes florals (Oñate & Munné-Bosch, 2010), la viabilitat i la producció de llavors (Müller *et al.*, 2014), així com canvis en els patrons d'expressió gènica i metilació del DNA (Day *et al.*, 2002; Mencuccini *et al.*, 2014).

Per si l'equació no fos ja prou complicada, el fet de viure a la natura, deixa les plantes exposades als estressos ambientals com ara el dèficit hídric i la sequera (Flexas & Medrano, 2002; Munné-Bosch *et al.*, 2003; Hernández *et al.*, 2004; Hernández *et al.*, 2006; Müller *et al.*, 2006), estrès salí (Tounekti *et al.*, 2011), temperatures extremes (Yu *et al.*, 2002; Lee & Lee, 2000; Asensi-Fabado *et al.*, 2013), i/o acció d'herbívors i patògens. L'efecte d'aquests estressos comporta l'augment dels nivells de ROS que les plantes intentaran revertir prenent un seguit de mesures que afectaran l'estat fisiològic d'aquestes.

Indubtablement una planta va acumulant canvis en el desenvolupament al llarg de la seva vida que alteraran el seu estat fisiològic a mesura que va envellint. Tot i així, sembla ser que el factor mida és més important com a factor determinant en la inducció d'aquests canvis que no pas l'edat de la planta (Matsuzaki *et al.*, 2005; Mencuccini *et al.*, 2005; Peñuelas, 2005;

Vanderklein *et al.*, 2007; Oñate & Munné-Bosch, 2008; Müller *et al.*, 2014). A causa de tota aquesta controvèrsia, fins al dia d'avui encara no s'ha pogut resoldre si les plantes perennes envelleixen. No obstant, aquests estudis posen de manifest la importància de la determinació de mesures a nivell fisiològic, com per exemple l'estrès oxidatiu, per a una aproximació més exacta de l'edat fisiològica de la planta, en contraposició a l'edat cronològica.

2.3. Estrès oxidatiu i edat

L'estrès oxidatiu en plantes pot estar causat per molts motius, tant per factors intrínsecs del propi metabolisme, com per factors extrínsecs, biòtics o abiòtics. Amb el pas del temps, la repetició d'aquests episodis d'estrès i la conseqüent exposició a un dany oxidatiu poden ser causants d'un efecte deleteri en les plantes, induint-ne una degeneració fisiològica amb el pas del temps. Aquest fet es coneix com a *teoria dels radicals lliures de l'envelliment* (proposada per Harman el 1956). Tot i la seva importància, existeixen pocs estudis que investiguin com pot afectar la possible acumulació d'efectes deleteris en l'organisme amb l'edat, en resposta a estressos ambientals.

Escasses són les evidències que relacionen l'estrès oxidatiu amb l'edat. Munné-Bosch & Alegre (2002b) varen observar un augment de l'estrès oxidatiu en el cloroplast amb l'edat en l'espècie *Cistus clusii*, una planta arbustiva perenne. Les plantes d'edats més avançades presentaven nivells més elevats de peroxidació lipídica juntament amb una disminució de pigments fotosintètics i dels nivells d'antioxidants. Estudis posteriors realitzats amb la mateixa espècie mostren l'augment de la susceptibilitat d'aquest arbust a l'estrès oxidatiu amb l'edat quan els individus es troben sota condicions ambientals adverses de sequera (Munné-Bosch & Lalueza, 2007) i/o excés de llum (Hernández *et al.*, 2011). En el primer estudi es va observar que a mesura que passava el temps, les plantes de *C. clusii* incrementaven la mida, però les fulles emergents en les plantes més velles

mostraven taxes de creixement reduïdes, una àrea menor i un augment de la taxa de massa per àrea (LMA) en comparació a les plantes més joves. Per altra banda, els individus de més edat també presentaven símptomes de fotoinhibició i nivells més baixos de pigments i antioxidants. En el segon estudi es va concloure que un excés de llum incrementava els nivells endògens de flavonoides en *C. clusii*, un procés que augmenta amb l'edat a causa de l'estrès per excés de llum.

En l'altre extrem estan els estudis realitzats amb *Borderea pyrenaica*, una espècie herbàcia perenne que sembla eludir l'envelliment (Oñate *et al.*, 2011; Morales *et al.*, 2013). Al comparar individus amb edats cronològiques molt allunyades (juvenils de menys de 50 anys amb plantes madures d'entre 100 i 300 anys) no presentaven símptomes d'un major dany oxidatiu amb l'edat. Els nivells de clorofil·les, l'eficiència màxima del PSII i els nivells de peroxidació lipídica, tots ells indicadors de dany oxidatiu, es van mantenir constants independentment del sexe i l'edat, el que suggereix l'absència d'estrès oxidatiu associat a l'edat a nivell d'organisme.

Encara són molts els estudis necessaris per poder arribar a esclarir una mica més com pot arribar afectar a l'estrès oxidatiu a tota la planta sencera al llarg del temps. Centrant-nos en la teoria dels radicals lliures, en aquesta tesi es suggereix la mesura de l'estrès oxidatiu com a possible indicador de l'edat fisiològica de la planta, centrant-nos sobretot en el paper del MDA, sota la premissa que un increment en els nivells d'estrès oxidatiu indicarà una major edat fisiològica.

3. Dimorfisme sexual en plantes

En les plantes dioiques els òrgans reproductors femenins i masculins apareixen en individus separats. Tant mascles com femelles generen flors, però les femelles generalment han d'invertir més recursos que els mascles a causa de la formació posterior de llavors. Aquestes variacions en els costs reproductius causen un seguit de diferències, tant morfològiques, fisiològiques com en els trets adquirits al llarg de la vida de la planta,

relacionades amb el sexe. Aquests trets que diferencien els mascles i les femelles són considerats com a dimorfisme sexual.

3.1. Esforç reproductiu

Els components més importants per a l'aptitud biològica d'una planta són: el creixement i manteniment d'un individu capaç de competir pels recursos (en què la mida vegetativa n'és determinant), les estratègies per evitar els predadors (que es pot aconseguir mitjançant la inversió en defenses) i reproduir-se (que està relacionada amb l'èxit assolit per les altres dues). Cadascuna d'aquestes activitats requereix una despesa d'energia provinent d'una font limitada; per tant, la inversió en una d'elles resultarà en pèrdues del potencial d'inversió en les altres. Si la planta empra l'energia i els recursos en un dels processos, disminuirà l'assignació de recursos per les altres dues (Obeso, 2002). Aquest fet es coneix com el "principi d'assignació", proposat per Levins (1968). En base a aquest principi, l'esforç reproductiu ha estat definit com les pèrdues del futur potencial reproductiu com a conseqüència de la inversió actual en reproducció (Jönsson, 2000).

Per tal d'assolir la competència per a reproduir-se, serà necessària l'adquisició i emmagatzematge d'energia (Bond, 2000), a més d'una posterior despesa energètica pels costs de formació de les estructures relacionades amb la reproducció (Obeso, 2002). Per aquest motiu, l'esforç reproductiu està estretament lligat al model estratègic de supervivència (anuals, biennals o perennes). Les plantes anuals i biennals gestionen els recursos de manera que durant el primer any inverteixen tota l'energia en creixement i després tots els recursos que han anat acumulant s'utilitzen per a la reproducció. Per contra, les plantes perennes distribueixen els recursos d'una forma més gradual. Durant el període juvenil els recursos es reparteixen entre creixement i defensa fins a arribar a una certa mida on aleshores una part es destinaran a la reproducció, però no tots, de tal manera que la planta pot seguir creixent contínuament.

Són molts els estudis que demostren el cost que suposa la reproducció (Obeso, 2002). En el cas de les plantes dioiques sembla ser que les femelles presenten un major esforç reproductiu com a conseqüència de la producció de llavors i fruits, mentre que els mascles només produeixen flors (Hancock & Bringhurst, 1980; Leigh & Nicotra, 2003; Zunzunegui *et al.*, 2006). Generalment, això genera diferències entre sexes en un seguit de trets no només pel que fa a les característiques reproductives sinó també a nivell vegetatiu. Existeixen evidències que demostren que la major inversió de nutrients en la reproducció en femelles es dóna a expenses del creixement vegetatiu, i per tant, les femelles presenten una productivitat més baixa que els mascles (Gross & Soule, 1981; Ågren, 1988; Korpelainen, 1992; Cipollini & Whigham, 1994; Zluvova *et al.*, 2010). Així i tot, altres estudis han demostrat l'existència d'excepcions, com és el cas de les herbàcies perennes, que no es comporten seguint la hipòtesi de l'assignació de recursos, ja que les femelles generalment són el sexe més vigorós. Aquestes diferències entre plantes llenyoses i herbàcies podrien estar relacionades amb pressions de selecció diferent (Obeso, 2002). D'altra banda, també s'han trobat casos en els quals simplement no s'observen diferències entre sexes (Harris & Pannell, 2008; Barrett & Hough, 2013).

Tot i la gran controvèrsia deguda a la diversitat de resultats obtinguts en l'estudi de l'esforç reproductiu, Tuomi *et al.* (1983) van postular que els costs reproductius no tenien perquè ser necessàriament observats, ja que els organismes reproductors podrien desenvolupar mecanismes compensatoris per reduir aquests costs. L'augment de la taxa fotosintètica en les fulles dels individus femella respecte els mascles els podria ajudar a suportar la demanda de nutrients necessària per a la reproducció. Obeso *et al.* (1998) van observar però, que aquesta major taxa fotosintètica es donava en les branques no reproductives de la planta mentre que en les reproductives era més baixa, posant de manifest la importància de l'estudi de l'esforç reproductiu a nivell de mòdul (brots). Un altre possible mecanisme compensatori podria ser la capacitat fotosintètica de les

estructures reproductives, contribuint al seu propi manteniment, però els casos en els quals la taxa fotosintètica neta és positiva són molt pocs (Watson & Casper, 1984; Williams *et al.*, 1985; Galen *et al.*, 1993). La disponibilitat de meristemes i l'arquitectura de la planta també poden estar implicats com a mecanismes compensatoris. La disponibilitat dels meristemes és un paràmetre important que afecta la distribució de recursos i els costs de reproducció. Si la planta desenvolupés estructures vegetatives a partir dels seus meristemes o els mantingués inactius, aquesta podria augmentar les seves fonts de carboni per poder cobrir els costs de la reproducció (Watson, 1984; Geber, 1990). Un altre dels mecanismes utilitzats per les femelles és la diferència en la regulació del temps invertit en creixement i en reproducció. Mentre les femelles dediquen molt més temps en la inversió de recursos en la fase de reproducció (ja que aquesta també inclou la fase de fructificació), els macles alhora van augmentant el seu creixement vegetatiu. Les femelles però, compensen les diferències en creixement quan arriba l'etapa de floració produint més fulles (Ågren, 1988; Popp & Reinartz, 1988; Delph, 1990). Per últim, un dels mecanismes compensatoris proposats és la reabsorció de nutrients de les estructures reproductores senescents (Goldman & Willson, 1986; Chapin, 1989; Ashman, 1994).

3.2. Resposta a estressos ambientals

Com hem vist en l'apartat anterior, les plantes dioiques presenten dimorfisme sexual a causa dels costs de l'esforç reproductiu, generalment en detriment de les femelles. Per tant, no és estrany pensar que també podrien existir diferències en les capacitats de cada gènere per afrontar possibles condicions ambientals adverses. Seria d'esperar que a causa d'una major inversió dels recursos en la reproducció per part de les femelles, sota condicions d'estrès, els mascles estiguessin afavorits (Korpelainen, 1999).

Alguns estudis en plantes dioiques han provat la resposta específica de gènere contra tot un seguit d'estressos ambientals. Creixent en condicions

adverses, s'ha suggerit que les femelles pateixen uns efectes negatius majors i presenten una capacitat de tolerància menor que els mascles. En el cas de l'estrès per sequera els mascles presenten un creixement i una capacitat fotosintètica major que en les femelles, així com una major eficiència en l'ús de l'aigua (Li *et al.*, 2004; Varga & Kytoviita, 2008; Xu *et al.*, 2008; Rozas *et al.*, 2009; Chen *et al.*, 2010a).

En contraposició, estudis duts a terme amb plantes de *Pistacia lentiscus* mostren un ús més eficient de l'aigua per part de les femelles, com a possible mecanisme per contrarestar l'alt requeriment hídric per a la formació dels fruits (Correia & Barradas, 2000).

Altres estudis fets amb la mateixa espècie coincideixen amb l'anterior en establir que les femelles presenten una major sensibilitat a l'estrès per sequera (Correia *et al.*, 1992; Jonasson *et al.*, 1997; Barradas & Correia, 1999).

Xu *et al.* (2008) van demostrar que les femelles de *Populus cathayana* eren més susceptibles a patir més danys en termes de creixement, fotosíntesi i estrès oxidatiu sota condicions de sequera i altes temperatures. En aquesta mateixa espècie, Zhang *et al.* (2011) també van observar una resposta diferencial a nivell morfològic, fisiològic i bioquímic entre sexes en condicions de baixes temperatures. Els mascles estressats per baixes temperatures presentaven una millor capacitat d'ajust osmòtic, major activitat d'enzims antioxidants així com de contingut de pigments en comparació amb les femelles sota les mateixes condicions d'estrès.

Tot i que la majoria d'estudis semblen indicar que les femelles són més sensibles als estressos ambientals com la sequera (Li *et al.*, 2004; Varga & Kytoviita, 2008; Xu *et al.*, 2008; Rozas *et al.*, 2009), les baixes temperatures (Zhang *et al.*, 2011), la salinitat (Chen *et al.*, 2010a), l'enriquiment de CO_2 atmosfèric (Wang & Curtis, 2001), l'augment de radiació ultraviolada-B (Xu *et al.*, 2010), la deficiència de nutrients (Montesinos *et al.*, 2012), l'excés de manganès i en la combinació de varis

estressos (Chen *et al.*, 2010b; Zhang *et al.*, 2010), altres estudis en perennes herbàcies indiquen que les femelles poden ser igual o més resistents que els mascles (Oñate & Munné-Bosch, 2009; Morales *et al.*, 2013).

3.3. Estrès oxidatiu i dimorfisme sexual

Malgrat l'evidència d'una resposta diferencial entre sexes en condicions d'estrès per una banda, i l'efecte dels estressos ambientals en l'augment de dany oxidatiu en les plantes per l'altra, pocs són els estudis que han intentat determinar l'existència de diferències lligades al sexe en resposta a l'estrès oxidatiu.

La major inversió en reproducció per part de les femelles ha estat associada generalment a un desavantatge, que fins i tot en alguns casos pot provocar un augment de l'estrès oxidatiu i lesions cel·lulars, sobretot en condicions adverses (Xu *et al.*, 2008; Chen *et al.*, 2010a,b; Zhang *et al.*, 2010, 2011). En l'estudi dut a terme per Xu *et al.* (2008) les femelles presentaven continguts més alts de MDA que els mascles en sequera, suggerint que els mascles posseeixen una major resistència ja que mostraven una habilitat antioxidant més elevada. En un estudi fet pel mateix grup, els mascles tornaven a presentar major resistència en termes d'eficiència antioxidant i contingut d'antocians que les femelles, en ser exposades a llum ultraviolada-B (Xu *et al.*, 2010). Una major capacitat dels mecanismes antioxidants dels mascles en condicions adverses (com per exemple en l'activitat dels enzims del cicle de l'ascorbat-glutatió o de la SOD) podria explicar les variacions en la capacitat d'eliminació de ROS en els dos sexes així com un major estrès oxidatiu observat en femelles indicat pels nivells de peroxidació lipídica (Chen *et al.*, 2010b; Zhang *et al.*, 2011).

La resposta diferencial entre sexes a l'hora de combatre l'estrès oxidatiu però, no sempre en resulta amb un desavantatge per a les femelles, sinó que s'han observat casos on aquestes poden ser tant o més resistents que els mascles. Morales *et al.* (2013) van investigar les possibles diferències

relacionades amb el sexe en l'estrès oxidatiu i la defensa antioxidant en *Borderea pyrenaica*. Sorprenentment van observar que les femelles amb edats superiors a 100 anys mostraven un millor rendiment en resposta a la dessecació severa, com indiquen un millor estat hídric i la major retenció de pigments fotosintètics i de nivells de xantofil·les.

Tot i els estudis realitzats, encara avui no som capaços d'entendre els possibles mecanismes diferencials entre sexes pels quals les plantes s'enfronten als estressos ambientals i combaten l'estrès oxidatiu. La gran diversitat de resultats, no tan sols entre hàbitats i condicions ambientals, sinó també entre espècies, dificulta molt l'elaboració d'un patró general vàlid per a tots els casos.

4. Models d'estudi

Pistacia lentiscus L.

La *Pistacia lentiscus* L., coneguda vulgarment amb el nom de llentiscle, és una espècie perenne de la família de les Anacardiaceae. Aquesta espècie té una gran importància ja que ha estat molt utilitzada des de l'antiguitat per la resina que se'n pot extreure i les propietats antioxidants de les seves fulles (Bozorgi *et al.*, 2013).

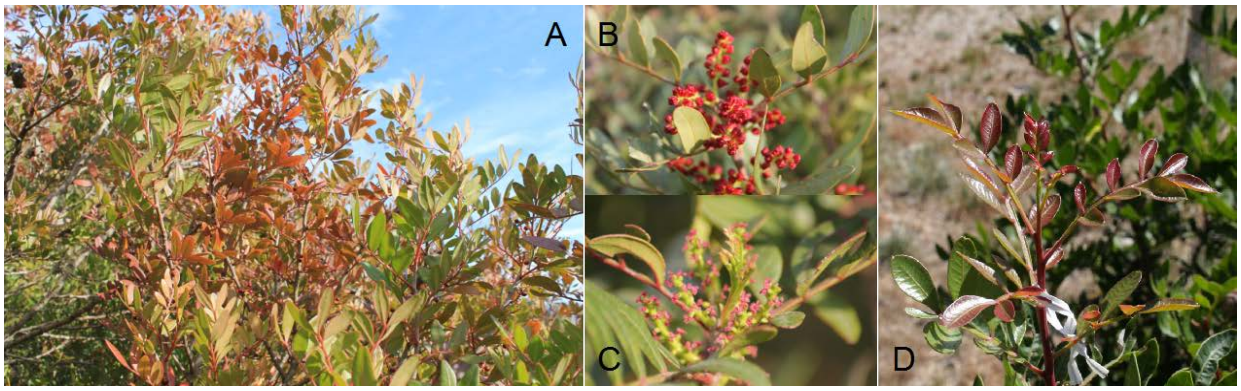

Figura 5: (A) Arbusts de *Pistacia lentiscus* L. en els camps experimentals de la Facultat de Biologia, Universitat de Barcelona (Catalunya). Flors masculina (B) i femenina (C) i fulles emergents (D) de plantes de *P. lentiscus*.

Forma arbustos que són capaços d'assolir més de 2 metres d'alçada en les zones més humides i protegides. El llentiscle està extensament distribuït al llarg de la conca Mediterrània, generalment en boscos escleròfils i brolles, molt abundant en zones de sotabosc en pinars i alzinars. Són molt característiques les fulles escleròfil·les compostes amb folíols coriacis que surten a la primavera i a la tardor. En el moment que broten les noves fulles aquestes adopten una coloració vermella molt particular (**Figura 5**). La longevitat de les fulles crescudes en condicions climàtiques mediterrànies sol ser més o menys d'un any, on la senescència foliar serveix com a remobilització de nutrients des de les fulles més velles a les més joves (Diamantoglou & Kull, 1988). Aquest fet contribueix a la supervivència de la planta sencera durant condicions climatològiques adverses (Munné-Bosch & Peñuelas, 2003).

El llentiscle és una espècie dioica que floreix a principis de la primavera, al març. Les inflorescències apareixen primer en els mascles, petites i vermelles, que es mantindran a les plantes fins al maig. En les femelles, aquestes sorgeixen al març i els fruits es desenvolupen de juny a febrer.

Aquesta espècie és un bon model de planta perenne per poder estudiar com afecta l'estrès oxidatiu causat per condicions ambientals adverses a l'estat fisiològic de les fulles i plantes de diferents edat. Però també ens proporciona la possibilitat d'investigar l'efecte de l'esforç reproductiu a les defenses de la planta en condicions d'estrès.

Fagus sylvatica L.

El faig és un arbre caducifoli de la família Fagaceae que forma boscos en obagues humides, encara que també es pot trobar barrejat en altres tipus de boscos (pinedes, avetoses, rouredes...) (**Figura 6**). Pot arribar a assolir els 30 m d'alçada i una longevitat de més de 900 anys (Thomas, 2013). Les seves fulles són ovato-el·líptiques, translúcides, ondulades i ciliades al marge i l'època de floració és del març al maig.

A Europa és la principal espècie d'arbre de fulla ample en els boscos de l'Europa occidental i central, que comprèn 12 milions d'hectàrees. El faig es distribueix des del bosc boreal d'Escandinàvia fins a les muntanyes de la regió del sud de la Mediterrània. Els hiverns freds i la sequera a l'estiu són els principals factors d'estrès per a aquesta espècie en el seu hàbitat (García-Plazaola & Becerril, 2001). Es tracta d'un arbre capaç d'alterar la seva arquitectura i creixement en funció de les condicions adverses a les que pot estar sotmès, com per exemple la poca disponibilitat de llum (Messier *et al.*, 1999). D'una banda, aquest tipus de creixement pot millorar la seva capacitat competitiva i permetre als arbres sobreviure en ambients desfavorables. D'altra banda, aquests ajustos poden donar-se en detriment del seu estat fisiològic. Per aquest motiu, el faig és un bon exemple per estudiar com l'estrès oxidatiu repercuteix en la fisiologia de la planta sencera.

Figura 6: Arbres de faig (*Fagus sylvatica*) de la Fageda d'en Jordà (Girona, Catalunya).

Objectius

OBJECTIUS

L'objectiu principal d'aquest treball ha estat aprofundir en el possible efecte de l'edat i la dioècia en els nivells d'estrès oxidatiu en plantes perennes en condicions naturals de camp.

Per dur a terme l'objectiu principal es varen proposar els següents objectius específics:

- Analitzar l'efecte de l'edat a nivell d'òrgan en el grau d'estrès oxidatiu utilitzant com a model l'estudi del desenvolupament foliar.

- Estudiar la influència de l'edat de la planta en els nivells d'estrès oxidatiu utilitzant com a model plantes juvenils de *Pistacia lentiscus* en condicions d'estrès per sequera.

- Estudiar com afecta l'edat de la planta a l'estrès oxidatiu utilitzant com a model arbres moribunds de *Fagus sylvatica*.

- Determinar la repercussió del sexe en femelles i mascles de plantes dioiques en els nivells d'estrès oxidatiu.

- Investigar el possible efecte del factor modular en plantes dioiques comparant brots reproductius i no reproductius.

Informe dels directors de tesi

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Barcelona, 22 de maig de 2014

El Dr. Sergi Munné Bosch i la Dra. Maren Müller, com a directors de la Tesi Doctoral titulada "**Edat cronològica, edat fisiològica i sexe: factors determinants de l'estrès oxidatiu en plantes**" presentada per la doctoranda Marta Juvany Canovas,

INFORMEN sobre el factor d'impacte i la participació de la doctoranda en cadascun dels articles inclosos en la memòria d'aquesta Tesi Doctoral

Capítol 1. Article "**Leaves of field-grown mastic trees suffer oxidative stress at the two extremes of their lifespan**", publicat a la revista *Journal of Integrative Plant Biology*, índex d'impacte (2012) de 3.750. En aquest treball es descriu la importància de l'edat de les fulles com a condicionant de l'estrès oxidatiu en plantes de llentiscle, amb un èmfasi especial en els processos d'estrès oxidatiu en relació a les condicions climàtiques en clima mediterrani. Cal destacar l'aproximació experimental original en que s'analitzen diversos marcadors d'estrès així com els nivells hormonals durant tot el desenvolupament de les fulles en condicions naturals. Es descriu de forma original que no només les fulles velles durant la senescència pateixen estrès oxidatiu, sinó que les fulles molt joves també poden patir un grau d'estrès oxidatiu important. La doctoranda ha realitzat tot el mostreig, les anàlisis de les mostres, el tractament estadístic i l'elaboració dels resultats, i a més ha participat en el disseny experimental i discussió dels resultats, així com en la redacció de l'article, constant per tant com a primera autora del treball. La doctoranda ha demostrat una gran capacitat de treball, així com un

excel·lent maneig en els mostrejos i una excel·lent predisposició en la introducció a l'ús de la cromatografia líquida acoblada a espectrometria de masses en tàndem (LC-MS/MS) per a les anàlisis d'hormones. La doctoranda demostra també una gran capacitat d'anàlisi i interpretació dels resultats.

Capítol 2. Article "**Plant age-related changes in cytokinins, leaf growth and pigment accumulation in juvenile mastic trees**", publicat a la revista *Environmental and Experimental Botany*, índex d'impacte (2012) de 2.578. En aquest treball s'avalua la importància de les citocinines en relació al creixement de fulles joves i la seva acumulació de pigments (tant pigments fotosintètics com antocians), amb un èmfasi especial en els estadis inicials del creixement foliar, considerant a més les diferències entre dos grups de plantes de diferents edats. Cal destacar la importància de l'estudi en quan als marcadors d'estrès utilitzats en plantes de llentiscle, així com en la regulació hormonal del creixement per citocinines. La doctoranda ha realitzat tot el mostreig, les anàlisis de les mostres, el tractament estadístic i l'elaboració dels resultats, i a més ha participat en el disseny experimental i discussió dels resultats, així com en la redacció de l'article, constant per tant com a primera autora del treball. La doctoranda ha demostrat una gran capacitat de treball, així com una excel·lent predisposició a la millora dels experiments realitzats.

Capítol 3. Article "**Bud vigor, budburst lípid peroxidation and hormonal regulation of bud dormancy release in healthy and moribund beech (*Fagus sylvatica* L.) trees**", enviat per a la seva publicació a la revista *American Journal of Botany*, índex d'impacte (2012) de 2.586. En aquest treball es descriu la capacitat dels arbres moribunds de faig de produir noves gemmes foliars, amb un èmfasi especial en l'àcid malondialdehid com a marcador de vigor de les gemmes, així com en el paper de les hormones vegetals en la regulació del trencament de la dormició en gemmes foliars del faig. Cal destacar l'aproximació experimental,

amb un caire molt més ambiental que en els anteriors capítols, original i amb un alt valor científic, ja que es demostra per primera vegada que tot i que els arbres moribunds presenten un menor vigor en la producció de gemmes foliars, els arbres vells són capaços d'establir mecanismes de supervivència de gran interès fisiològic. La doctoranda ha realitzat tot el mostreig, les anàlisis de les mostres, el tractament estadístic i l'elaboració dels resultats, i a més ha participat en el disseny experimental i discussió dels resultats, així com en la redacció de l'article, constant per tant com a primera autora del treball. La doctoranda demostra una gran capacitat d'anàlisi i interpretació dels resultats, a més d'un excel·lent maneig al laboratori amb l'ús de la LC-MS/MS per a les anàlisis d'hormones.

Capítol 4. Article "**Sex-related differences in lipid peroxidation and photoprotection in *Pistacia lentiscus***", publicat a la revista *Journal of Experimental Botany*, índex d'impacte (2012) de 5.242. En aquest darrer treball experimental es descriu la importància del sexe com a determinant de l'estrès oxidatiu en plantes dioiques, amb un èmfasi especial en la peroxidació lipídica, així com en les seves causes a nivell bioquímic i cel·lular. Es descriu per primera vegada que les femelles de plantes de llentiscle són més susceptibles a l'estrès oxidatiu en períodes de gran esforç reproductiu, però a la vegada es mostra en aquest cas com l'estrès oxidatiu es desenvolupa com un mecanisme de resistència per fer front a l'excés de llum. A més, es correlaciona per primera vegada en aquesta espècie el grau d'acumulació d'àcid malondialdehid amb l'activitat lipoxigenasa i els nivells de citocinines endògens en fulles. La doctoranda ha realitzat tot el mostreig, les anàlisis de les mostres, el tractament estadístic i l'elaboració dels resultats, i a més ha participat en el disseny experimental i discussió dels resultats, constant per tant com a primera autora del treball. La doctoranda ha demostrat una gran capacitat de treball i ha participat també molt activament en la redacció de l'article. La doctoranda demostra un excel·lent grau de maduresa científica.

Annex 1. Article "**Photo-oxidative stress in emerging and senescing leaves: a mirror image?**", publicat a la revista *Journal of Experimental Botany*, índex d'impacte (2012) de 5.242. En aquest darrer treball, en aquest cas amb un enfocament teòric realitzant una revisió de la literatura, es descriu la importància de l'edat de la fulla com a determinant del grau d'estrès oxidatiu en plantes. En relació a la idea postulada ja en el Capítol 1, la doctoranda elabora un assaig utilitzant tota la bibliografia existent, en que es descriu per primera vegada de forma robusta que les fulles molt joves, igual que les molt velles, pateixen un grau important d'estrès oxidatiu, analitzant-ne a més les seves possibles causes. La doctoranda ha demostrat una gran capacitat de treball i de síntesi, elaborant tot el treball. La doctoranda confirma el seu excel·lent grau de maduresa científica.

I, per que així consti als efectes oportuns,

Dr. Sergi Munné Bosch Dra. Maren Müller

Resultats

CAPÍTOL 1

Les fulles de llentiscle presenten estrès oxidatiu en els dos extrems del seu desenvolupament

CHAPTER 1

Leaves of field-grown mastic trees suffer oxidative stress at the two extremes of their lifespan

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RESUM DEL CAPÍTOL 1

La senescència foliar és un fenomen complex que es dóna en totes les espècies vegetals, però encara avui en dia es coneix poc en plantes que creixen en condicions mediterrànies de camp i que estan ben adaptades a condicions climàtiques adverses. Per entendre millor els processos fisiològics subjacents a la senescència foliar en llentiscle (*Pistacia lentiscus* L.), es varen avaluar el creixement foliar, el contingut d'aigua i nitrogen, l'eficiència fotoquímica del fotosistema II (PSII), la peroxidació lipídica i els nivells de pigments fotosintètics, antioxidants, àcid abscísic, àcid salicílic i àcid jasmònic al llarg de tota la vida foliar, des de la seva expansió fins als estadis finals de senescència, en relació a les condicions climàtiques naturals en el camp. Mentre les fulles madures varen patir un dèficit tant d'aigua com de nitrogen durant la primavera i l'estiu, tant les fulles joves (emergents) com les velles (senescents) varen ser més sensibles a l'estrès fotooxidatiu, indicat per reduccions de la relació F_v/F_m i l'increment de la peroxidació lipídica a finals de la tardor i durant l'hivern. Les reduccions de la relació F_v/F_m varen estar associades amb baixos nivells d'α-tocoferol (vitamina E), mentre les fulles senescents més velles varen mostrar addicionalment una dràstica reducció dels nivells d'antocians. Es va concloure que tant les fulles joves (emergents) com les velles (senescents) de plantes de llentiscle patien estrès oxidatiu, el que podria estar lligat en part a les temperatures subòptimes a les que varen estar sotmeses durant el final de la tardor i l'hivern així com amb els baixos nivells de vitamina E.

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Research Article

Leaves of Field-Grown Mastic Trees Suffer Oxidative Stress at the Two Extremes of their Lifespan

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Abstract

Leaf senescence is a complex phenomenon occurring in all plant species, but it is still poorly understood in plants grown in Mediterranean field conditions and well-adapted to harsh climatic conditions. To better understand the physiological processes underlying leaf senescence in mastic trees (*Pistacia lentiscus* L.), we evaluated leaf growth, water and N content, photosystem II (PSII) photochemistry, lipid peroxidation and levels of photosynthetic pigments, antioxidants, abscisic acid, and salicylic acid and jasmonic acid during the complete leaf lifespan, from early expansion to late senescence in relation to natural climatic conditions in the field. While mature leaves suffered from water and N deficit during late spring and summer, both young (emerging) and old (senescing) leaves were most sensitive to photo-oxidative stress, as indicated by reductions in the F_v/F_m ratio and enhanced lipid peroxidation during late autumn and winter. Reductions in the F_v/F_m ratio were associated with low α-tocopherol (vitamin E) levels, while very old, senescing leaves additionally showed severe anthocyanin losses. We have concluded that both young (emerging) and old (senescing) leaves suffer oxidative stress in mastic trees, which may be linked in part to suboptimal temperatures during late autumn and winter as well as to low vitamin E levels.

Keywords: Leaf senescence; lentisc (*Pistacea lentiscus* L.); oxidative stress; phytohormones; seasonal effects.

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Introduction

Leaf development, from bud break to death, is a finely controlled process at the molecular, biochemical and physiological levels. The two extremes of leaf lifespan are characterized by rapidly growing, young emerging leaves on one hand, and very old, senescing leaves on the other. While studies of senescing leaves are common, their physiology is still poorly understood. Furthermore, there are few experiments to better understand the processes underlying what happens in emerging young leaves. Leaf senescence is characteristic of the latest stages of development and involves nutrient remobilization to other plant parts and some degenerative changes that lead these organs to death. It is usually characterized by three phases: (i) an initiation phase triggers the process in fully-expanded young leaves (also called mature, non-senescing leaves). This is followed by (ii) a remobilization phase, which is governed at the molecular level and allows nutrient remobilization accomplishing one of the most important functions of the senescing process in leaves. Finally, (iii) a terminal phase occurs when nuclei and the nucleic acid-related machinery is destroyed and the leaf can no longer accomplish any physiological role (Munné-Bosch and Alegre 2004; Lim et al. 2007; van Doorn 2011; Fischer 2012).

Reactive oxygen species (ROS) generation is common to all aerobic organisms including plants, and occurs in the plant's response to various stresses, including water deficit, high light, low temperatures or salinity, etc., as well as during leaf senescence. The process of protein degradation during leaf senescence, which occurs to a high extent in chloroplasts, is initiated by ROS and involves the action of proteolytic enzymes (Bhattacharjee 2005; Khanna-Chopra 2011).

Chloroplasts function at high oxygen tensions and in the light, therefore displaying a high photoprotective demand and a strong photo-oxidative potential. They can produce superoxide anions as a consequence of the direct reduction of oxygen by ferredoxin in the so-called Mehler reaction in PSI, but can also give rise to the more reactive singlet oxygen as a result of the interaction of oxygen with triplet excited chlorophylls ($^3Chl^*$). Indeed, it has recently been shown that singlet oxygen is one of the most potentially damaging ROS molecules in chloroplasts (Triantaphylidès et al. 2008). Superoxide anions can be rapidly converted by superoxide dismutases to hydrogen peroxide, which is in turn metabolized to water by the so-called ascorbate-glutathione cycle (Wang et al. 2010; Foyer and Noctor 2011). In contrast, singlet oxygen can be destroyed by the concerted action of α-tocopherol (vitamin E) and carotenoids, mainly β-carotene (pro-vitamin A), in photosystem II (PSII) reaction centers (Trebst 2003), therefore avoiding photodamage to PSII. Another mechanism that is thought to protect leaves from photo-oxidative stress is the accumulation of anthocyanins, which might act as a light-barrier and therefore avoid over-excitation of the photosynthetic apparatus (Steyn et al. 2002). However, the direct role of anthocyanin accumulation, which occurs generally in emerging and senescing leaves of several species, is presently under debate (Gould et al. 2009).

On the other hand, abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA), which are known to play a major role in plant responses to environmental stress, are also generally involved in senescing processes in the leaves of several species. It has been shown both in the model plant *Arabidopsis thaliana* and in other plant species that ABA, JA, and SA are involved in promoting the senescing process, both in developmentally-regulated and stress-induced leaf senescence (He et al. 2002; Buchanan-Wollaston et al. 2005; Lim et al. 2007; Schippers et al. 2007; Abreu and Munné-Bosch 2009; Lee et al. 2011; Fischer 2012). However, little is known about the possible role of these phytohormones in very young, emerging leaves, although it has been known for a long time that bud break is associated with a physiological stress. And that a decrease in ABA levels is needed in dormant buds so that leaves can emerge (Wright 1975).

We have previously shown that mastic trees activate mechanisms of photo- and antioxidative protection, presumably for maintaining chloroplast function during the first stages of leaf senescence, while antioxidant defenses are lost during the latest stages of senescence (Munné-Bosch and Peñuelas 2003). On the other hand, it has been shown in other species that young, emerging leaves show a high photoprotective demand and are therefore very sensitive to photo-oxidative stress (Jiang et al. 2005). However, it is still unknown whether this is the case in young leaves accumulating high anthocyanin levels, such as those of mastic trees. The study of the physiology of mastic tree leaves at the two extremes of the leaf lifespan is very important and is a good example to better understand the ecophysiology of Mediterranean species well-adapted to survive harsh climatic conditions. In this study, we aimed at evaluating to what extent (i) young, emerging leaves might be as sensitive as old, senescing leaves to oxidative stress in this species, (ii) whether this is linked to specific climatic variables that change over the seasons under Mediterranean field conditions, and (iii) can this be associated with intrinsic changes in the levels of some photoprotectants, such as anthocyanins, carotenoids and tocopherols, or to endogenous levels of the phytohormones ABA, SA and JA.

Results

Climatic conditions during the experimental period (from November 2009 to November 2010) were typical of the Mediterranean climate (**Figure 1**). Suboptimal growth temperatures (mean monthly maximum and minimum temperatures below 20 °C and 10 °C, respectively) were combined with abundant precipitation (387 mm between October and March) during autumn and winter, while the summer (particularly between June and August) was characterized by scarce rainfalls (94 mm during this period) and maximum mean monthly temperatures above 30 °C in July. The maximum diurnal photosynthetically-active photon flux density (PPFD) and monthly precipitation occurred in May (**Figure 1**). Taking into account these changing climatic conditions over the course of the seasons, we followed the physiological processes underlying the development of mastic tree leaves from very young (emerging) leaves in November 2009 to very old, senescing leaves in November 2010. Very small, emerging leaves were marked and the same leaves were followed throughout the seasons until their deaths. Therefore, observed changes in physiological parameters were caused by the interaction of both the leaf's age and the climatic conditions that the plants were exposed to over the year. The lifespan of leaves was of approximately 12 months, with some variability between plants. It is worth noting that sampling during November 2010 was performed on the plants for which leaves still persisted, since some of the leaves had already dropped by that time and could not be sampled. Therefore, this sampling point represents leaves in a very advanced developmental stage, just before leaf abscission occurred (here called very old, senescing leaves).

Leaf growth, water, and nutrient content

Leaf growth (estimated as dry mass) increased sharply from 14.1 mg to 43.8 mg during the first stages, particularly between January and May, but only very slightly between November and January. Maximum leaf biomass was attained in May, after which it remained constant (**Figure 2**). The leaf mass per area

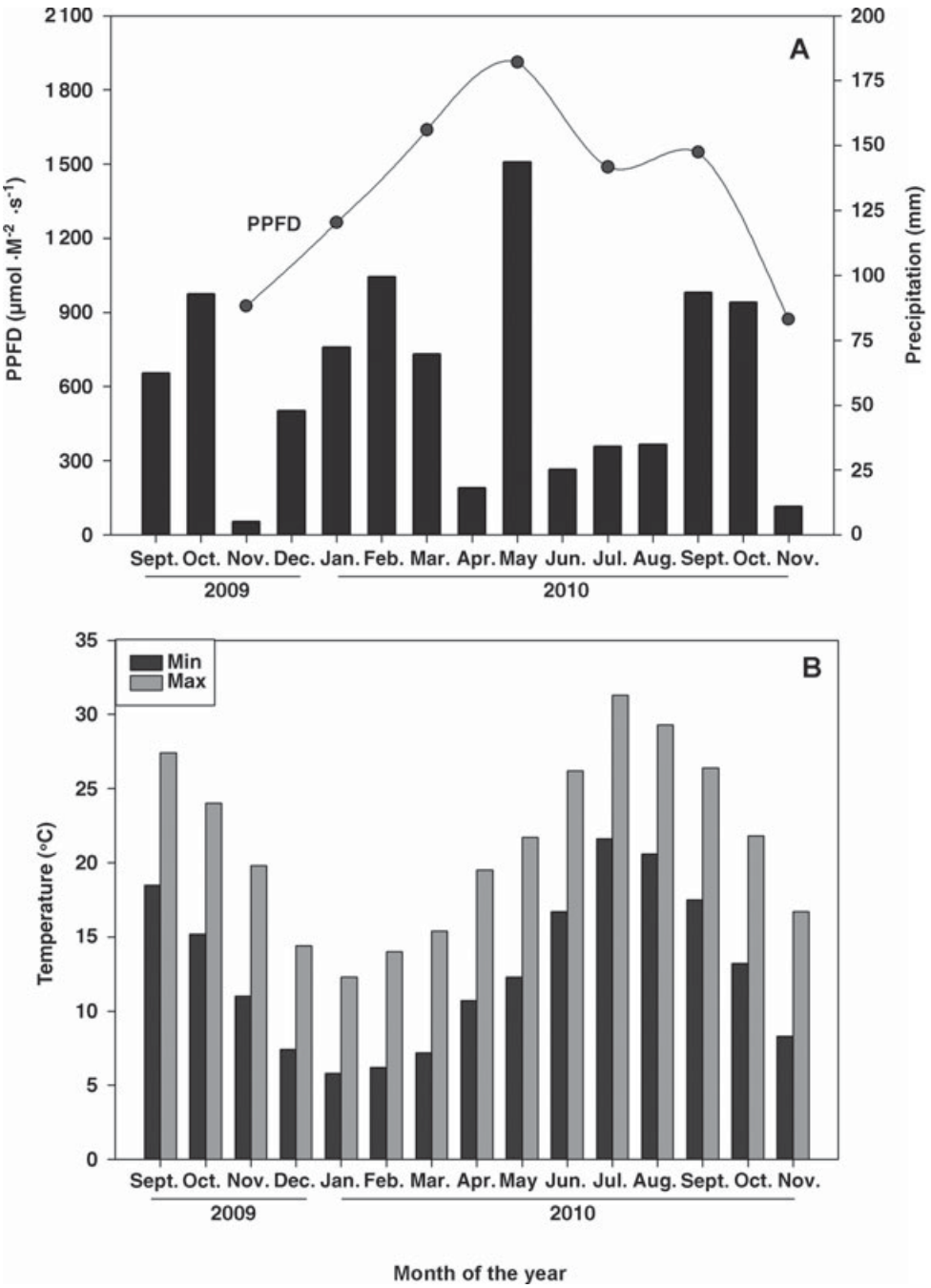

Figure 1. Climatological conditions during the experimental period at the Experimental Fields of the Faculty of Biology of the University of Barcelona, where mastic trees were growing under Mediterranean field conditions.

(A) Monthly precipitation and photosynthetically-active photon flux density (PPFD) during the measurement days.
(B) Maximum and minimum Monthly temperatures during the experiment.

(LMA) ratio increased sharply during early stages of growth from November to May, and most notably between January and March, to decline later in July and increase again in November of the next year. The relative water content (RWC) increased slightly during the first stages of development to decline later in May. RWC values remained above 90% in January and March and close to 80% between May and September, with a slight decline at the end of the experiment (**Figure 2**). The marked decrease in the RWC between March and May was accompanied by a 35% decrease in the N content of leaves

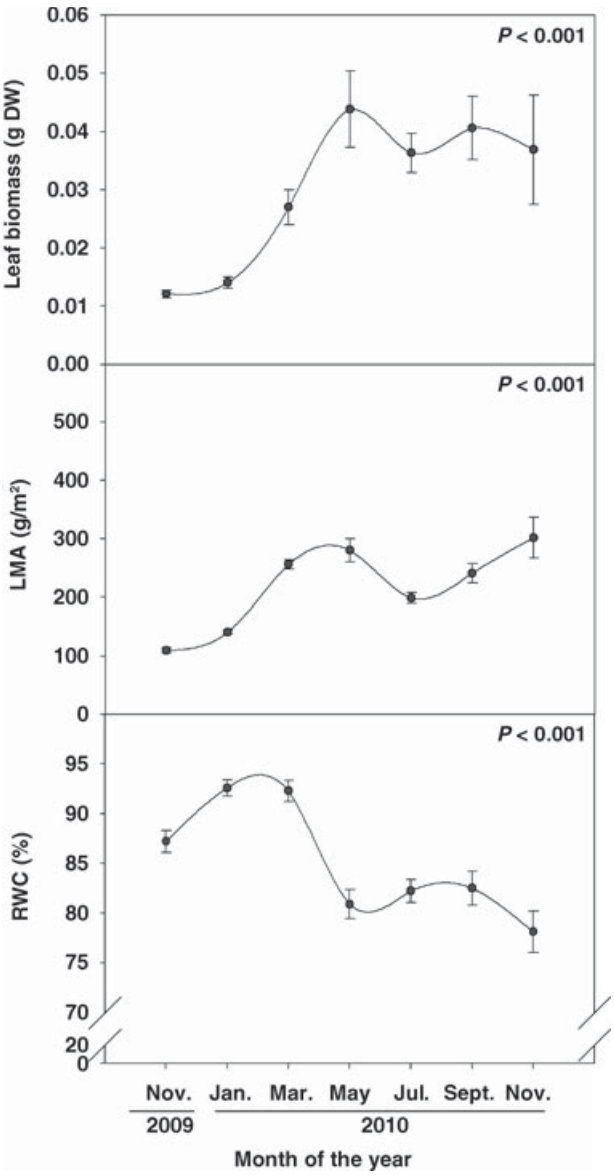

Figure 2. Biomass, dry mass per area ratio (LMA), and relative water content (RWC) of mastic tree leaves during their complete lifespan.

New, emerging leaves were marked in November 2009 and physiological parameters were followed once every two months just before their death, which occurred in December 2010. Data correspond to the mean ± *SE* of $n = 16–18$ individuals. Results of statistics, which indicate differences over time, are shown in the inserts (one-way ANOVA).

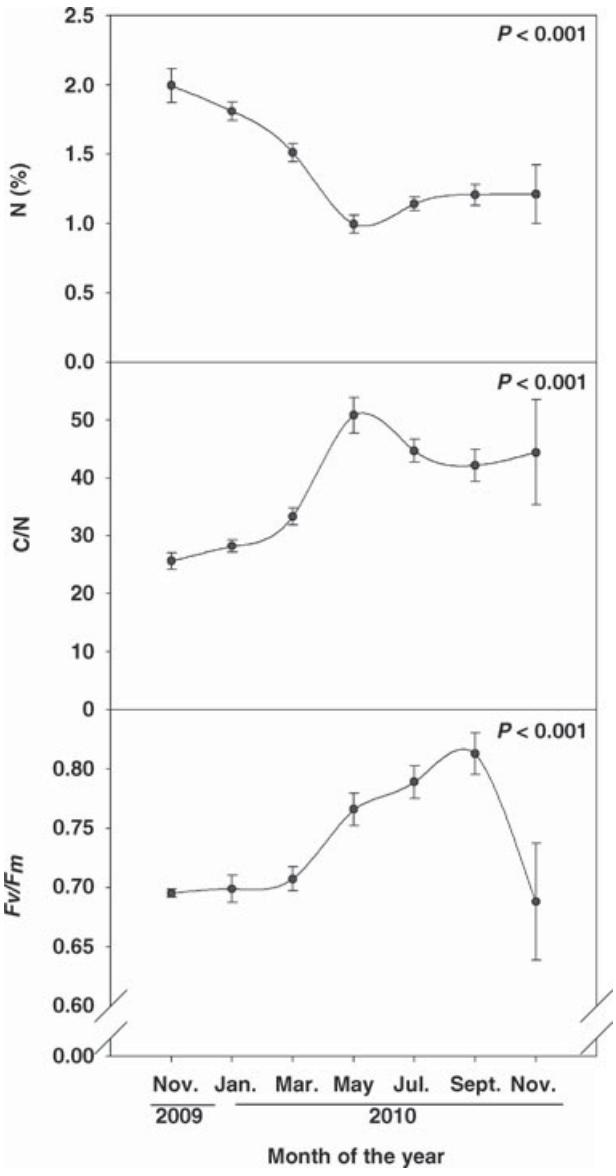

Figure 3. Total nitrogen (N) content, C/N ratio, and maximum efficiency of photosystem II (PSII) photochemistry (F_v/F_m ratio) of mastic tree leaves during their complete lifespan.

Data correspond to the mean ± *SE* of $n = 16–18$ individuals. Results of statistics, which indicate differences over time, are shown in the inserts (one-way ANOVA).

and a 52% increase in the C/N ratio (**Figure 3**). Indeed, N content decreased and the C/N ratio increased from the start of leaf development in November until May, and remained constant thereafter. C content remained unchanged throughout the experiment, with values ranging between 50.3% and 53.8% (data not shown).

PSII photochemistry and photosynthetic pigments

The F_v/F_m ratio followed a marked variation throughout the seasons, with minimum values at the two extremes of the

leaf lifespan. Values below 0.75 were attained both at the beginning of leaf development (between November and March, with the lowest values of 0.69 attained during November and January), and at the very end of the leaf lifespan (with values of 0.68 during November 2010, **Figure 3**). The F_v/F_m ratio remained above 0.75 between May and September, with maximum values of 0.81 attained in September. It is interesting to note that the lowest F_v/F_m values were observed during late autumn and winter and at the two extremes of the leaf lifespan. However, these variations did not correlate with the contents of photosynthetic pigments. Chlorophyll a+b levels decreased from the onset of leaf development, and most particularly during the first months of leaf development. The minimum chlorophyll levels were attained in July and thereafter remained constant until the end of the leaf lifespan (**Figure 4**). In contrast, the chlorophyll a/b ratio followed a different pattern. This ratio increased during the early stages of leaf growth to decrease sharply from March to May, and later remained constant until the end of the leaf lifespan. Carotenoid levels followed a similar pattern of variation of chlorophyll, but the decline started later (**Figure 4**).

Antioxidants and lipid peroxidation

Aside from the lipid-soluble carotenoids (**Figure 4**), we analyzed other antioxidant molecules including tocopherols, and anthocyanins, and evaluated the extent of lipid peroxidation by estimating the levels of malondialdehyde (MDA) (**Figure 5**). Among the tocopherols analyzed (α, β, γ and δ-tocopherol), only the α homolog could be properly quantified in mastic tree leaves. α-Tocopherol levels increased gradually with leaf development, attaining maximum levels of 63.3 μg (per g dry weight (DW)) during September, 6.2-fold higher than at the beginning of the experiment. However, the levels of this antioxidant were drastically reduced (by 93%) in very old, senescing leaves (from September to November). The values in these leaves were even 55% lower than those observed at the beginning of the experiment in recently emerged leaves. The lowest values in anthocyanin levels were also observed in very old, senescing leaves. However, in this case, recently emerged leaves accumulated large amounts of these compounds, 4.6-fold higher than in very old, senescing leaves. It is worth noting that anthocyanin accumulation in young leaves was maintained throughout late autumn and winter. On the other hand, anthocyanin levels suffered a drastic decrease not only during spring (between March and May), but also during late autumn in old leaves (September to November), concomitantly with drastic reductions in tocopherol levels (**Figure 5**). Interestingly, MDA levels peaked at the two extremes of the leaf lifespan (**Figure 5**), opposite to the variations in the F_v/F_m ratio (**Figure 3**). The highest MDA levels correlated with the lowest F_v/F_m values, except for September, in which

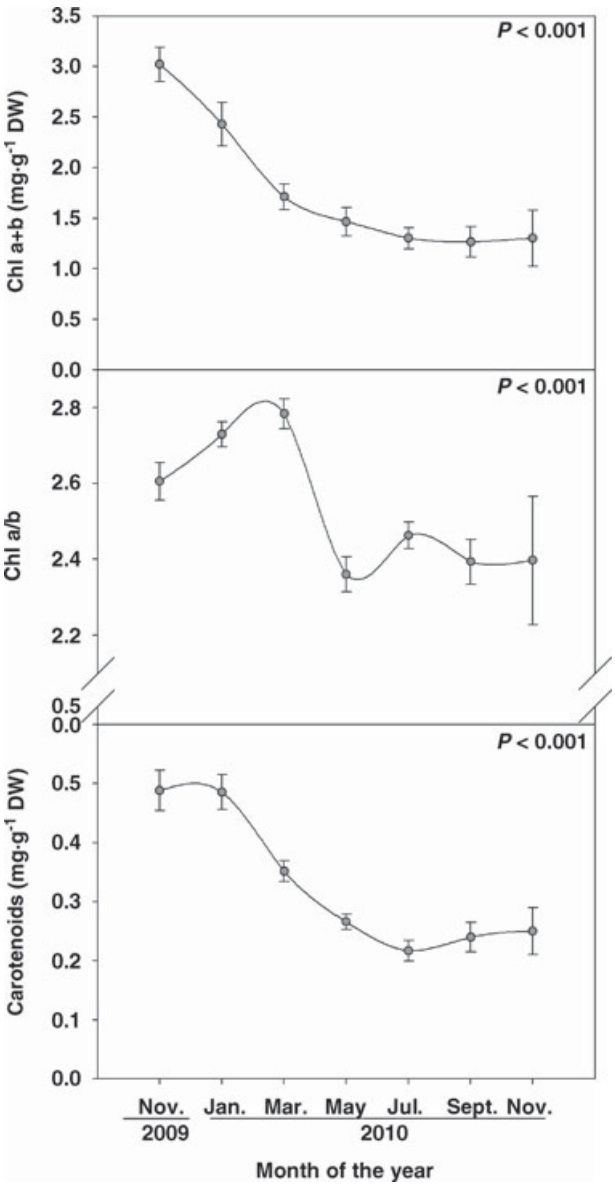

Figure 4. Chlorophyll (Chl) a + b levels, Chl a/b ratio, and the levels of total carotenoids in mastic tree leaves during their complete lifespan.

Data correspond to the mean ± *SE* of $n = 12$ individuals. Results of statistics, which indicate differences over time, are shown in the inserts (one-way ANOVA).

lipid peroxidation increased (**Figure 5**) without any effect on the F_v/F_m ratio (**Figure 3**). It is also worth noting that the extent of lipid peroxidation gradually decreased in developing young leaves, reaching minimum levels during May and July. Furthermore, when comparing the two extremes of the lifespan, we observed that MDA levels were 1.7-fold higher in recently emerged leaves when compared with very old, senescing leaves (**Figure 5**).

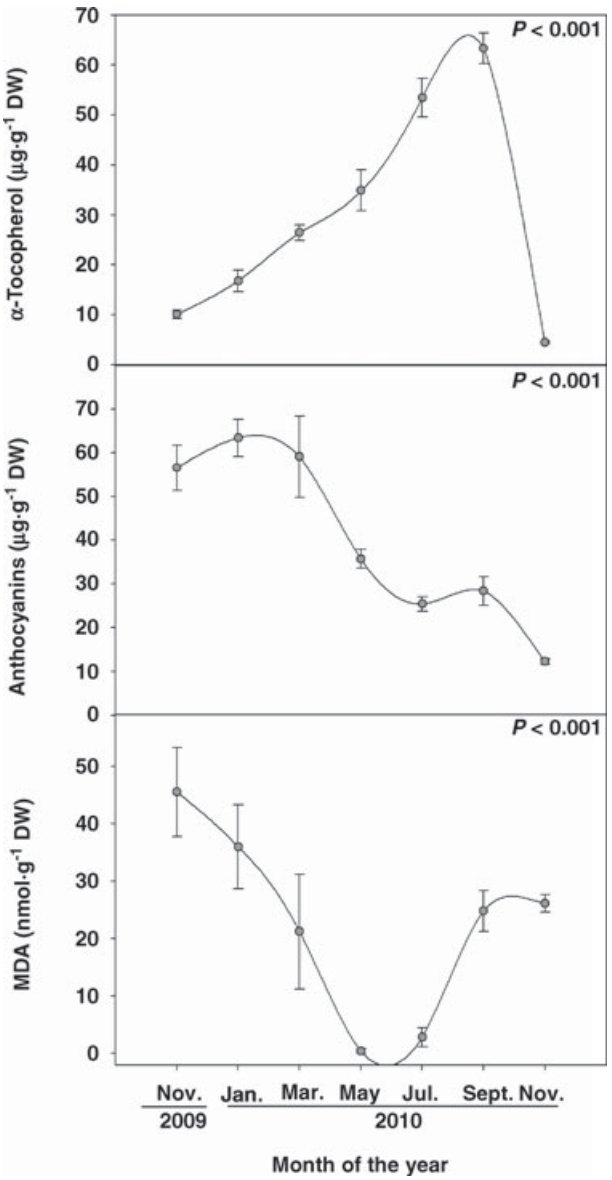

Figure 5. Levels of α-tocopherol, total anthocyanins and malondialdehyde (MDA), an indicator of lipid peroxidation, in mastic tree leaves during their complete lifespan.

Data correspond to the mean ± *SE* of $n = 12$ individuals. Results of statistics, which indicate differences over time, are shown in the inserts (one-way ANOVA).

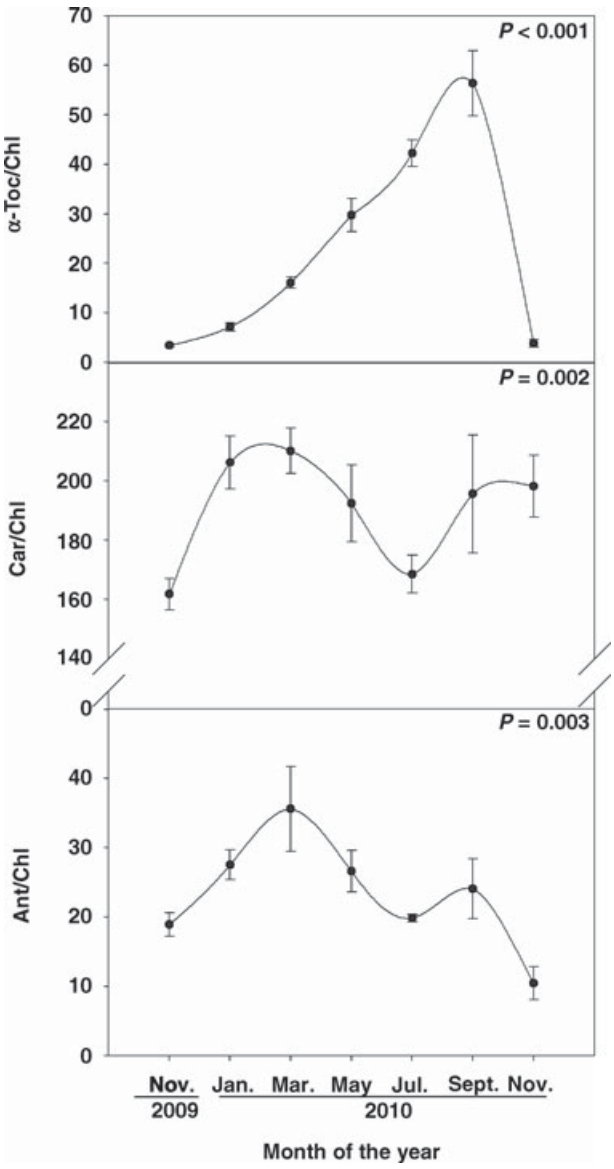

Figure 6. Levels of α-tocopherol (α-Toc), total carotenoids (Car) and anthocyanins (Ant) per unit of chlorophyll (Chl) a + b in mastic tree leaves during their complete lifespan.

Data correspond to the mean ± *SE* of $n = 12$ individuals. All data are given in mg of compound per g of Chl a + b. Results of statistics, which indicate differences over time, are shown in the inserts (one-way ANOVA).

The levels of antioxidants per unit of chlorophyll (**Figure 6**) revealed a different pattern of variation compared to that given when values were expressed in dry mass (**Figures 4** and **5**), particularly for carotenoids and anthocyanins. Recently emerged leaves showed a 28% increase in carotenoids per chlorophyll unit from November to January (due to the reduction of chlorophylls but not of carotenoids), concomitantly with increases in tocopherol and anthocyanins per chlorophyll unit, although in these two latter increases lasted until March. Young, emerging leaves (November 2009) showed low levels of antioxidants (tocopherol, carotenoids and anthocyanins) per chlorophyll unit, at least compared to fully expanded leaves (May), while the lowest levels of antioxidants (in this case tocopherol and anthocyanins, but not carotenoids) per

590 Journal of Integrative Plant Biology Vol. 54 No. 8 2012

chlorophyll unit were observed again in very old, senescing leaves (November 2010).

Phytohormones

Endogenous ABA, SA, and JA content were also followed during the entire leaf lifespan throughout the year (**Figure 7**). The most striking differences were again observed in young, emerging leaves, particularly between November and January during which ABA, SA, and JA decreased. However, the major changes were observed for ABA, which decreased by 74% during this period. It is also interesting to note a 7-fold increase in SA levels between March and May, which occurred concomitantly with a 50% increase in ABA levels. Finally, it is worth mentioning that ABA, SA, and JA all showed no significant increase during autumn in very old, senescing leaves (**Figure 7**).

Discussion

Mastic tree (*P. lentiscus*) is a deep-rooted, evergreen, dioecious shrub or small tree of up to 5 m tall that produces new leaves both in spring and autumn. Autumn leaves were selected here to monitor physiological changes throughout the year, and most particularly at the two extremes of their lifespan, when leaves are exposed to similar climatic conditions of autumn/winter and can therefore suffer photo-oxidative stress due to suboptimal temperatures – an aspect poorly investigated thus far in Mediterranean plants. It should be noted that the age-related changes in physiological parameters shown here reflect the changes in the so-called autumn leaves, which appear in late summer/early autumn. Spring leaves (appearing during late winter/early spring), which were not investigated here, may not necessarily follow the same physiological changes during leaf development, an aspect that warrants further research. Another important point is that the effects of leaf age cannot be separated from the influence of climatic conditions. Although this is somewhat limiting in establishing a causal relationship, we believe this approach is essential to understand the eco-physiology of this species. With this approach we can observe what really occurs to the leaves of this plant species during its complete lifespan in natural field conditions. Of particular interest, it was shown that leaves at the two extremes of their lifespan show stress symptoms (most particularly oxidative stress), and also important differences in terms of phytohormones (mainly ABA) and antioxidants (mainly tocopherols and anthocyanins).

Clear symptoms of oxidative stress were observed in emerging leaves, as reflected by MDA accumulation, an indicator of the extent of lipid peroxidation (Hodges et al. 1999), and F_v/F_m values below 0.75, which are generally indicative of damage to PSII photochemistry (Takahashi and Murata 2008). However, it is possible that low F_v/F_m ratios were associated with an immature chloroplast, and not necessarily a damaged PSII in rapidly growing, emerging leaves, which requires further investigations. Interestingly, the F_v/F_m ratio stayed close to 0.70 throughout late autumn and winter (between November

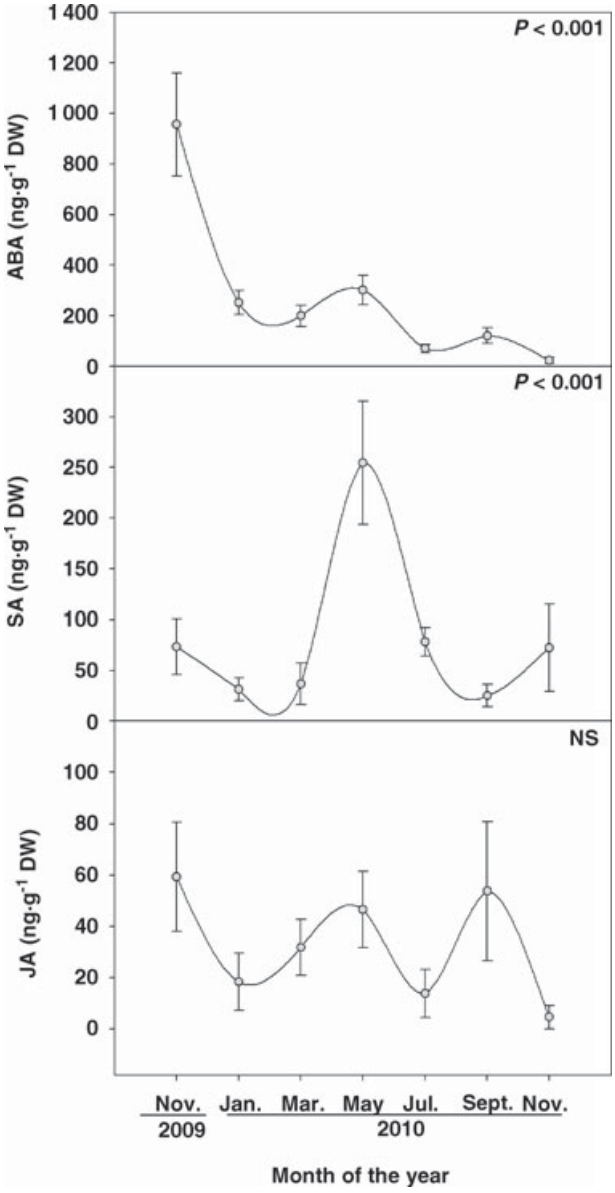

Figure 7. Endogenous contents of the phytohormones, abscisic acid (ABA), salicylic acid (SA), and jasmonic acid (JA) in mastic tree leaves during their complete lifespan.

Data correspond to the mean ± *SE* of $n = 12$ individuals. Results of statistics, which indicate differences over time, are shown in the inserts (one-way ANOVA). NS indicates not significant.

and March), while MDA concentrations decreased gradually during the same period, suggesting that reduced F_v/F_m values are probably linked to low temperatures and/or immature chloroplasts, and the degree of lipid peroxidation is reduced as leaves expand and develop. In other words, it appears that the youngest, emerging leaves are the most sensitive to lipid peroxidation under the suboptimal low temperatures typical of late autumn and winter. It is interesting to note that the F_v/F_m ratio also decreased in very old, senescing leaves, which also encountered similar low temperatures typical of late autumn in the Mediterranean climate, suggesting a direct effect of low temperatures on the induction of photoinhibition in this species. Indeed, this is in agreement with Nikiforou et al. (2011), who showed that despite accumulating anthocyanins, leaves of this species are sensitive to cold-induced photoinhibition during the winter. Therefore, it appears that accumulating anthocyanins, which might act as light screens or directly as antioxidants (Gould et al. 2009), does not confer young, emerging leaves complete photoprotection. In this study, it is shown that recently emerged leaves accumulate anthocyanins, but have low levels of carotenoids and tocopherols per chlorophyll unit, therefore displaying a low photoprotective capacity in relation to the potential amount of light absorbed by chlorophyll molecules, an aspect considered very important to counteract excess light (Kyparissis et al. 1995; Munné-Bosch and Alegre 2000; Munné-Bosch et al. 2001). It therefore appears that recently emerged leaves are not only in a physiological state with reduced photosynthetic capacity as previously shown (Nikiforou et al. 2011), but also exhibit a reduced capacity for photoprotection by carotenoids and tocopherols, which are essential to maintain the integrity of PSII (Trebst 2003). It is still unknown whether or not these young, emerging leaves of mastic trees would suffer a higher degree of photo-oxidative stress if they did not accumulate anthocyanins.

Similar to recently emerging leaves, senescing leaves also experience oxidative stress, but with some important differences. In this case, lipid peroxidation increased first, well before the F_v/F_m ratio was significantly reduced. This indicates that oxidative stress is induced in senescing leaves and that photo-oxidative damage occurs in these leaves, but at the latest stages only, concomitantly with a strong depletion of tocopherols and anthocyanins. Oxidative stress therefore appears not to be induced (or at least not to be solely induced) by high light, since mature leaves exposed to very high PPFDs showed the lowest MDA accumulation over the entire leaf lifespan. It has previously been shown that this species is very resistant to summer drought typical of the Mediterranean climate, by inducing low osmotic potential and by reducing transpiration rates, partly due to the presence of thick leaf cuticles (Zohary 1962). It is therefore not surprising that plants could maintain RWC values above 80% throughout the leaf lifespan, except in very old, senescing leaves, in which values fell just slightly below this threshold which is considered essential to keep full cell turgor. It is likely that the combination of drought and high light typical of the Mediterranean summer are triggering the senescing process, as suggested previously (Munné-Bosch and Peñuelas 2003). In this previous study, however, drought during the summer was more severe, which induced a higher degree of oxidative stress and accelerated the senescence process compared to the present study. It is also worth mentioning that the MDA increases in senescing leaves occurred before α-tocopherol levels dropped, thus indicating that a decrease in this antioxidant is not the only cause of oxidative stress, but probably the consequence of increased oxidative stress within the senescing cell. Further research is therefore required to unravel the signaling events and molecular players leading to MDA accumulation in senescing leaves of this species.

Abscisic acid levels were very high in recently emerged leaves, decreasing sharply from November to January. It is worth noting that ABA decreases did not correlate with leaf biomass increases; the depletion of ABA levels occurred earlier than the increase in leaf biomass. It is also noteworthy that the highest MDA levels coincided with the lowest F_v/F_m ratio and the highest ABA levels in recently emerged leaves only, but not during leaf expansion. A decrease in ABA levels may be a prerequisite for leaf growth, since ABA is generally considered a growth inhibitor (Zhou et al. 2003). Furthermore, it is well known that phytohormones are involved in the regulation of leaf senescence, but their specific activities can vary depending on the species and very little is known about leaf senescence in natural field conditions, despite its importance to better understand the ecophysiology of plants. ABA, SA, JA levels did not increase during the progression of senescence in mastic tree leaves. This is not in agreement with previous findings that support an effect of ABA, SA, and JA as promoters of leaf senescence in several species (Morris et al. 2000; Buchanan-Wollaston et al. 2005; Abreu and Munné-Bosch 2008, 2009). It therefore appears that leaf senescence in this species is not triggered by any of these senescence-promoting compounds.

We conclude that both young (emerging) and old (senescing) leaves suffer oxidative stress in mastic trees, which may be linked to suboptimal temperatures during late autumn and winter as well as to low vitamin E levels, among other factors, in both leaf types.

Materials and Methods

Plant material, growth conditions and sampling

This study was conducted using *Pistacia lentiscus* L., an evergreen species widely distributed along the Mediterranean basin. Fifty-five juvenile plants with a height between 40 and 110 cm were purchased in Bioriza (Cornellà de Terri, Girona, Spain) in the spring of 2009 and were homogeneously

transplanted in an area of 30 m^2 to the experimental fields of the Faculty of Biology at the University of Barcelona (Barcelona, Spain). Plants were grown under Mediterranean climatic conditions and received water exclusively from rainfall during the study period. All new, emerging leaves of four newly emerged shoots of each plant were labeled (28 and 29 October 2009) to know the leaf age at the moment of the measurements. Leaf samples were collected from the labeled leaves every 2 months from November 2009 to November 2010 at midday (at maximum incident diurnal PPFD). For analysis of MDA, photosynthetic pigments, anthocyanins, tocopherols and phytohormones, samples were collected, immediately frozen in liquid nitrogen, and stored at −80 °C until analysis. Despite destructive analyses, the large number of leaves labeled at the beginning of the experiment allowed us to follow the exact age of the leaves throughout the experiment.

Leaf biomass, RWC, LMA and elemental analyses

Leaf biomass was measured by weighing the samples before and after drying to constant weight at 80 °C. The relative water content (RWC) was determined as (FW-DW)/(TW-DW), where FW is the fresh matter, TW is the turgid weight after hydrating the leaves with distilled water for 24 h at 4 °C, and DW is the dry matter after oven-drying the samples. Leaf area was estimated by using a flatbed scanner (model CX- 5400; Epson Stylus, Nagano, Japan) and an image-processing program. Leaf mass area (LMA) was measured as the ratio of leaf area per DW. Total C and N concentrations in leaves were measured by the Dumas elemental analysis method, using a Thermo EA 1108 analyzer (Thermo Scientific, Milan, Italy).

Photosynthetic pigments, anthocyanins, and tocopherols

Leaf samples (100 mg) were ground in liquid nitrogen and extracted with 100% methanol using ultrasonication. After centrifuging at 16 000 × *g* for 10 min at 4 °C, the pellet was re-extracted with the same solvent and supernatants were pooled. Chlorophylls and carotenoids were estimated spectrophotometrically, and specific absorption coefficients for chlorophyll a, chlorophyll b, and total carotenoids reported by Lichtenthaler (1987) were used. A molecular weight of 570 for carotenoids was used for calculations. Anthocyanin content was determined following extract acidification with concentrated HCl, and an absorption coefficient of 30/mM per cm at 530 nm was used according to Gitelson et al. (2001).

For the extraction of tocopherols, leaf samples (50 mg) were ground in liquid nitrogen and extracted with 100% methanol using ultrasonication for 45 min at 4 °C. The samples were then centrifuged for 15 min at 4 °C and the supernatants were transferred to vials for analysis. The high performance liquid chromatography (HPLC) analysis was carried out as described (Amaral et al. 2005). In brief, the HPLC equipment consisted of an integrated system with a Waters 600 controller pump, a Waters 714 plus auto-sampler, and an FP-1520 fluorescence detector (Jasco, Essex, UK). Tocopherols were separated on an Inertsil 100A (30 × 250 mm, 5 μm, GL Sciences Inc. Torrance, CA, USA) normal-phase column operating at room temperature. The mobile phase used was a mixture of *n*-hexane and 1,4-dioxane (95.5:4.5, v/v) at a flow rate of 0.7 mL/min, and the injection volume was 10 μL. Detection was carried out for excitation at 295 nm and emission at 330 nm. Among the analyzed tocopherols (α-, β-, γ- and δ-tocopherol) only α-tocopherol could be quantified based on the fluorescence signal response compared with an authentic standard (Sigma-Aldrich, St. Louis, MO, USA).

Lipid peroxidation and chlorophyll fluorescence

The extent of lipid peroxidation was estimated by measuring the amount of MDA in leaves by the method described by Hodges et al. (1999), which takes into account the possible influence of interfering compounds in the thiobarbituric acid-reactive substances (TBARS) assay.

Measurements of the maximum efficiency of photosystem II photochemistry (F_v/F_m ratio) were made by using a pulse modulated fluorimeter Imaging PAM (Walz, Effeltrich, Germany) after 2 h of dark adaptation in leaves collected at midday. The F_v/F_m ratio was calculated as $(F_m\text{-}F_0)/F_m$, where F_m and F_0 are the maximum and basal fluorescence yields, respectively, of dark adapted leaves as described earlier (van Kooten and Snel 1990).

Phytohormone analyses

The extraction and analyses of endogenous concentrations of ABA, SA, and JA were carried out as described by Müller and Munné-Bosch (2011). Deuterium labeled phytohormones (d_6-ABA, d_4-SA, and d_5-JA) were used as internal standards.

Statistical analyses

Seasonal variations were evaluated using the analysis of variance (ANOVA) and were considered significant at a probability level of $P < 0.05$.

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CAPÍTOL 2

Canvis en els nivells de citocinines, creixement foliar i acumulació de pigments causats per l'edat en arbres juvenils de llentiscle

CHAPTER 2

Plant age-related changes in cytokinins, leaf growth and pigment accumulation in juvenile mastic trees

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RESUM DEL CAPÍTOL 2

L'edat de la planta és un factor clau per entendre el rendiment i supervivència de qualsevol espècie en el camp, tot i així, és poc el coneixement que es té actualment sobre la seva influència en els nivells endògens de citocinines en individus juvenils de plantes perennes. En aquest estudi es va hipotetitzar que l'edat de la planta en arbres de llentiscle juvenils pot afectar els nivells de citocinines i conduir a canvis significatius en processos relacionats amb aquestes, com el creixement foliar i l'acumulació de pigments. Dos grups de plantes juvenils de 2 i 5 anys es van cultivar al camp sota condicions típiques del clima mediterrani per tal d'avaluar les diferències relacionades amb l'edat en els nivells endògens de citocinines i d'auxines, en la biomassa i àrea foliar, en els nivells de pigments i en l'eficiència fotoquímica del PSII (relació F_v/F_m). A més a més, es varen dur a terme anàlisis de regressions entre els nivells de citocinines i diversos indicadors d'estrès en plantes juvenils de llentiscle. Els resultats mostraren que els nivells endògens de citocinines, principalment el 2-isopentenil adenina (2iP), augmentaven durant el període d'intens creixement foliar. Tanmateix, l'edat de la planta no va afectar els nivells de 2iP, però sí els de zeatina (Z); les plantes de 2 anys varen mostrar uns nivells de Z dos vegades més elevats comparat al de les plantes de 5 anys, tot i que aquestes últimes tenien 10 vegades més biomassa total. Les reduccions de citocinines relacionades amb l'edat de la planta no estaven associades amb reduccions del creixement foliar en els individus més grans, però sí amb un increment de la relació clorofil·la a/b. Nous anàlisis de regressions varen revelar que tot i que l'increment de la relació clorofil·la a/b predisposa a les plantes a patir un major estrès fotooxdatiu, com indicava l'increment dels nivells d'antocians i els baixos valors de F_v/F_m en les plantes juvenils de llentiscle, els individus d'edats diferents mostraven un grau de tolerància a l'estrès semblant. En aquest estudi es va concloure que l'edat de la planta pot alterar la composició endògena de citocinines i el

patró d'acumulació de pigments en les fulles, però aquest fet no redueix el creixement foliar o la tolerància a l'estrès en aquesta espècie.

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Plant age-related changes in cytokinins, leaf growth and pigment accumulation in juvenile mastic trees

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ABSTRACT

Despite plant age is a key factor to understand the performance and survival of any given species in the field, little is known about its influence on the endogenous levels of cytokinins in juvenile individuals of perennial plants. In this study, we hypothesized that plant age in juvenile mastic trees may affect cytokinin levels and lead to significant changes in cytokinin-related processes, such as leaf growth and pigment accumulation. Two groups of juvenile plants aged 2 and 5 years old were grown under Mediterranean field conditions to evaluate the age-related differences in the endogenous levels of cytokinins and auxins, leaf biomass and area, pigments levels and the efficiency of PSII photochemistry (F_v/F_m ratio). Furthermore, regression analyses between cytokinin levels and several indicators of stress performance were carried out in a population of juvenile mastic trees. Results showed that endogenous levels of cytokinins, particularly 2-isopentenyladenine (2-iP), increased during periods of intense leaf growth. However, plant age did not affect 2-iP levels, but those of *trans*-zeatin (Z); 2-year-old plants showing 2-fold higher levels of Z compared to 5-year-old plants, the latter having 11-fold higher total plant biomass. Plant age-related reductions in cytokinins were not associated with reductions in leaf growth in the largest individuals, but with an increased chlorophyll (Chl) a/b ratio. Further regression analyses revealed that although enhanced Chl a/b ratios predispose leaves to suffer photooxidative stress, as indicated by increased anthocyanins levels and lowered F_v/F_m ratios in juvenile mastic trees, individuals of different ages showed a similar degree of stress tolerance. It is concluded that plant age may alter the endogenous composition of cytokinins and the pattern of pigment accumulation in leaves, but this does not reduce leaf growth or stress tolerance in this species.

1. Introduction

Plant age is a key factor to understand the performance and survival of any given species in the field. However, very little information is available on the influence of the age of individuals on ecophysiological traits in juvenile individuals of perennial plants. The mortality rate by age is usually defined by a cubic smoothing spline, in which mortality is highest among very young and very old individuals; and when models effectively capture the extremely high mortality rates of very young plants observed in seedling studies mortality rates at very early stages of plant development can be as high or even higher than at very old ages (Pierson and Turner, 1998). In trees, mortality rate is also generally modeled as a U-shape function of diameter. Smaller individuals present high mortality rates as a result of competition from overstory trees. Large trees can also have high mortality rates owing to senescence and susceptibility to insect attacks or windthrows (Harcombe, 1987; Vieilledent et al., 2009). Although very little is known about the ecophysiology of juvenile plants of different ages, particularly how they perform under field conditions and the intrinsic characteristics underlying their survival rates, this information is essential if we aim at better understanding the causes of death in natural populations, the relationship of within- and between-population variation in the rate of aging, and the physiological basis of variation in the rate of aging in natural populations (Ricklefs, 2008). To get insight into this, we were interested in exploring the possibility that plant age might influence the endogenous levels of the phytohormones, cytokinins and auxins, which are essential regulators of plant growth and development.

Leaf development, from bud break to death, is a finely controlled process at the molecular, biochemical and ecophysiological levels. Cytokinins, in cooperation with auxin, play a key role in the control of leaf growth. Cytokinins, mainly the active forms 2-isopentenyladenine (iP) and *trans*-zeatin (Z), play a key role in

Abbreviations: 2-iP, 2-isopentenyl adenine; Chl, chlorophyll; DHZ, dihydrozeatin; DHZR, dihydrozeatin riboside; DW, dry matter; F_v/F_m, maximum efficiency of photosystem II photochemistry; FW, fresh matter; IPA, isopentenyladenosine; RWC, relative leaf water content; Z, zeatin; ZR, zeatin riboside.

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the regulation of cell division modulating the progression in the cell cycle through the regulation of cyclin-dependent kinase activity (Hirose et al., 2008). Auxin cooperates with cytokinins in the regulation of cell cycle progression and is also an essential regulator of cell expansion (Perrot-Rechenmann, 2010; Jurado et al., 2010). Initial studies with these phytohormones also showed that cytokinins may stimulate the maturation and replication of plastids during leaf growth (Stetler and Laetsch, 1965; Boasson et al., 1972; Lichtenthaler and Buschmann, 1978), as well as the biosynthesis of photosynthetic pigments, plastid proteins and nucleic acids (Feierabend and de Boer, 1978; Kinoshita et al., 1979; Naito et al., 1979). Furthermore, it was shown that chloroplast replication is favored and chloroplast degradation reduced in the presence of cytokinins in the light (Thimann et al., 1977). Later, several studies focused on the role of cytokinins in *in vitro* growth cultures and de-etiolation, and it has been shown that exogenous cytokinins play a key role in de-etiolation and chloroplast formation (Chory et al., 1994). More recently, several studies have been focused on the molecular biology underlying cytokinin responses (Frébort et al., 2011), but to our knowledge nothing is known about the influence of plant age on the accumulation of these important phytohormones in juvenile trees.

Taking advantage of mastic trees (*Pistacia lentiscus* L.), a dominant tree of the coastal areas of Mediterranean basin which matures after 5 or 6 years and can survive for more than 100 years, the influence of plant age on the ecophysiology of leaves during early phases of growth was investigated, with a particular focus on cytokinins. We hypothesized that increased plant age may exert an effect on endogenous cytokinin levels and affect leaf growth. More specifically, we evaluated to what extent (i) the growth of young, emerging leaves might be regulated by cytokinins and auxins in this species, (ii) this may be influenced by plant age, and (iii) age-related changes in cytokinin levels may alter cytokinin-regulated processes, such as leaf growth and pigment accumulation, in juvenile mastic trees.

2. Materials and methods

2.1. Plant material, growth conditions and sampling

2.1.1. Experiment 1

A population of 60 juvenile mastic trees (*P. lentiscus* L.), which were growing in a Calcic Luvisol (FAO) at the experimental fields of the Faculty of Biology at the University of Barcelona (Barcelona, Spain) together with *Cistus albidus* L., were used for experiments. Plants of different species were distributed in different areas, but in close proximity (separated by 3 m only). Mastic trees differed in size, with a height ranging between 50 and 100 cm, and with all individuals still in a juvenile stage. All plants were exposed to Mediterranean field conditions and received water exclusively from rainfall both prior and during the experiments. For experiments, new, emerging leaves of each plant were labeled during the autumn of 2009 and samples (labeled leaves) were collected every two months from January to July 2010. Leaf biomass, leaf area and endogenous levels of cytokinins and auxin were measured as described below. At each sampling point, leaf area and leaf biomass were measured from 16 randomly selected individuals, while phytohormones were measured from leaves of 10 randomly chosen plants.

2.1.2. Experiment 2

This study was conducted in parallel to the previous one but using a different set of plants, which included two plant groups (aged 2 and 5 years old, respectively). Both plant groups were obtained from the same seeds, but seeds from 2-year-old plants were induced to germinate 3 years later. After germination, all plants were grown in a glasshouse under controlled watering conditions until the spring of 2009, when both plant groups were transferred to the experimental fields of the University of Barcelona. Twenty-five individuals of each group were transplanted at the experimental fields (Calcic Luvisol, FAO) in two plots of 30 m^2. Each plot was separated by 2 m and all plants were exposed to Mediterranean field conditions until the experiment started and during the whole experiment, receiving no additional watering. At the time of experiments (2010), both groups were in a juvenile stage but differed in size (Table 1). New, emerging leaves were also labeled during the autumn of 2009 and samples collected once growth has already started (January 2010) and at maximum rates of cell division (May 2010). Leaf biomass, leaf area, endogenous levels of cytokinins and auxin, photosynthetic pigments levels and the efficiency of PSII photochemistry (F_v/F_m ratio) were measured as described below. At each leaf growth stage, six randomly chosen plants were used for measurements.

Table 1
Growth parameters of 2- and 5-year-old plants at the end of the experiment (July 2010). DW, dry weight. P, photosynthetic biomass. NP, non-photosynthetic biomass. Data correspond to the mean ± SE of $n = 4$ individuals.

Parameters	2-year old	5-year old
Total biomass (g DW)	56.1 ± 2.8	619.4 ± 22.6*
Photosynthetic biomass (g DW)	24.1 ± 6.8	180.0 ± 28.2*
Aerial non-photosynthetic biomass (g DW)	20.4 ± 2.9	282.0 ± 37.8*
Roots biomass	11.5 ± 2.6	157.4 ± 21.1*
P/NP	0.71 ± 0.10	0.41 ± 0.02*
Shoot/root biomass	4.3 ± 0.9	3.0 ± 0.3

* Significant differences between 2- and 5-year-old plants (Student's *t*-test, $P \leq 0.05$).

In addition, the two set of plants (2- and 5-year-old plants) were exposed to summer drought and mature leaves were collected prior (18 May, control) and during summer drought (29 July, stress) to compare the physiology of leaves in terms of water contents, photosynthetic pigment and anthocyanin levels and the F_v/F_m ratio. Monthly rainfall during June and July was of 25.2 and 34.1 mm, respectively, so that a summer drought typical of the Mediterranean climate occurred during the experiment. Mortality rates were measured in these two plant groups after the summer.

Additionally, leaves from a mixture of the juvenile plants described for Experiments 1 and 2 were used for regression analyses to evaluate the relationship between endogenous Z levels and pigment contents, as well as physiological stress indicators, including the levels of total anthocyanins, the F_v/F_m ratio, total N contents and the relative water content (RWC). A minimum of 36 individuals were taken for each regression analyses.

For all experiments, all samples were collected at midday (at maximum incident diurnal PPFD). For analysis of endogenous levels of phytohormones and photosynthetic pigments, samples were collected, immediately frozen in liquid N_2, and stored at −80 °C until analysis. Despite destructive analysis, the large number of leaves labeled at the beginning of each experiment allowed us to follow the exact development stage of the leaves throughout the experiments.

2.2. Leaf biomass, area and estimation of growth rates

Leaf biomass was measured by weighing the samples before and after drying to constant weight at 80 °C. Leaf area was estimated by using a flatbed scanner (model CX-5400; Epson Stylus, Nagano, Japan) and an image-processing program. Leaf growth rates were estimated by considering either the leaf biomass or area at each sampling point and the time elapsed between them. At the end of the experiment, total plant biomass, considering photosynthetic

and non-photosynthetic parts were measured from 4 individuals per age group as described by Oñate and Munné-Bosch (2010).

2.3. Phytohormone analysis

The extraction and analysis of endogenous concentrations of cytokinins and auxin were performed as described by Müller and Munné-Bosch (2011). Deuterium labeled phytohormones were used as internal standards.

2.4. Pigments and chlorophyll fluorescence

Chlorophylls (Chls) were estimated spectrophotometrically from methanolic extracts as described by Lichtenthaler (1987). Later, the same extracts were acidified with HCl and total anthocyanins measured spectrophotometrically as described by Gitelson et al. (2001). Measurements of the maximum efficiency of photosystem II photochemistry (F_v/F_m ratio) were performed by using a pulse modulated fluorimeter Imaging PAM (Walz, Effeltrich, Germany) after 2 h of dark adaptation. The F_v/F_m ratio was calculated as $(F_m - F_0)/F_m$, where F_m and F_0 are the maximum and basal fluorescence yields, respectively, of dark adapted leaves (van Kooten and Snel, 1990).

2.5. Water and N contents

The RWC was determined as (FW – DW)/(TW – DW), where FW is the fresh matter, TW is the turgid weight after hydrating the leaves with distilled water for 24 h at 4 °C, and DW is the dry

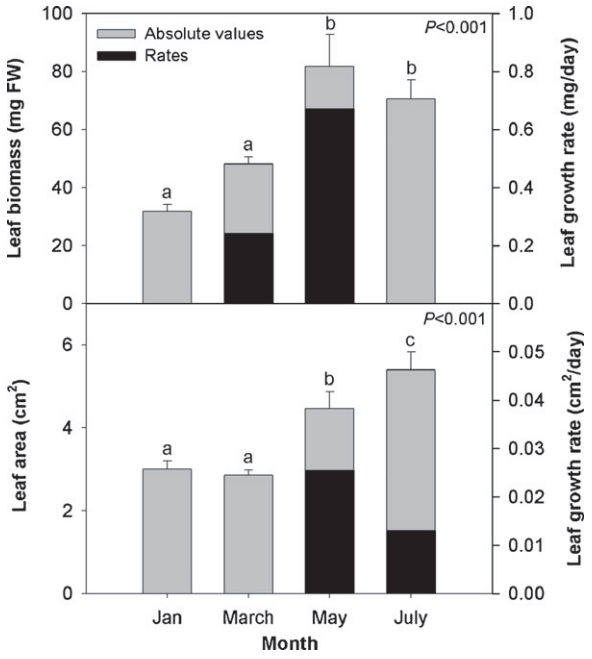

Fig. 1. Leaf biomass, leaf area and leaf growth rates (expressed both as mg fresh weight/day and cm^2/day) in mastic tree leaves during early phases of leaf development from January to July. Data correspond to the mean ± SE of $n = 16$ individuals (Experiment 1, see Section 2 for details). Results of statistics, which indicate differences over time, are shown in the inserts (one-way ANOVA). Different letters indicate significant differences between sampling points using DMS posthoc analyses.

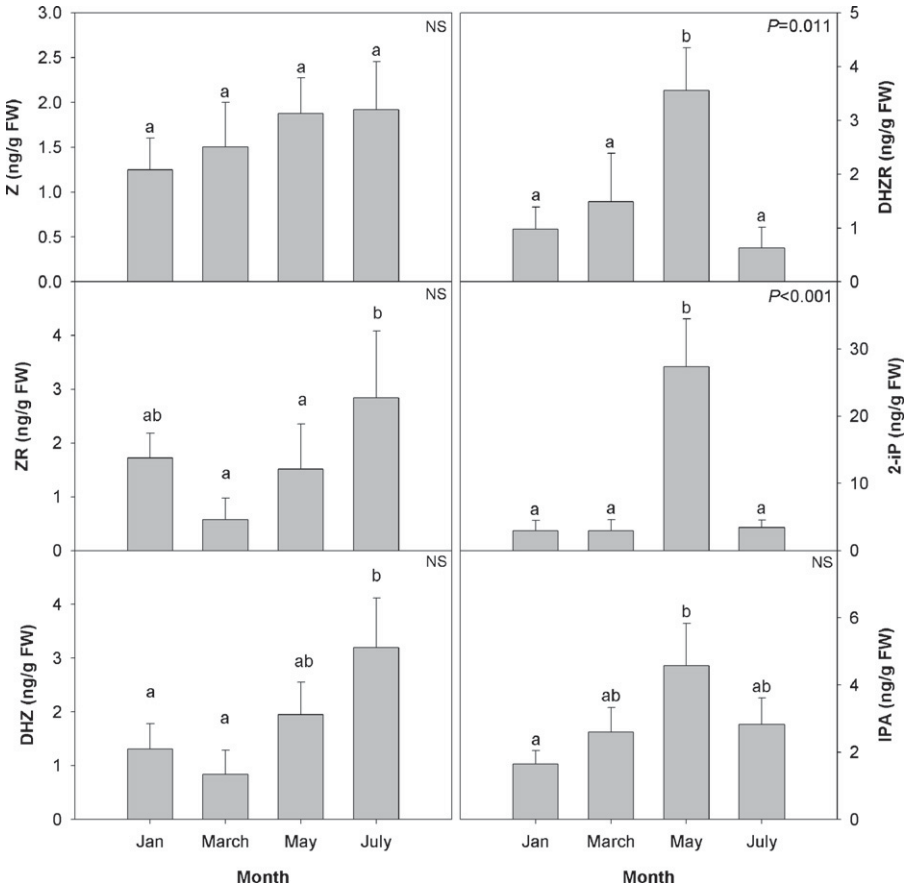

Fig. 2. Endogenous levels of cytokinins in mastic tree leaves during early phases of leaf development from January to July. Data correspond to the mean ± SE of $n = 10$ individuals (Experiment 1, see Section 2 for details). Results of statistics, which indicate differences over time, are shown in the inserts (one-way ANOVA). Different letters indicate significant differences between sampling points using DMS posthoc analyses.

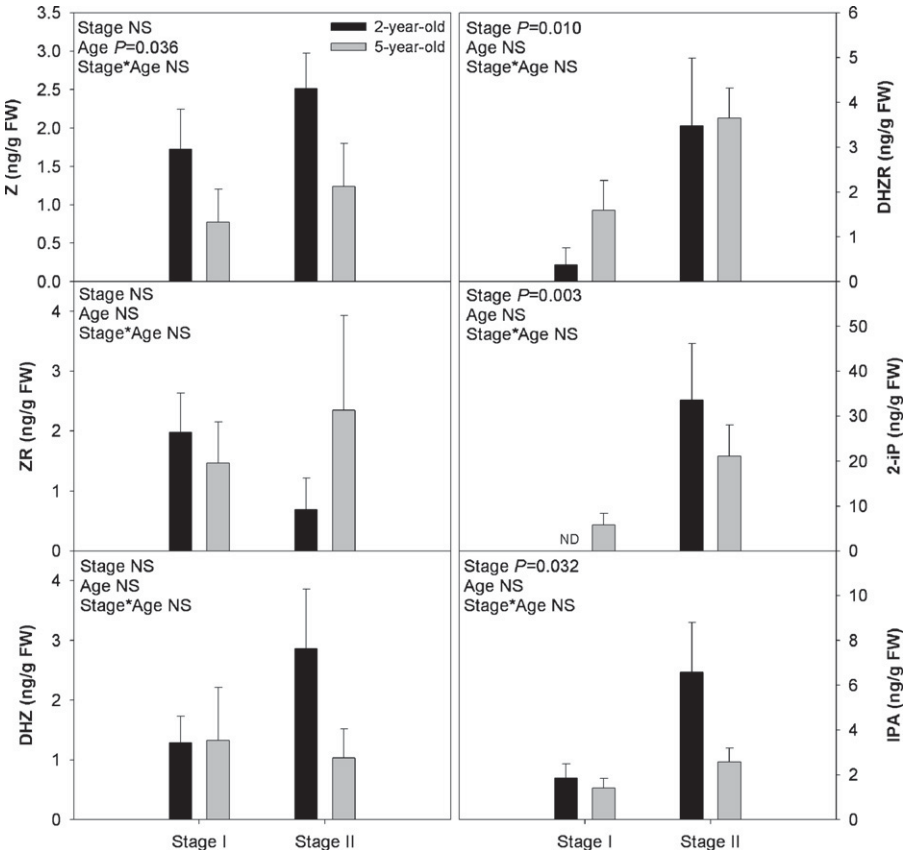

Fig. 3. Endogenous levels of cytokinins in leaves of 2- and 5-year-old mastic trees during early phases of leaf development from January (stage I, start) to May (stage II, maximum growth). Data correspond to the mean ± SE of $n = 6$ individuals. Results of statistics, which indicate differences over time (between stages) and plant groups (age), are shown in the inserts (two-way ANOVA).

matter after oven-drying the samples. Total N concentrations in leaves were measured by the Dumas elemental analysis method, using a Thermo EA 1108 analyzer (Thermo Scientific, Milan, Italy).

2.6. Statistical analyses

Differences with time, leaf developmental stage and/or plant age were evaluated using the analyses of variance (ANOVA). Regression analyses were used to evaluate the relationship between zeatin or Chl a/b and different parameters. Effects of different factors were considered significant at a probability level of $P < 0.05$, giving specific P values in the figure inserts in each case.

3. Results

3.1. Cytokinins, auxins and leaf growth

Cytokinins, auxins and leaf growth were analyzed in the first experiment from a population of juvenile mastic trees grown under Mediterranean field conditions. Leaf biomass and leaf area of young emerging leaves increased significantly 2.6-fold and 1.5-fold from January to May, respectively, resulting in the highest leaf growth rate in May (Fig. 1). From May to July, however, leaf biomass remained constant, while leaf area increased significantly, thus indicating a period of cell expansion. Levels of endogenous cytokinins increased during the first phases of growth (Fig. 2). Particularly, 2-iP levels increased 9.2-fold from January to May and decreased again to the initial level during July. Similar increases as for 2-iP could be observed for isopenetenyladenosine (IPA) though to a lesser extent (2.9-fold) and dihydrozeatin riboside (DHZR, 3.6-fold). In contrast, levels of Z, zeatin riboside (ZR), as well as levels of the auxin, indole-3-acetic acid (IAA) remained constant during both the periods of cell division and expansion. Dihydrozeatin (DHZ) levels increased slightly during the latest phases of leaf growth (Fig. 2, Fig. S1, Supporting Information). Significant changes on leaf growth (both leaf area and biomass $P < 0.001$) were accompanied with significant changes in endogenous cytokinin levels (2-iP, $P < 0.001$ and DHZR, $P = 0.011$), whereas levels of auxin did not change significantly, except when expressed per leaf unit (Fig. 1, Fig. 2 and Supplementary Fig. S1).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2012.09.007.

3.2. Plant age-related changes in cytokinins

Parallel to the first experiment two different plant age groups of juvenile mastic trees were analyzed focusing on age-related changes in cytokinin levels. Two- and 5-year-old plants differed significantly in size (5-year-old plants showed 86% higher photosynthetic, 92% higher root and 90% higher total biomass compared to 2-year-old plants, Table 1). Endogenous cytokinin levels in leaves of the groups (2- and 5-year-old juvenile mastic trees) measured at the start (stage I, January) and at maximum growth (stage II, May) revealed significant age-related effects (Fig. 3), but only for Z. Within cytokinins only Z showed significant changes for the two plant age groups. Interestingly, 2-year-old plants had 2-fold higher Z levels compared to 5-year-old plants both at the start and maximum growth (stage I and II). In contrast, IAA levels did not differ between both plant age groups (Supplementary Fig.

S2). However, the higher levels of Z in 2-year-old plants did not result in significant higher leaf growth compared to 5-year-old plants (Fig. 4). Therefore, growth of individual leaves was similar between both age groups, while 5-year-old plants accumulated much higher leaf biomass per plant compared to 2-year-old plants (Table 1). Age-related changes in Z were associated with variations in the Chl contents and the F_v/F_m ratio (Fig. 5). Whereas 5-year-old plants showed 11.7% lower Chl a + b levels than 2-year-old plants (although changes were only significant at stage I and when expressed per fresh weight, but not leaf area), the Chl a/b ratio increased 4.1% with plant age both at stages I and II. The F_v/F_m ratio showed significant changes with plant age but only at stage II.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2012.09.007.

3.3. Correlative analysis between zeatin and pigment contents

To confirm differences between endogenous Z levels and photosynthetic pigment contents and the F_v/F_m ratio, leaves from a mixture of both populations used in previous experiments were analyzed together with physiological stress indicators, including the levels of total anthocyanins, total N contents and the RWC. A significant correlation was observed between Z levels and the Chl a/b ratio, but not with Chl a + b contents or the F_v/F_m ratio. High levels of Z were associated with reductions in Chl a/b ratios as it was observed for 2-year-old plants, while low levels of Z were associated with increasing Chl a/b ratios as observed for 5-year-old plants (Fig. 6). Neither total anthocyanins, total N nor RWC correlated significantly with Z levels (Fig. 7). Further regression analyses showed a significant positive correlation between Chl a/b ratios and

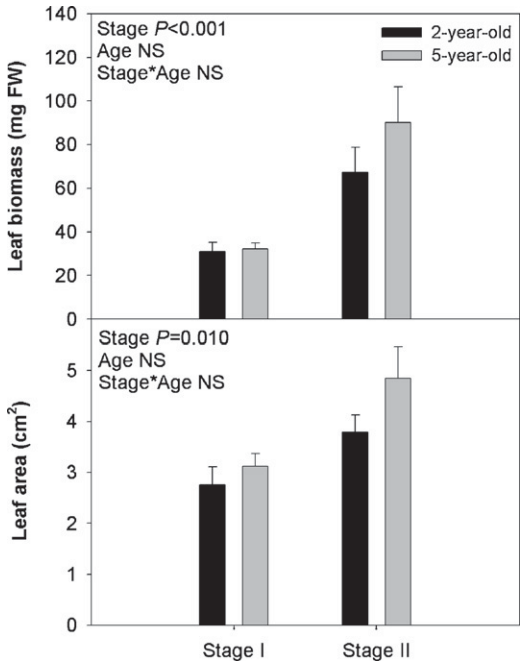

Fig. 4. Leaf biomass and area of 2- and 5-year-old mastic trees during early phases of leaf development from January (stage I, start) to May (stage II, maximum growth). Data correspond to the mean ± SE of $n = 6$ individuals. Results of statistics, which indicate differences over time (between stages) and plant groups (age), are shown in the inserts (two-way ANOVA).

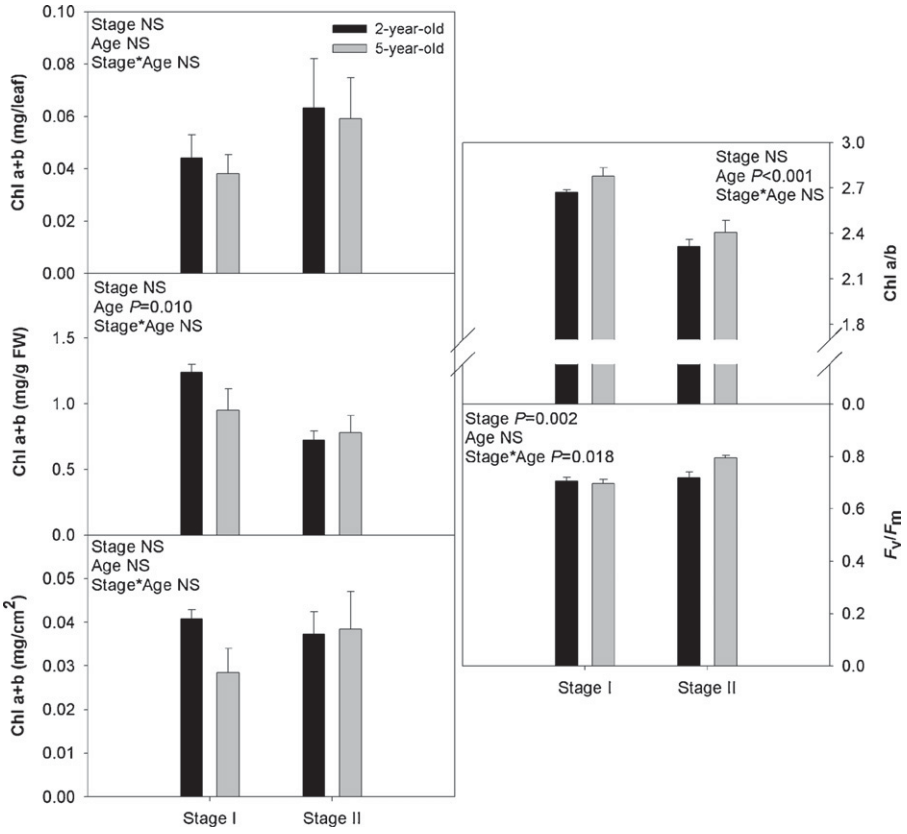

Fig. 5. Foliar levels of chlorophyll (Chl) a + b (expressed per leaf, fresh weight and area), the Chl a/b ratio and the maximum efficiency of PSII photochemistry (F_v/F_m ratio) of 2- and 5-year-old mastic trees during early phases of leaf development from January (stage I, start) to May (stage II, maximum growth). Data correspond to the mean ± SE of $n = 6$ individuals. Results of statistics, which indicate differences over time (between stages) and plant groups (age), are shown in the inserts (two-way ANOVA).

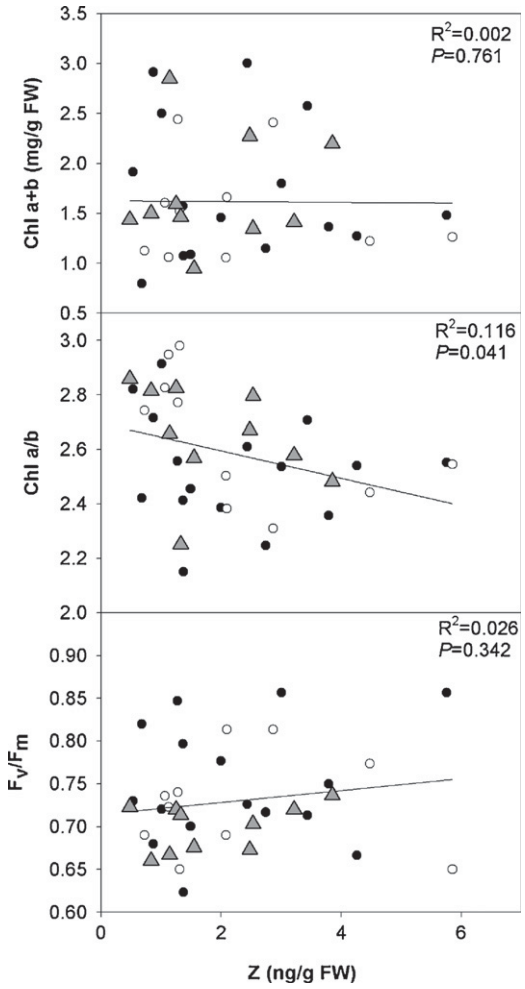

Fig. 6. Regression analyses between endogenous zeatin levels and chlorophyll (Chl) a + b contents (expressed per fresh weight), the Chl a/b ratio and the maximum efficiency of PSII photochemistry (F_v/F_m ratio) of juvenile mastic trees during early phases of leaf development. Data of Experiment 1 (triangles) and Experiment 2 (circles) are shown together, indicating 2- and 5-year-old plants in black and white, respectively. Results of statistics, which indicate significance of the regression analyses, are shown in the inserts.

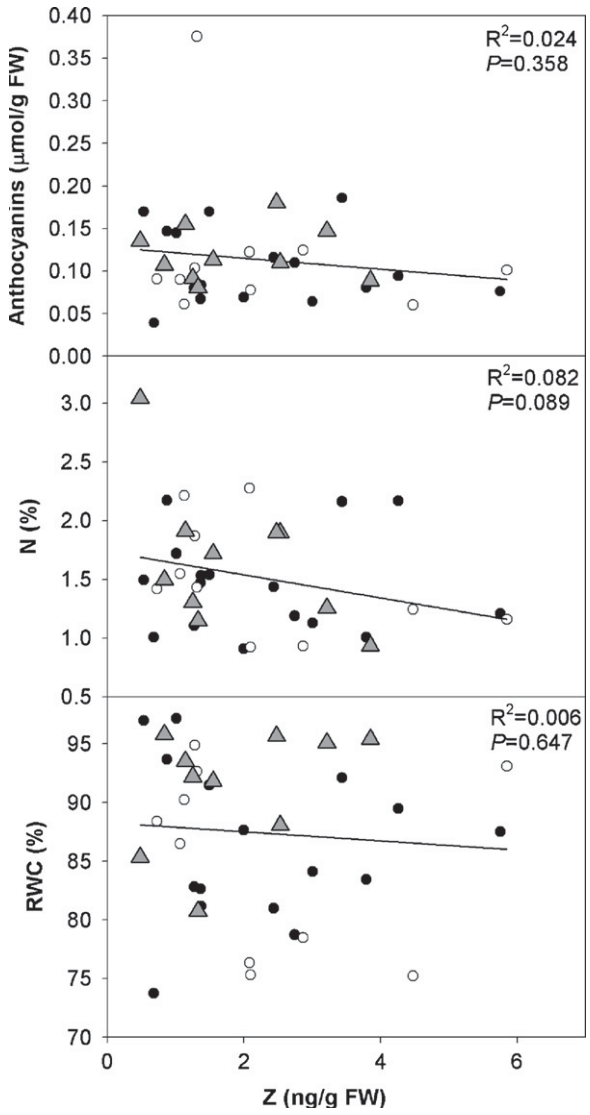

Fig. 7. Regression analyses between endogenous zeatin levels and anthocyanins accumulation, total N content, and relative water content (RWC) of juvenile mastic trees during early phases of leaf development. Data of Experiment 1 (triangles) and Experiment 2 (circles) are shown together, indicating 2- and 5-year-old plants in black and white, respectively. Results of statistics, which indicate significance of the regression analyses, are shown in the inserts.

anthocyanin levels as well as total N contents, and a marginally significant negative correlation with the F_v/F_m ratio (Fig. 8).

3.4. Plant response to summer drought was not influenced by plant age

To evaluate to what extent the age-related differences in cytokinin levels and the Chl a/b ratio influence the plant response to summer drought, several markers of photooxidative stress were evaluated in mature leaves of both 2- and 5-year-old plants prior (May, considered as controls) and during summer drought (July, stressed plants). Results showed constant foliar RWC values, Chl a + b levels, and the Chl a/b and F_v/F_m ratios, during the summer drought (Fig. 9). Anthocyanin levels decreased during drought but did not differ between age groups. Despite absence of symptoms of age-related photooxidative stress in leaves, the mortality rate in 2-year-old plants was of 11.43%, while no plants died in the oldest group.

4. Discussion

Several ecophysiological important traits are hormonally mediated, such as the integration of environmental signals, phenotypic plasticity and development processes. Several studies have investigated the hormonal physiology of plants growing in a variety of complex environmental situations (Voesenek and Blom, 1996; Mercier and Endres, 1999; Amzallag, 2001; Wang et al., 2012). In the present study, it is shown how an important ecological and physiological trait, such as plant age, affects the endogenous levels of cytokinins in juvenile mastic trees.

Cytokinins play an important role in plant growth and development by regulating a number of processes, including meristem size and activity (Werner and Schmülling, 2009), root proliferation (Werner et al., 2001), reproductive organ development (Corbesier et al., 2003; Bartrina et al., 2011) or leaf senescence (Mok and Mok, 1994; Gan and Amasino, 1995; Kim et al., 2006), and they may also have a role in controlling chloroplast number and differentiation within the cell (Okazaki et al., 2009). Results show that the highest concentrations of 2-iP were associated with the maximum leaf growth rate in May. Although Z and ZR are generally considered the major cytokinin active forms, these results suggest that 2-iP is the active form promoting cell division in this species. This is an

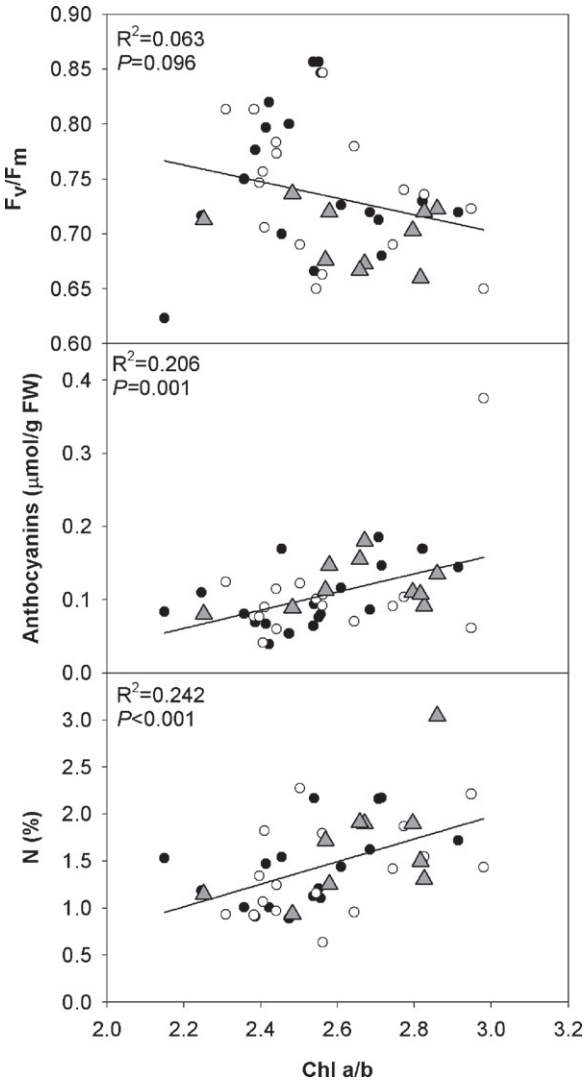

Fig. 8. Regression analyses between the chlorophyll (Chl) a/b ratio and the maximum efficiency of PSII photochemistry (F_v/F_m ratio), anthocyanins accumulation, and total N content of juvenile mastic trees during early phases of leaf development. Data of Experiment 1 (triangles) and Experiment 2 (circles) are shown together, indicating 2- and 5-year-old plants in black and white, respectively. Results of statistics, which indicate significance of the regression analyses, are shown in the inserts.

important finding for studies aimed at propagating mastic trees *in vitro*, as those described recently in which micropropagation can be used as an ideal technique for cloning this dioecious woody plant, in which male trees are preferred due to higher economical value (Yildirim, 2012).

Many phenotypic traits of juvenile plants change dramatically over the course of plant growth and development with age. For example, total biomass, in different plant parts, such as leaves and roots, clearly increases with overall growth (Coleman et al., 1994). There are several studies that demonstrate the importance of plant age in determining several ecophysiological traits of mature plants, from leaf growth to reproductive competence, and other that demonstrate the ecological significance of reaching plant maturity (Poorter and Pothmann, 1992; Romero and Marañon, 1996; Lyon and Barnes, 1998; Mencuccini et al., 2007; Oñate et al., 2011; Oñate and Munné-Bosch, 2010). However, very little studies have examined the effects of plant age on juvenile plants, except in some epiphytes in which size is considered to be a key factor modulating photosynthesis and growth (Zotz, 1997; Schmidt and Zotz, 2001). To our knowledge, this is the first study demonstrating a plant-age related effect on cytokinins in juvenile plants. We observed that a difference of 3 years in age led to a 10-fold increase in total biomass with a decrease in the ratio of shoot/root biomass, the youngest plant group still being established in the soil. Establishment of individuals in communities is very important since it is one of the major causes of mortality among young trees in several ecosystems, including the Mediterranean macchia. To reverse land degradation processes, restoration in the Mediterranean Basin had been frequently obtained by planting indigenous shrubs and trees, such as rosemary and mastic trees, which are more efficient than allochthonous in restoring degraded soils (Pariente, 2002; Sarah and Rodeh, 2004). Usually, plantation is successful but mortality increases in these species after summer, independently of the plantation treatments, likely due to summer drought (De Dato et al., 2009). Indeed, an increased mortality was observed in the youngest group of plants; the mortality rate in 2-year-old plants was of 11.43%, while no plants died in the oldest group. Other studies have also shown that mortality and growth are related with plant age, showing a higher mortality and lower growth rates for juvenile trees (Davies, 2001).

Previous studies show that plant form, physiology, and sites of hormone action and tissue sensitivity also change throughout plant development, as well as environmental changes (Amzallag, 2001). In the present study, we observed significant reductions in the levels of Z in 5-year-old plants compared with 2-year-old plants. Aside from essential modulators of plant growth, cytokinins are known to affect photosynthesis and control chloroplast biogenesis and Chl biosynthesis, the differentiation of chloroplasts and their number in the cell, and upregulate the production of two important photosynthetic proteins: the small subunit of rubisco (rbcS) and the major Chl a/b-binding polypeptide of the light-harvesting complex in the chloroplast (Zubo et al., 2008; Okazaki et al., 2009). Here, it was shown that the reductions in Z levels with plant age (5-year-old plants showed 2-fold higher levels compared to 2-year-old plants) were associated with slight but significant increases (5%) in the Chl a/b ratio, but not with any changes in leaf growth. Cytokinin applications in tobacco induce an increase in grana stacking and a lowering of the Chl a/b ratio in these chloroplasts (Wilhelmova and Kutik, 1995), thus suggesting that plant age-related changes in cytokinins in juvenile mastic trees led to differences in chloroplast maturation and photosynthetic pigment accumulation rather than to any damage to the photosynthetic apparatus, since the F_v/F_m ratio kept constant of even slightly higher (at stage II) in 5- compared to 2-year-old plants. In addition, regression analyses in this species revealed that Z levels correlated with the Chl a/b ratio, but not with the F_v/F_m ratio. Are however such differences in the Chl a/b ratio leading to any alteration in the physiological ecology of plants? It has been described that reductions in Chl, and more specifically increases in the Chla/b ratio may be an adaptive feature in plants growth under extreme climatic conditions by reducing the amount of light intercepted by leaves and at the same time reduces the possibility of further damage to the photosynthetic machinery by the formation of activated oxygen under high light (Kyparissis et al., 1995; Munné-Bosch and Alegre, 2000). However, there were no age-related changes in Chl contents and the F_v/F_m ratio was not negatively affected in 5-year-old plants. Therefore, changes in the Chl a/b ratio between ages must be related with an effect of the cytokinins in the chloroplast maturation.

Further regression analyses showed that although enhanced Chl a/b ratios predispose leaves to suffer photooxidative stress, as indicated by increased anthocyanins levels and lowered F_v/F_m ratios in juvenile mastic trees, plants of different ages show a similar degree of foliar stress tolerance. During the summer drought, mature leaves kept constant foliar RWC values, Chl a + b levels, and the Chl a/b and F_v/F_m ratios, thus confirming absence of photooxidative stress in leaves with increasing age. It is very likely

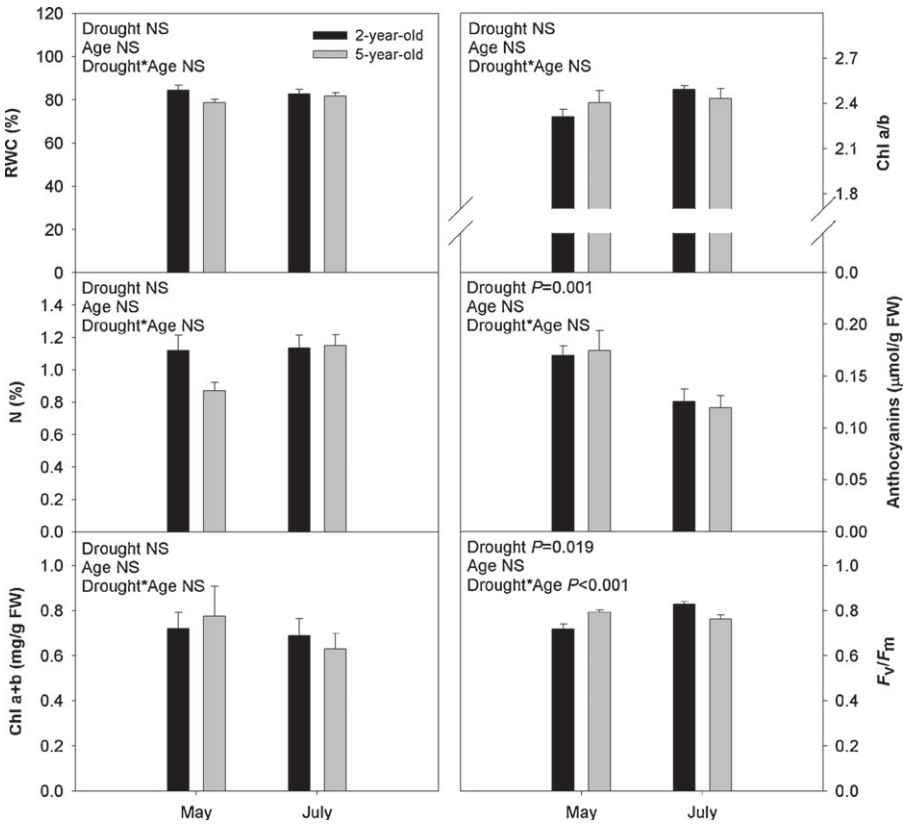

Fig. 9. Relative leaf water content (RWC), N contents, and foliar levels of chlorophyll (Chl) a + b, the Chl a/b ratio, anthocyanin accumulation and the maximum efficiency of PSII photochemistry (F_v/F_m ratio) of 2- and 5-year-old mastic trees prior (May, considered as controls) and during summer drought (July, stressed plants). Data correspond to the mean ± SE of $n = 6$ individuals. Results of statistics, which indicate differences over time (drought effects, between May and July) and plant groups (age), are shown in the inserts (two-way ANOVA).

that the increased mortality in the youngest individuals is related to establishment processes in the soil. Indeed, previous studies have shown a strong positive relationship between survival and maximum rooting depth, as well as between survival and soil moisture in Mediterranean woody species (Padilla and Pugnaire, 2007). Therefore, the root depth, rather than age-related changes in cytokinin levels in leaves appear to be crucial in determining the survival of the plant when this is being established in the soil.

It is concluded that plant age may alter the endogenous composition of cytokinins in leaves of juvenile mastic trees. However, this was not associated with reductions in leaf growth, but with a differential pigment accumulation. Although changes in the Chl a/b ratio correlated with markers of photooxidative stress in this species, the youngest individuals did not suffer from increased foliar stress during the summer drought. Enhanced mortality rates in the youngest individuals may be due to establishment processes in the root-soil interface, rather than to age-related differences in foliar cytokinin levels.

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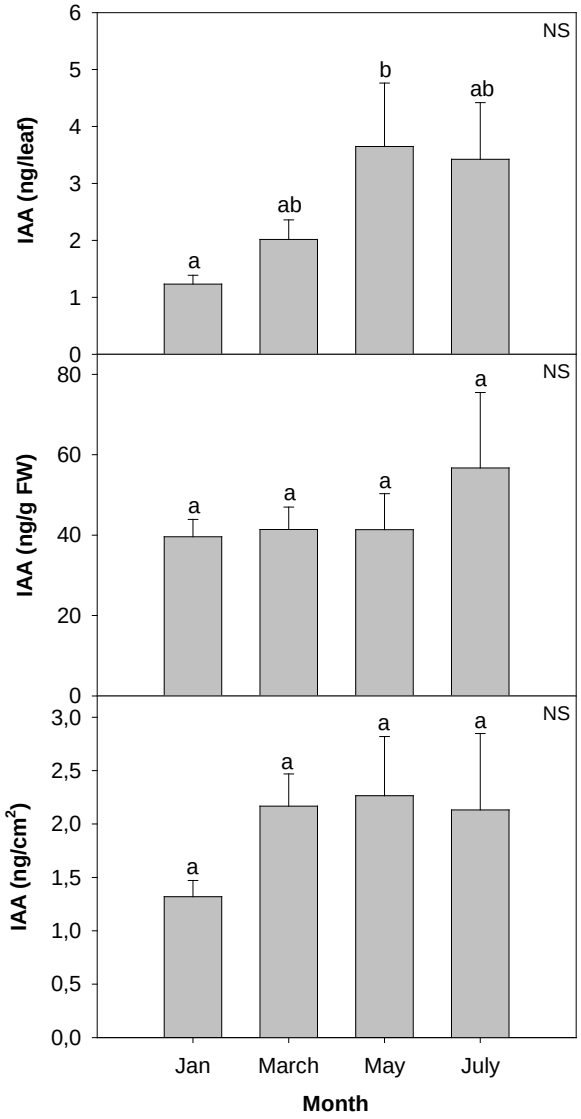
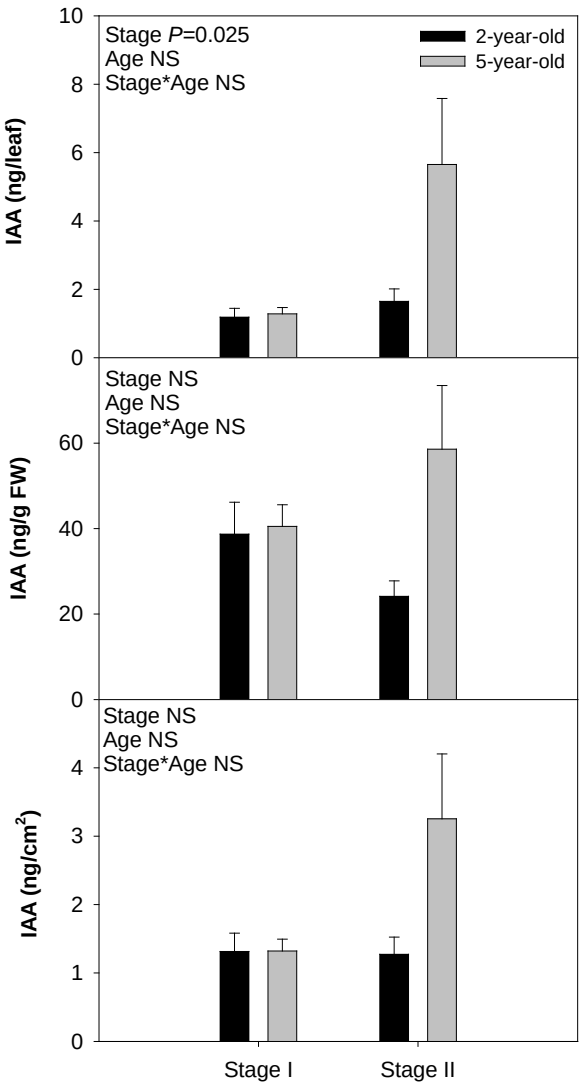
Supplementary data



Supplementary Fig. S1. Endogenous levels of the auxin, índole-3-acetic acid (IAA) in mastic tree leaves during early phases of leaf development from January to July. Data correspond to the mean ± SE of n=10 individuals. Results of statistics, which indicate differences over time, are shown in the inserts (one-way ANOVA). Different letters indicate significant differences between sampling points using DMS poshoc analyses.



Supplementary Fig. S2. Endogenous levels of the auxin, índole-3-acetic acid (IAA) in leaves of 2- and 5-year-old mastic tree during early phases of leaf development from January (stage I, start) to May (stage II, maximum growth). Data correspond to the mean ± SE of n=6 individuals. Results of statistics, which indicate differences over time (between stages) and plant groups (age), are shown in the inserts (two-way ANOVA).

CAPÍTOL 3

Diferències en el vigor de les gemmes, la peroxidació lipídica i la regulació hormonal durant el trencament de la dormició entre arbres sans i moribunds de faig (*Fagus sylvatica* L.)

CHAPTER 3

Bud vigor, budburst lipid peroxidation and hormonal regulation of bud dormancy release in healthy and moribund beech (*Fagus sylvatica* L.) trees

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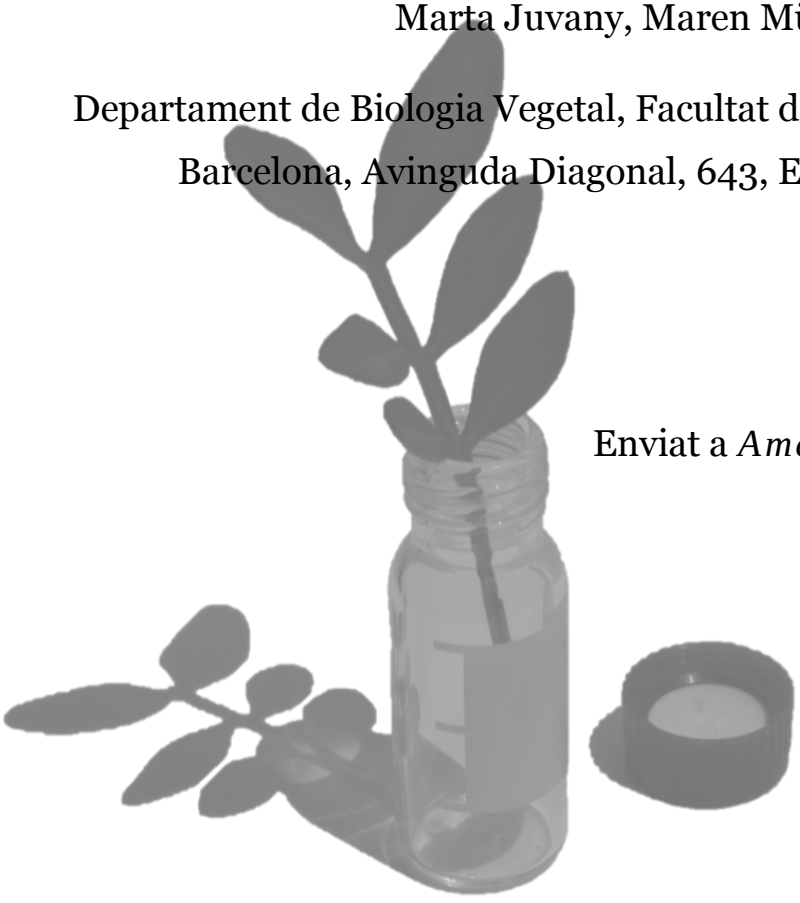

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RESUM DEL CAPÍTOL 3

Un millor coneixement dels factors que regulen el vigor i el trencament de la dormició de les gemmes així com dels mecanismes subjacents és essencial per a la gestió dels arbres de fulla ample, essent els roures i els faigs uns dels més importants. En aquest estudi es va investigar les possibles diferències en el vigor, trencament de la dormició i germinació de les gemmes en arbres de faigs sans i moribunds. Les gemmes vegetatives de les branques situades a l'ombra en el sotabosc d'una fageda natural a Catalunya es varen utilitzar per fer les comparacions. Els resultats varen mostrar una disminució del vigor de les gemmes en els arbres moribunds en comparació amb els sans. També en els moribunds es va observar un increment de la peroxidació lipídica. Tot i que les gibberel·lines, citocinines i auxines modularen el trencament de la dormició en ambdós tipus d'arbres, les gemmes dels arbres moribunds mostraven una dinàmica clarament diferent referent a la concentració endògena d'auxines, àcid abscísic i del precursor de l'etilè, l'àcid 1-aminociclopropà-1-carboxílic, durant el trencament de la dormició de les gemmes. Es va concloure que (i) les gibberel·lines, citocinines i auxines regulaven el trencament de la dormició en arbres de faig, (ii) que existia una dinàmica hormonal diferent en el trencament de la dormició en els arbres moribunds, (iii) els arbres moribunds presentaven un menor vigor i una major peroxidació lipídica que els arbres sans.

Bud vigor, budburst lipid peroxidation and hormonal regulation of bud dormancy release in healthy and moribund beech (*Fagus sylvatica* L.) trees

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Abstract

A better understanding of the factors governing bud vigor, bud dormancy release and budburst and their underlying mechanisms is essential for management of broad-leaved stands, of which oaks and beeches are among the most important species. This study investigated possible differences in bud vigor, bud dormancy release and budburst in healthy and moribund beech trees. Leaf buds on shaded branches found in the understory of a natural beech stand in Catalonia (NE Spain) were used for comparison. Results showed less bud vigor in moribund trees than in healthy ones. In the former, there was increased lipid peroxidation at budburst. Although gibberellins, cytokinins and auxin modulated bud dormancy release in both tree types, buds of moribund trees showed markedly different dynamics in the endogenous concentrations of auxin, abscisic acid and the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid, during bud dormancy release and budburst. We conclude that (i) gibberellins, cytokinins and

Abbreviations: ABA: abscisic acid, ACC: 1-amino-cyclopropane-1-carboxyic acid, DHZ: dihydrozeatin, DHZR: dihydrozeatin riboside, GA: gibberellin, JA: jasmonic acid, IAA: indole-3-acetic acid, 2iP: 2-isopentenyladenine, IPA: isopentenyladenosine, JA: jasmonic acid, MDA: malondialdehyde, RWC: relative water content, SA: salicylic acid, Z: zeatin, ZR: zeatin riboside.

auxin regulate bud dormancy release in beech trees, (ii) there is a distinct hormonal dynamic of bud dormancy release in moribund trees, and (iii) moribund trees have less bud vigor and greater lipid peroxidation at budburst than healthy trees.

Introduction

Factors governing bud vigor, bud dormancy release and budburst, and their underlying mechanisms in broad-leaved stands, of which oaks and beeches are among the most important species, have been the subject of intensive research in recent decades, due to their important implications for forest management (Huttunen et al. 2001). In established beech stands, the formation of epicormic branches or their persistence on the tree is often associated with (i) stand thinning, leading to the formation of "light suckers" or (ii) increased shading, leading to the formation of "agony branches" or "shade suckers" (Nicolini et al. 2001). In addition, epicormic branch formation may be caused by other abrupt changes in a tree's environment, such as severe abiotic constraints (drought, extreme cold), storms or insect attacks (Meier et al. 2012). In general, any phenomenon that interferes with the basipetal transport of auxin from the apical bud of the main shoot favors epicormic branch formation due to a loss of apical dominance within the tree (Cline, 2000). In consequence, epicormic branches from moribund trees, according to Kraft's definition (1884, in Lanier 1986), share space and compete for water, nutrients and light with branches from healthy, young trees, which are being established in the understory of natural beech stands. Although epicormic branch formation has been intensively researched in recent years (reviewed by Meier et al. 2012), the physiology of buds found in epicormic branches of broad-leaved stands has received very little attention. To our knowledge, no studies have compared the physiology of the buds derived from epicormic branches with those from healthy trees found in equivalent climatic conditions in the understory of beech stands.

Epicormic branches from moribund trees generally result from damage to the tree, particularly at the apex of the main shoot (Nicolini et al. 2001). Therefore, tissues derived from epicormic branches will probably show some signs of physiological deterioration. However, theory predicts that a meristem keeps totipotency and the capacity to form new organs almost indefinitely (Verdeil et al. 2007). Given that epicormic branches come from latent buds that were previously undamaged for several years, it is also possible that epicormic branches give rise to tissues with exactly the same physiological status as those formed from healthy trees under the same climatic conditions. Indeed, the formation of epicormic branches serves for the regeneration of the entire tree. Which then of these two will prevail: the meristem (regeneration) effects providing intact tissues or a physiological deterioration linked to the physiology of other parts of the moribund tree? To respond to this question, our research focused on evaluating bud vigor and the extent of lipid peroxidation during budburst, as markers of a possible physiological deterioration, by comparing healthy and moribund individuals in a natural beech stand.

Furthermore, hormones play a role in epicormic branch formation and bud dormancy release (Meier et al. 2012). Auxins are the hormones considered to be most important in controlling bud dormancy release, since the basipetal transport of auxin in the main shoot prevents lateral shoot development (Cline, 2000). But the hormonal balance, rather than a single hormone, is what will control the development of epicormic branches, as well as bud dormancy release and budburst in these branches or in those of healthy, young trees sharing the understory of beech stands. Unfortunately, however, the role of hormones in the regulation of these physiological processes in trees is mostly limited to auxins, gibberellins and cytokinins. Gibberellins are generally recognized as essential regulators of bud break, particularly in buds responding to chilling and photoperiod, such as beeches (Heide 1993; Falusi and Calamassi 2003). As cytokinins play a crucial role in the regulation of cell division and sink strength (Roitsch and

Ehneβ 2000), high cytokinin levels will be required in the developing bud for dormancy release and burst. Kinetin applications did not, however, result in increased bud break in beech (Falusi and Calamassi 2003). Unfortunately, the roles of other cytokinins and growth regulators, such as abscisic acid, ethylene, jasmonates or salicylates have been explored far less. Their effects on bud dormancy release and budburst in beeches are, to our knowledge, still unknown.

In the present study, we hypothesized that bud vigor, lipid peroxidation at budburst and hormonal regulation of bud dormancy release may differ between branches of moribund, old trees and healthy, young ones, the latter showing in general a better physiological status despite the totipotency of meristems. To test this hypothesis, we compared bud vigor for two years, calculated the extent of lipid peroxidation at budburst and evaluated the variations in the endogenous concentrations of phytohormones during bud dormancy release in branches of both tree types in the understory of a natural beech stand.

Materials and methods

Study site, plant material and sampling

The study site was La Fageda d'en Jordà, a beech stand located in la Garrotxa near the village of Santa Pau (42°9'N 2°30'E at 565 m.a.s.l., Girona, Catalonia, NE Spain). This beech stand is characterized by its growth in volcanic soil. The climatic conditions of the field site are typically continental Mediterranean, with abundant precipitation mostly during autumn and winter and dry, hot summers. Within this beech stand, we distinguished two different types of trees for experiments: (i) healthy, young trees (7-25 cm trunk diameter) and (ii) moribund, adult trees (65-140 cm diameter), all of them producing branches in the understory (Fig. 1). Moribund trees accounted for 10.5% of the beeches at the sample site. For measurements, twelve healthy and twelve moribund trees were

randomly selected and leaf buds were selected from branches growing 1.5-2 m above the soil surface, so that all buds were always exposed to the same light conditions in the understory. To obtain buds at different developmental stages, two samplings were conducted at midday. Dormant (latent) buds were sampled on 20 March 2012 (≈400 μmol m^{-2} s^{-1}, 17ºC, 21% relative humidity), while non-dormant (both closed -swollen- and open) buds were collected on 17 April 2012 (≈500 μmol m^{-2} s^{-1}, 19ºC, 24% relative humidity, Fig. 1).

Bud vigor was measured during 2012, 2013 and 2014, while all other measurements (bud mass and water content, lipid peroxidation and hormones) were performed during 2012. For biochemical analysis, samples were collected, immediately frozen in liquid nitrogen and stored at -80ºC until analyses.

Bud vigor, biomass and relative water content

Successful bud development was considered a measurement of bud vigor. Bud vigor (percentage of buds that reached burst) was measured in all buds of three branches of five trees for each tree type.

For biomass and water status calculation, buds were transported in a saturated atmosphere to the lab and immediately weighed to obtain the fresh weight (FW). Then, buds were saturated with distilled water for 24h at 4ºC in darkness to measure their turgid weight (TW). Dry weight (DW) was recorded after oven-drying the samples at 80ºC until constant weight was achieved. The relative water content (RWC) was determined as (FW-DW/TW-DW) × 100.

Lipid peroxidation

The extent of lipid peroxidation was calculated by measuring the amounts of malondialdehyde (MDA) equivalents, following Hodges *et al.* (1999). Bud samples (100 mg) were ground in liquid nitrogen and repeatedly extracted with ice-cold 80% ethanol (v/v), using ultrasonication for 45 min.

The supernatants were pooled and an aliquot was mixed with the same volume of either –TBA solution containing 20% trichloroacetic acid and 0.01% BHT or +TBA solution containing the above plus 0.65% TBA. Samples were vortexed and heated at 95ºC for 25 min. They were then cooled and absorbance was read at 440, 532 and 600 nm. MDA equivalents were calculated as $[(A–B)/157000)] \times 10^6$, where $A=[(Abs\ 532_{+TBA})–(Abs\ 600_{+TBA})–(Abs\ 532_{–TBA}–Abs\ 600_{–TBA})]$ and $B=[(Abs\ 532_{–TBA}–Abs\ 600_{–TBA}) \times 0.0571]$.

Phytohormone analyses

Endogenous concentrations of gibberellins (GAs), cytokinins, auxin, abscisic acid (ABA), the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), salicylic acid (SA) and jasmonic acid (JA) were measured by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS), following the method described by Müller and Munné-Bosch (2011). Deuterated standards were used to calculate recovery rates for each sample.

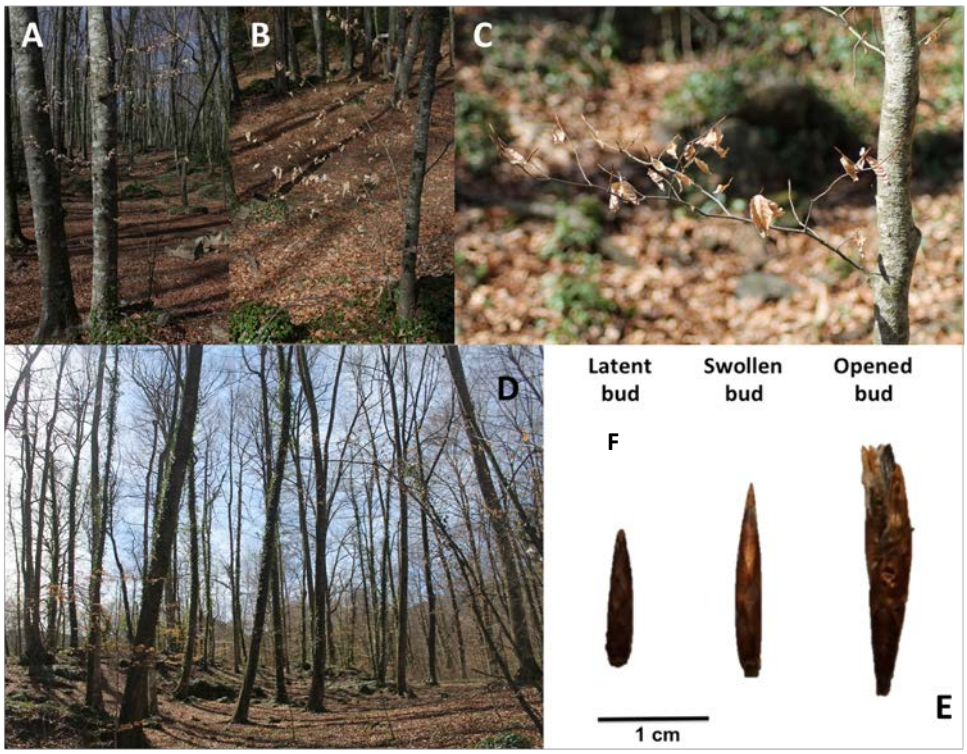
Statistical analysis

Statistical analyses were performed with the SPSS package (Chicago, IL). Differences between years or bud developmental stages, and tree types were found by two-way factorial analyses of variance (ANOVA), followed by DMS post-hoc tests. When data followed unequal variances (i.e. RWC, MDA, GA_{24}, GA_9, GA_4, GA_{19}, all cytokinins except zeatin, and JA), the Games-Howell post-hoc test was applied. Statistical significance was recognized at $P<0.05$.

Results

Moribund trees show less bud vigor and more lipid peroxidation at budburst than healthy trees

Bud vigor was higher in healthy, young trees than in moribund, adult trees during the three years of study. Maximum differences in bud vigor between tree types were observed during 2012, in which healthy trees had bud vigor above 80%, while this dropped below 60% in moribund trees (Fig. 1).



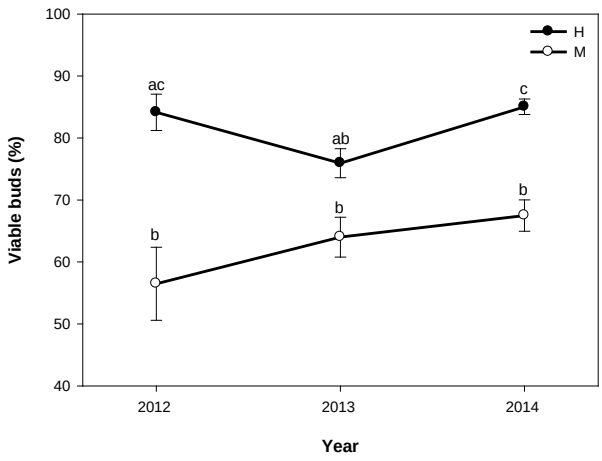


Figure 1: (**A**-**E**) Photographs illustrating details of the site, trees and buds used for experiments. **A**) Moribund tree, **B**) Healthy tree, **C**) Branch from which some buds were sampled, **D**) Study site, La Fageda d'en Jordà, **E**) Bud developmental stages sampled in this study. (**F**) Viability of leaf buds in healthy (H) and moribund (M) beech trees during 2012, 2013 and 2014. Data are the mean ± SE of n=5 individuals, with all buds from 3 branches per individual tree being analyzed. Different letters indicate significant differences (P<0.05).

Measurements of MDA levels during 2012 revealed increased lipid peroxidation at budburst in both healthy and moribund trees, but especially in the latter (Fig. 2). MDA levels increased from below 2 in dormant buds to 17 mmol (g DW)$^{-1}$ in open buds of healthy trees, while MDA equivalents reached 32 mmol (g DW)$^{-1}$ in open buds of moribund trees, thus indicating increased lipid peroxidation at budburst in the latter. Therefore, fewer buds reached burst in moribund trees (Fig. 1). The ones that did reach this stage showed increased lipid peroxidation (Fig. 2).

Bud mass and water content analyses did not reveal any difference between healthy and moribund trees (Fig. 3). Bud mass increased in closed, non-dormant buds more than in dormant buds, while it then remained stable until budburst (open and closed non-dormant buds did not differ in mass). The RWC remained at around 80% throughout bud development, indicating adequate bud water contents in both healthy and moribund trees (Fig. 3).

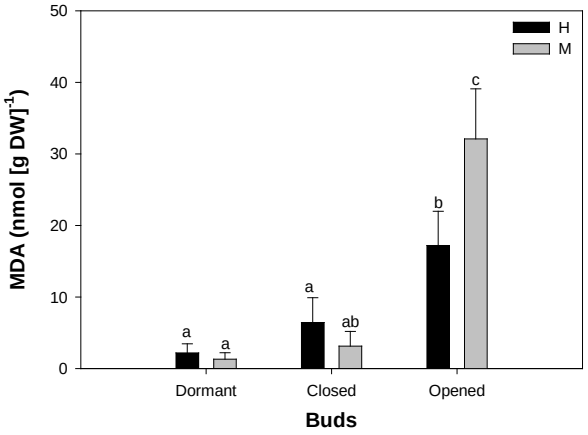


Figure 2: Levels of malondialdehyde (MDA), an indicator of lipid peroxidation in dormant and non-dormant buds of healthy (H) and moribund (M) beech trees. Non-dormant buds included closed and opened buds (the latter were collected just after budburst). Data correspond to the mean ± SE of n=12 individuals. Different letters indicate significant differences (P<0.05).

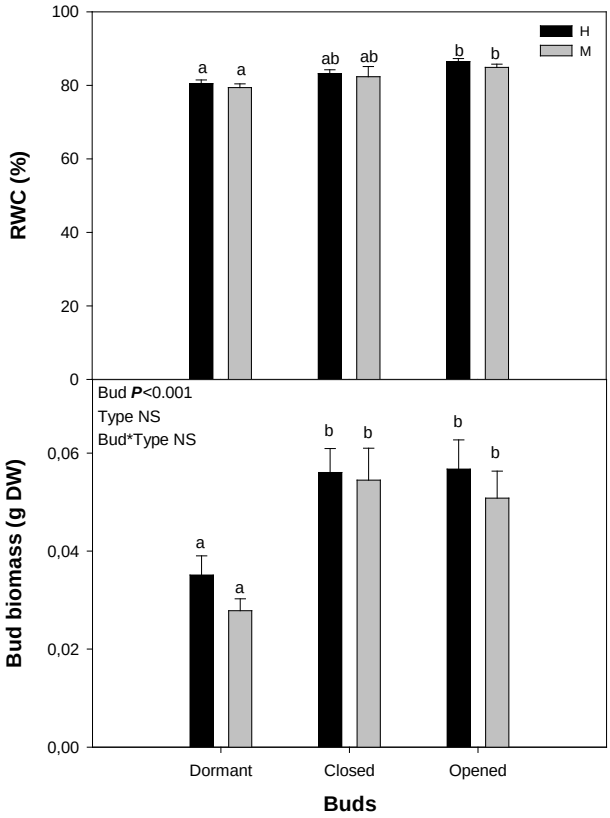


Figure 3: Bud biomass and relative water content (RWC) of dormant and non-dormant buds of healthy (H) and moribund (M) beech trees. Non-dormant buds included closed and opened buds (the latter were collected just after budburst). Data correspond to the mean ± SE of n=12 individuals. Different letters indicate significant differences (P<0.05).

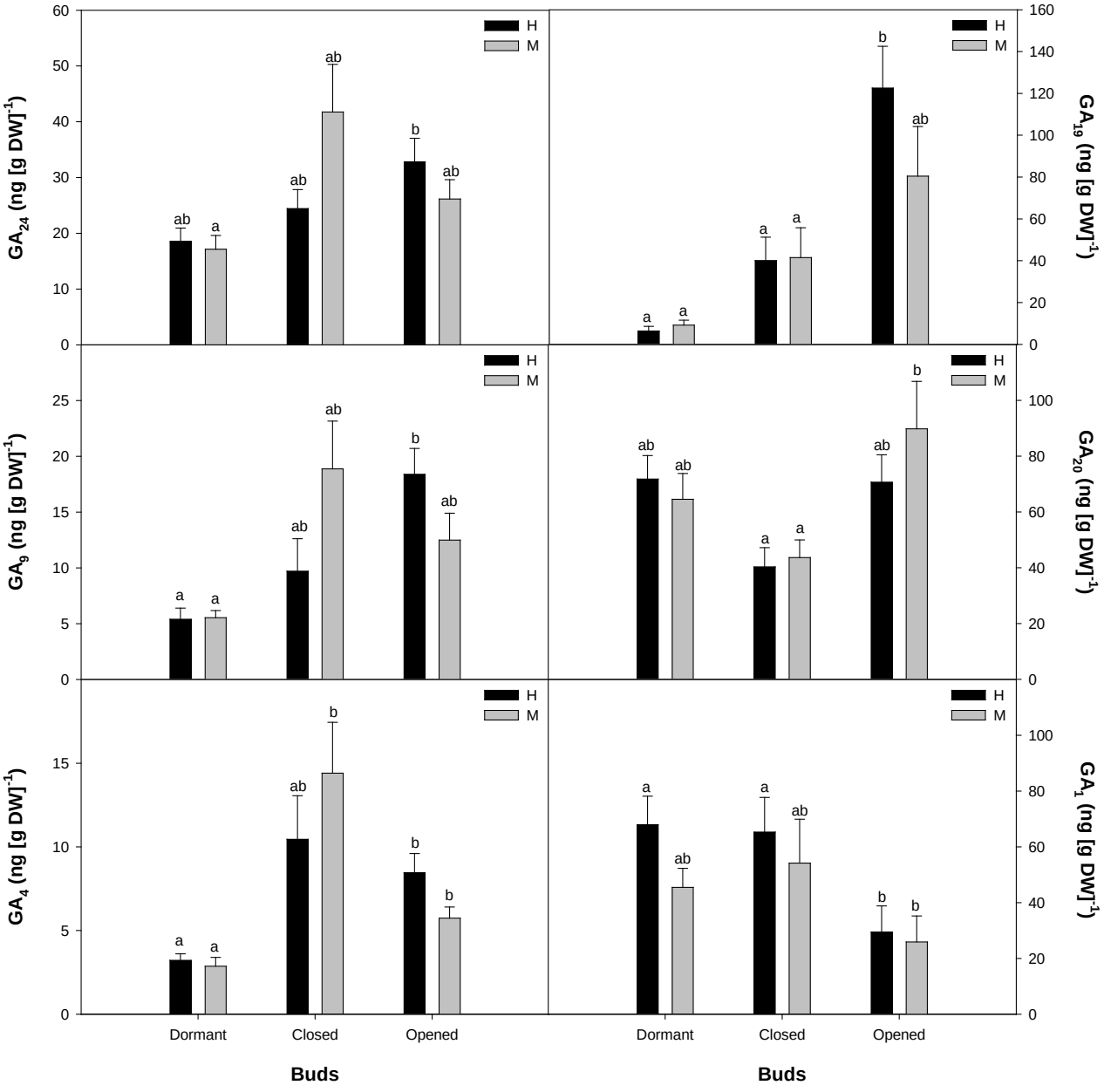


Figure 4: Endogenous concentrations of gibberellins of dormant and non-dormant buds of healthy (H) and moribund (M) beech trees. Non-dormant buds included closed and opened buds (the latter were collected just after budburst). Data correspond to the mean ± SE of n=12 individuals. Different letters indicate significant differences (P<0.05).

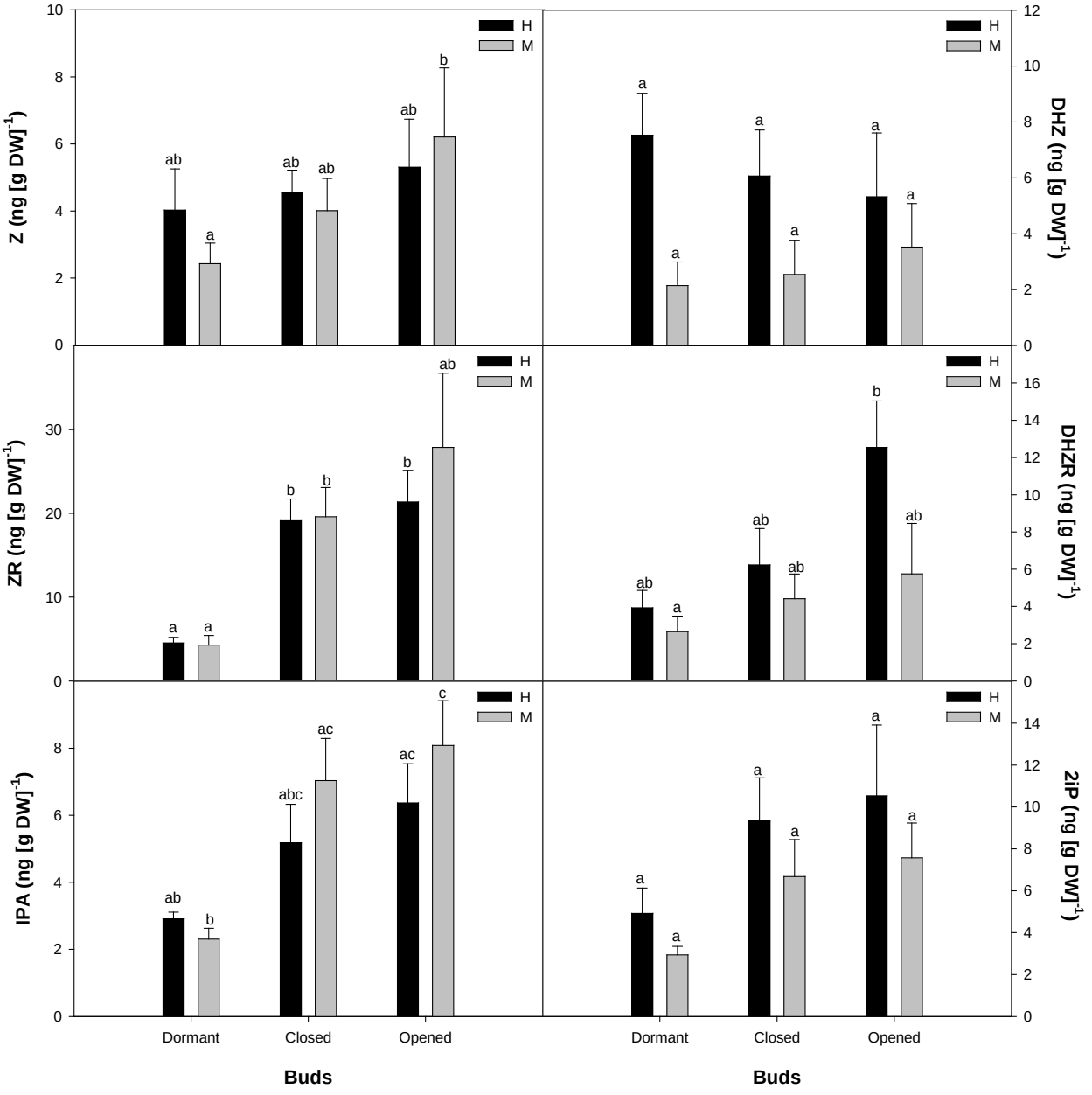


Figure 5: Endogenous concentrations of cytokinins of dormant and non-dormant buds of healthy (H) and moribund (M) beech trees. Non-dormant buds included closed and opened buds (the latter were collected just after budburst). Data correspond to the mean ± SE of n=12 individuals. Different letters indicate significant differences (P<0.05). Z, zeatin. ZR, zeatin riboside. IPA, isopentenyladenosine. DHZ, dihydrozeatin. DHZR, dihydrozeatin riboside. 2iP, 2-isopentenyladenine.

Hormonal regulation of bud dormancy release in moribund and healthy trees

GA profiling, which included the analysis of GA_4 and GA_1 and their immediate precursors GA_9 and GA_{24} (for GA_4) and GA_{20} and GA_{19} (for GA_1), revealed that GA_4 was the only GA showing significant increases after dormancy release (comparing closed, non-dormant buds with dormant buds, Fig. 4). GA_4 concentrations increased around 3-4 fold after bud dormancy release in both healthy and moribund trees (Fig. 4).

Cytokinin concentrations also increased after bud dormancy release, particularly those of zeatin riboside (ZR), whose levels were 5 times greater in closed, non-dormant buds than in dormant buds in both healthy and moribund trees (Fig. 5). The only difference in cytokinins between healthy and moribund trees was observed in the concentrations of dihydrozeatin (DHZ) in dormant buds and dihydrozeatin riboside (DHZR) at budburst, which were 3.7- and 2.2.-fold higher, respectively, in healthy trees than in moribund ones (Fig. 5).

In contrast to GAs and cytokinins, major differences in auxin concentrations were observed between healthy and moribund trees. The concentrations of indole-3-acetic acid (IAA) were 3.8-fold higher in dormant buds of healthy trees than in those of moribund trees (Fig. 6). The concentrations of auxin were still higher in healthy trees after bud dormancy release (in closed, non-dormant buds), but not at budburst.

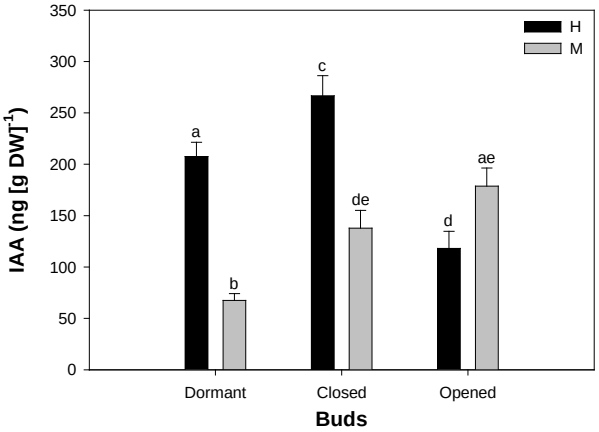


Figure 6: Endogenous concentrations of the auxin, indole-3-acetic acid (IAA), in dormant and non-dormant buds of healthy (H) and moribund (M) beech trees. Non-dormant buds included closed and opened buds (the latter were collected just after budburst). Data correspond to the mean ± SE of n=12 individuals. Different letters indicate significant differences (P<0.05).

Trends were reversed and IAA levels were 40% higher in open buds of moribund trees than in those of healthy trees (Fig. 6).

No significant differences in ABA concentrations during bud dormancy release were observed in healthy trees, but the levels of this hormone decreased significantly after bud break in moribund trees (Fig. 7). Furthermore, ACC levels increased significantly after budburst in moribund trees only (Fig. 7), indicating a difference in the hormonal regulation of bud dormancy release and budburst between healthy and moribund trees. Although JA levels increased sharply at budburst, no differences were

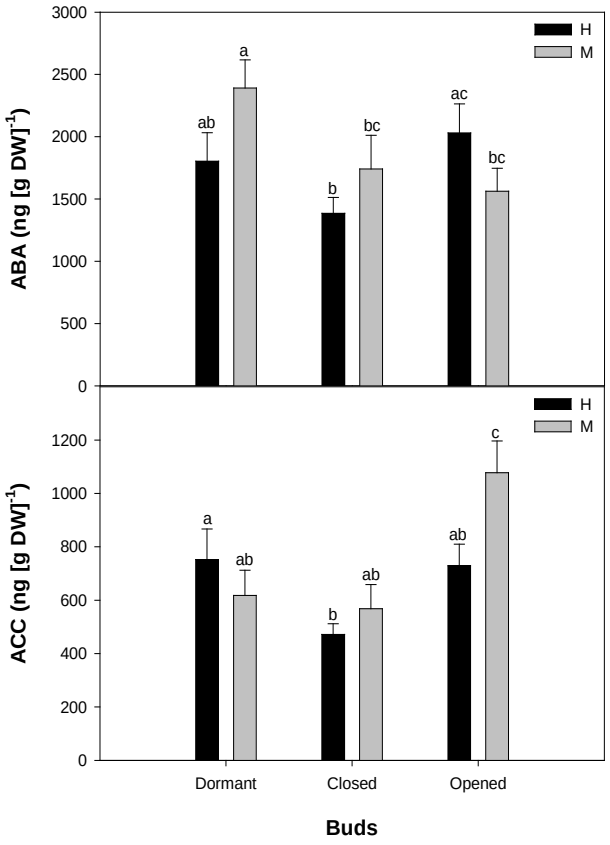


Figure 7: Endogenous concentrations of abscisic acid (ABA) and the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), in dormant and non-dormant buds of healthy (H) and moribund (M) beech trees. Non-dormant buds included closed and opened buds (the latter were collected just after budburst). Data correspond to the mean ± SE of n=12 individuals. Different letters indicate significant differences (P<0.05).

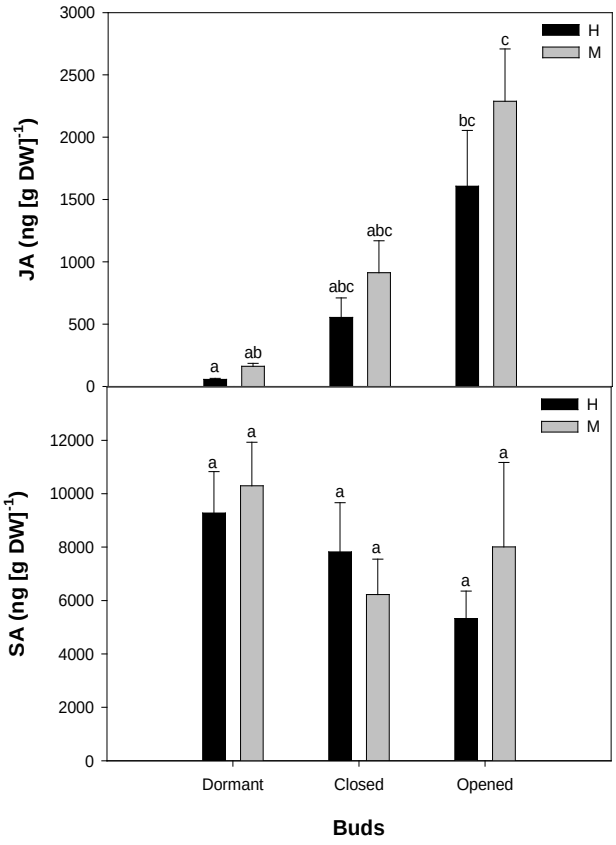


Figure 8: Endogenous concentrations of jasmonic acid (JA) and salicylic acid (SA) of dormant and non-dormant buds of healthy (H) and moribund (M) beech trees. Non-dormant buds included closed and opened buds (the latter were collected just after budburst). Data correspond to the mean ± SE of n=12 individuals. Different letters indicate significant differences (P<0.05).

observed between healthy and moribund trees (Fig. 8). SA concentrations did not change with bud development in either healthy or moribund trees (Fig. 8).

Discussion

Hormonal regulation of bud dormancy release

The most striking differences in hormonal levels during bud dormancy release were observed for GAs, cytokinins and auxin. The endogenous concentrations of the three hormone classes increased after bud break, which is consistent with a positive role for these compounds in the regulation of this important physiological process. Auxins are considered the most important hormones controlling bud dormancy release, since the basipetal transport of auxin in the main shoot prevents lateral shoot development (Cline 2000). Therefore, increases in auxin in leaf buds of lateral branches are essential to break dormancy. This is particularly important for moribund trees, since the main shoot is usually damaged in these trees. Indeed, auxin levels were lower in dormant buds of moribund trees than in those of healthy trees, but then auxin concentrations increased sharply at budburst, an indicator of the need to re-establish growth in epicormic branches of moribund trees. Interestingly, cytokinin and GA concentrations increased after bud break, but no differences were observed between moribund and healthy trees, which suggests that auxin is the main hormone determining budburst in moribund trees. Another important feature is that reductions in ABA concentrations with bud break occurred in buds of moribund trees only. As ABA regulates growth negatively (Zhou et al. 2003), a reduction in the levels of this hormone, together with GA, cytokinin and auxin increases, may favor bud dormancy release in moribund trees. Thus, the hormonal regulation of bud dormancy release clearly differs between healthy and moribund trees, with ABA and auxin

playing in the latter an additional role to that exerted by GA and cytokinins in healthy trees.

Another factor that merits attention is the role of the cytokinin and GA forms active in bud dormancy release. Although kinetin applications did not result in increased bud break in beech (Falusi and Calamassi 2003), results reported here show a role for cytokinins and suggest that zeatin riboside is the most important cytokinin modulating bud break. Similarly, GA profiling revealed that the most active GA in regulating bud break was GA_4. This information is very relevant, since it helps to identify the active hormones in the regulation of bud dormancy release. It can also help in further studies on hormonal applications in beech trees. Finally, it is interesting to note the differences in DHZ in dormant buds and DHZR in open buds of healthy and moribund trees, the latter showing lower levels than the former. DHZ and DHZR are thought to be inactive, reduced forms of Z and ZR, respectively, but specific roles for these compounds cannot be ruled out (Van der Krieken et al. 1990; Davies 2010). Further investigation is consequently required into this differential cytokinin metabolism during bud dormancy release and budburst in moribund and healthy trees. Finally, it is worth mentioning that JA and SA are associated with increased resistance to insects and fungi, respectively (Davies 2010; El-Wakeil et al. 2010). Therefore, increases in the levels of JA after budburst could play a role in increasing the bud survival.

Increased lipid peroxidation at budburst

Budburst increased the extent of lipid peroxidation. Not only JA concentrations, but also MDA levels increased after budburst in both healthy and moribund trees, thus indicating an increase in both enzymatic and non-enzymatic lipid peroxidation at budburst (Müller et al. 2008). It should be noted that significant increases in JA and MDA levels were observed in open buds, but not in non-dormant, closed buds, thus indicating that light helps promote lipid peroxidation. Indeed, previous

studies have linked photo-oxidative stress with lipid peroxidation in leaves (Demmig-Adams et al. 2013). Furthermore, moribund trees showed increased lipid peroxidation at budburst, indicating increased physiological deterioration. Not only fewer buds reached burst, but the ones that did had greater lipid peroxidation in moribund than in healthy trees. This is very interesting, since it clearly shows that epicormic branches of moribund trees are more physiologically deteriorated than newly formed branches of young, healthy trees. However, this increased lipid peroxidation and production of lipid-derived signals can be protective and defensive. Both JA and MDA have a role in leaves of protection against stress by modulating both biotic and abiotic stress-related genes (Weber et al. 2004; Müller et al. 2008). In this respect, it should be noted that not only lipid peroxidation-derived signals increased in moribund trees, but also the levels of the ethylene precursor, ACC. Taken together, these results indicate that lipid peroxide-derived signals may act in concert with ethylene to protect epicormic branches of moribund trees. Although results suggest that moribund, old trees are more sensitive to stress than young, healthy ones, our results clearly show that moribund trees activate several defense responses at the biochemical level that undoubtedly help epicormic braches to develop new functional leaves.

We conclude that (i) gibberellins, cytokinins and auxin regulate bud dormancy release in beech trees; (ii) there is a different hormonal dynamic of bud dormancy release in moribund trees; and (iii) moribund trees show less bud vigor and greater lipid peroxidation at budburst than healthy trees.

Acknowledgments

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CAPÍTOL 4

Efecte del sexe en peroxidació lipídica i fotoprotecció en plantes de *Pistacia lentiscus*

CHAPTER 4

Sex-related differences in lipid peroxidation and photoprotection in *Pistacia lentiscus*

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RESUM DEL CAPÍTOL 4

Les diferències relacionades amb el sexe en les respostes de les plantes dioiques alhora de combatre estressos abiòtics han estat molt poc estudiades fins al dia d'avui. Aquest estudi explora en quina mesura el sexe pot arribar a afectar les respostes de les plantes a l'estrès en *Pistacia lentiscus* L. (Anacardiaceae), un arbre adaptat a les condicions del clima mediterrani. Es va hipotetitzar que un major esforç reproductiu en les femelles podria incrementar l'estrès oxidatiu de les fulles, especialment quan les plantes es troben en condicions d'estrès abiòtic. Mesures de marcadors d'estrès oxidatiu al llarg de l'any varen revelar un increment de la peroxidació lipídica en femelles, però tant sols durant l'hivern. L'increment de la peroxidació lipídica en femelles estava associat a una menor fotoprotecció, com indicaven els baixos nivells de tocoferol i de l'extinció no fotoquímica (NPQ) de la fluorescència de les clorofil·les. Uns majors nivells de peroxidació lipídica en femelles també es va observar abans de l'alba, associats amb un increment de l'activitat de la lipoxigenasa a més d'una reducció dels nivells de citocinines. Un anàlisi comparatiu entre els brots reproductius i no reproductius en femelles va mostrar una capacitat fotoprotectiva major per part dels brots reproductius. Aquesta capacitat es va caracteritzar per un increment de l'NPQ i una major protecció antioxidant (increment dels nivells de carotenoides i tocoferols per unitat de clorofil·la) en els brots reproductius en comparació als no reproductius. Es va concloure que (i) les femelles presentaven una major peroxidació lipídica en les fulles respecte als mascles, però tant sols durant l'hivern (quan les diferències en l'esforç reproductius relacionades amb el sexe eren majors), (ii) aquest fet estava associat a una baixa capacitat fotoprotectiva al migdia, així com un increment de l'activitat lipoxigenasa i una reducció dels nivells de citocinines abans de l'alba, i (iii) els brots reproductius presentaven una capacitat fotoprotectiva major que els brots no reproductius en femelles.

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RESEARCH PAPER

Sex-related differences in lipid peroxidation and photoprotection in *Pistacia lentiscus*

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Abstract

Sex-related differences in the response of dioecious plants to abiotic stress have been poorly studied to date. This work explored to what extent sex may affect plant stress responses in *Pistacia lentiscus* L. (Anacardiaceae), a tree well adapted to Mediterranean climatic conditions. It was hypothesized that a greater reproductive effort in females may increase oxidative stress in leaves, particularly when plants are exposed to abiotic stress. Measurements of oxidative stress markers throughout the year revealed increased lipid peroxidation in females, but only during the winter. Enhanced lipid peroxidation in females was associated with reduced photoprotection, as indicated by reduced tocopherol levels and nonphotochemical quenching (NPQ) of chlorophyll fluorescence. Enhanced lipid peroxidation in females was also observed at predawn, which was associated with increased lipoxygenase activity and reduced cytokinin levels. An analysis of the differences between reproductive (R) and nonreproductive (NR) shoots showed an enhanced photoprotective capacity in R shoots compared to NR shoots in females. This capacity was characterized by an increased NPQ and a better antioxidant protection (increased carotenoid and tocopherol levels per unit of chlorophyll) in R compared to NR shoots. It is concluded that (i) females exhibit higher lipid peroxidation in leaves than males, but only during the winter (when sex-related differences in reproductive effort are the highest), (ii) this is associated with a lower photoprotective capacity at midday, as well as enhanced lipoxygenase activity and reduced cytokinin levels at predawn, and (iii) photoprotection capacity is higher in R relative to NR shoots in females.

Key words: Dioecy, mastic tree (*Pistacia lentiscus*), oxidative stress, photoprotection, reproductive effort, sexual dimorphism.

Introduction

Studies on dimorphism between sexes in dioecious plants have not only shown differential reproductive traits in males and females but also significant differences in vegetative traits generally associated with a greater reproductive effort in females (Barrett and Hough, 2013). There is evidence that females present a higher investment of nutrients in reproduction at the expense of vegetative growth, and therefore females show lower productivity than males (Gross and Soule, 1981; Ågren, 1988; Korpelainen, 1992; Cipollini and Whigham, 1994; Zluvova *et al.*, 2010). Consequently, environmental stress, caused by less-than-optimal light, nutrition, or water conditions, often favours maleness (reviewed by Korpelainen, 1999). Nevertheless, several exceptions exist, particularly in the case of herbaceous perennials (reviewed by Obeso, 2002). Therefore, more studies are needed to better understand the consequences of sex dimorphism in the adaptation of plants to environmental stresses. This information can provide helpful insights into better understanding the biology of dioecious plants and help to improve management practices in their natural habitat.

Reproductive effort implies that both sexes differ in a range of morphological, physiological, and life-history traits. Some studies in trees have tested the gender-specific response against a number of environmental stresses such as drought (Li *et al.*, 2004; Varga and Kytoviita, 2008; Xu *et al.*, 2008; Rozas *et al.*, 2009; Chen *et al.*, 2010), low temperatures (Zhang *et al.*, 2011),

salinity (Chen *et al.*, 2010), atmospheric CO_2 enrichment (Wang and Curtis, 2001), enhanced UV-B radiation (Xu *et al.*, 2010), nutrient deficiency (Montesinos *et al.*, 2012), excess manganese, and a combination of different stresses (Chen *et al.*, 2010; Zhang *et al.*, 2010). Although these studies conclude that in front of environmental stresses females seem to be more sensitive and usually experience greater negative effects, other studies in perennial herbs indicate that females are equally or even more resistant than males to environmental stresses, such as drought (Oñate and Munné-Bosch, 2009; Morales *et al.*, 2013). Due to the very limited amount of studies, it is still not clear whether such differential effects of sexual dimorphism on plant stress responses are species-specific or caused by specific ecological circumstances; therefore, further research in other species and habitats is needed to strengthen knowledge on this topic.

In Mediterranean-type ecosystems plants are exposed to marked seasonal variations throughout the year, which are usually characterized by a summer drought followed by a rainy autumn and lower temperatures during the winter. Even though sex-related differences in the response of dioecious Mediterranean plants to photo-oxidative stress have not been examined thus far, other studies have shown that dioecious plants differ in the physiological response to drought between sexes. Females of *Populus yunnanensis* growing in China exhibited more growth inhibition, gas exchange rate depression, reactive oxygen species (ROS) accumulation, and more damage to cell organelles than did males under drought (Chen *et al.*, 2010). Female individuals of *Populus cathayana* growing in China were also more responsive and showed greater negative effects (in terms of growth, net photosynthesis, and oxidative stress) than did males when grown under environments with increased drought stress and elevated temperature (Xu *et al.*, 2008). Therefore, this work hypothesized that females of *Pistacia lentiscus* L., a tree widely distributed along the Mediterranean basin, would also exhibit higher photo-oxidative stress than males, particularly during the summer. Indeed, previous studies already showed evidence for a higher sensitivity to drought stress in terms of leaf gas exchange in females (Correia *et al.*, 1992; Jonasson *et al.*, 1997; Barradas and Correia, 1999), but no studies have investigated sex-related differences in photo-oxidative stress in this or other Mediterranean dioecious plants. These studies can undoubtedly bring new insights into better understanding the biochemical mechanisms behind stress tolerance and provide clues to better understand sex biases in nature. Furthermore, to better understand photoprotection and photo-oxidative stress in fruit-bearing plants, this work specifically examined the effects of the reproductive effort in females by (i) analysing the differences between reproductive (R) and nonreproductive (NR) shoots, and (ii) conducting manipulative experiments by detaching fruits on R shoots.

Materials and methods

Plant material, growth conditions, and samplings

This study was conducted using *Pistacia lentiscus* L. (Anacardiaceae), a dioecious evergreen tree, typical of Mediterranean dry and semiarid climates. Fifty-five plants with a height between 40 and 110 cm were purchased in Bioriza (Cornella de Terri, Girona, Spain) in the spring of 2009 and were homogeneously transplanted in an area of 30 m^2 to the experimental fields of the Faculty of Biology at the University of Barcelona (Barcelona, Spain, 41° 22′ 59″ N 02° 06′ 44″ E, 60 m above sea level). Plants were grown under Mediterranean climatic conditions and received water exclusively from rainfall before and during the study. During March 2012, at flowering time, a group of 12 plants (six females and six males) were selected and tagged for experiments. Two different experiments were performed with these plants.

In the first experiment, leaves of both sexes were sampled at midday during spring (28 March), summer (28 July), autumn (29 October), and winter (24 January) of 2012–2013 to evaluate sex-related differences in seasonal variations of photo-oxidative stress markers. In males, inflorescences appeared during March and abscised during May, while in females inflorescences appeared in March and fruits developed from June to February. In this experiment, leaves from R and NR shoots were not differentiated.

In the second experiment, leaves from NR shoots of both males and females were sampled at midday at regular intervals between 29 October 2012 and 24 January 2013. During the same period, leaves from both R and NR shoots of females were used to specifically evaluate the effects of the reproductive effort in females during winter. Furthermore, a manipulative experiment on females was conducted during the same period by removing fruits at the start of the experiment in R shoots (without fruit) and its response was compared to leaves of R shoots (with fruit). Finally, to determine the possible diurnal variations in photo-oxidative stress markers during winter, all leaf types mentioned before were collected at predawn (1 h before sunrise), midday (at maximum diurnal solar radiation), and evening (3 h after sunset) during 25 January 2013. In this experiment, lipoxygenase activity and hormone levels (cytokinins, abscisic acid (ABA), and jasmonic acid (JA)) were additionally measured to better understand the causes of the sex-related differences in lipid peroxidation at predawn and midday.

Fully developed, young leaves were always selected for measurements. For biochemical analyses, samples were immediately frozen in liquid nitrogen and stored at –80 °C until measurements.

Climatological conditions and leaf water contents

Climatological conditions during experiments were monitored every 5 min by means of a weather station situated at 100 m from the experimental fields. Data was obtained by a photosynthetically active photon flux density (PPFD) pyranometer sensor CM11 (KIPP and ZONEN, Delft, The Netherlands) and a HMP35AC thermohygrometer (Vaisala, Helsinki, Finland). Leaf water status was estimated by measuring the relative water content (RWC) as $100 \times (FW - DW)/(TW - DW)$, where FW is the fresh weight, TW is the weight of the leaves rehydrated for 24 h at 4 °C in darkness, and DW corresponds to the dry weight obtained after overdrying the samples for 24 h at 80 °C.

Extent of lipid peroxidation

The extent of lipid peroxidation was estimated spectrophotometrically by the amount of malondialdehyde (MDA) in leaves, following the method described by Hodges *et al.* (1999), which takes into account the possible influence of interfering compounds in the thiobarbituric acid-reactive substances assay.

Photoinhibition and photoprotection

The maximum efficiency of PSII photochemistry (F_v/F_m), which is indicative of photoinhibition to PSII, was measured with a pulse-modulated fluorimeter Imaging PAM (Walz, Effeltrich, Germany) according to van Kooten and Snel (1990). Measurements were performed after 2 h of dark adaptation.

For pigment (chlorophyll (Chl) a+b, carotenoids, and anthocyanins) analysis, samples were ground in liquid nitrogen and repeatedly

extracted with ice-cold 100% methanol (v/v) using sonication until the extract was colourless. Chl and carotenoid contents were estimated spectrophotometrically. Specific absorption coefficients reported by Lichtenthaler (1987) were used. Anthocyanin content was determined with the acidification of the same extract with HCl and measured spectrophotometrically as described by Gitelson *et al.* (2001). Carotenoid and anthocyanin levels were given both on a dry weight and Chl basis, the latter indicating the capacity of photoprotection per unit of Chl.

For tocopherol analyses, samples were ground in liquid nitrogen and repeatedly extracted with ice-cold 100% methanol (v/v) using sonication until the extract was colourless. Tocopherols (including all four homologues) were determined by HPLC as described by Amaral *et al.* (2005). Since differences in α-tocopherol levels were found between sexes, this work further evaluated the oxidation state of α-tocopherol in the diurnal cycle by measuring α-tocopherol quinone (α-TQ). For α-TQ analyses, samples were extracted as described for tocopherols, but HPLC analyses were performed as described by Munné-Bosch and Alegre (2003). All tocopherol homologues and α-TQ were identified by coelution with authentic standards (Sigma, Steinheim, Germany), and identification of α-TQ was further confirmed by matching UV/visible spectra. Tocopherol levels were given both on a dry weight and Chl basis, the latter indicating the capacity of photoprotection per unit of Chl. α-TQ measurements allowed this work to additionally calculate the oxidation state of α-tocopherol, given as α-TQ/α-Tt, where α-Tt is the sum of α-tocopherol plus α-TQ.

Leaf gas exchange and Chl fluorescence measurements were additionally performed at the end of the experiment to examine light intensity-dependent curves of photosynthesis and photoprotection in terms of excess energy dissipation as heat (NPQ). All measurements were perfomed between 24 and 26 January 2013. Leaf gas exchange measurements coupled to Chl fluorescence were performed on attached leaves with a portable infrared gas analyser (LI-6400 system, LI-COR, Lincoln, NE, USA) with a leaf chamber fluorometer. To mimic environmental conditions at midday, leaves were maintained at 15 °C and the supplied CO_2 concentration inside the cuvette was set at 400 μmol mol^{-1} during measurements.

Table 1. *Climatological conditions during the measurement days of experiment 2*

Data correspond to measurements performed at maximum diurnal incident PPFD (at midday). PPFD, maximum photosynthetically active photon flux density; T_{air}, air temperature; RH, relative humidity.

Day	Time (weeks)	PPFD (μmol m^{-2} s^{-1})	Tair (°C)	RH (%)
29.10.2012	0	1152	14.6	35
04.12.2012	5	776	14.9	46
20.12.2012	8	290	15.1	57
08.01.2013	10	760	15.3	53
24.01.2013	12	876	10.0	52

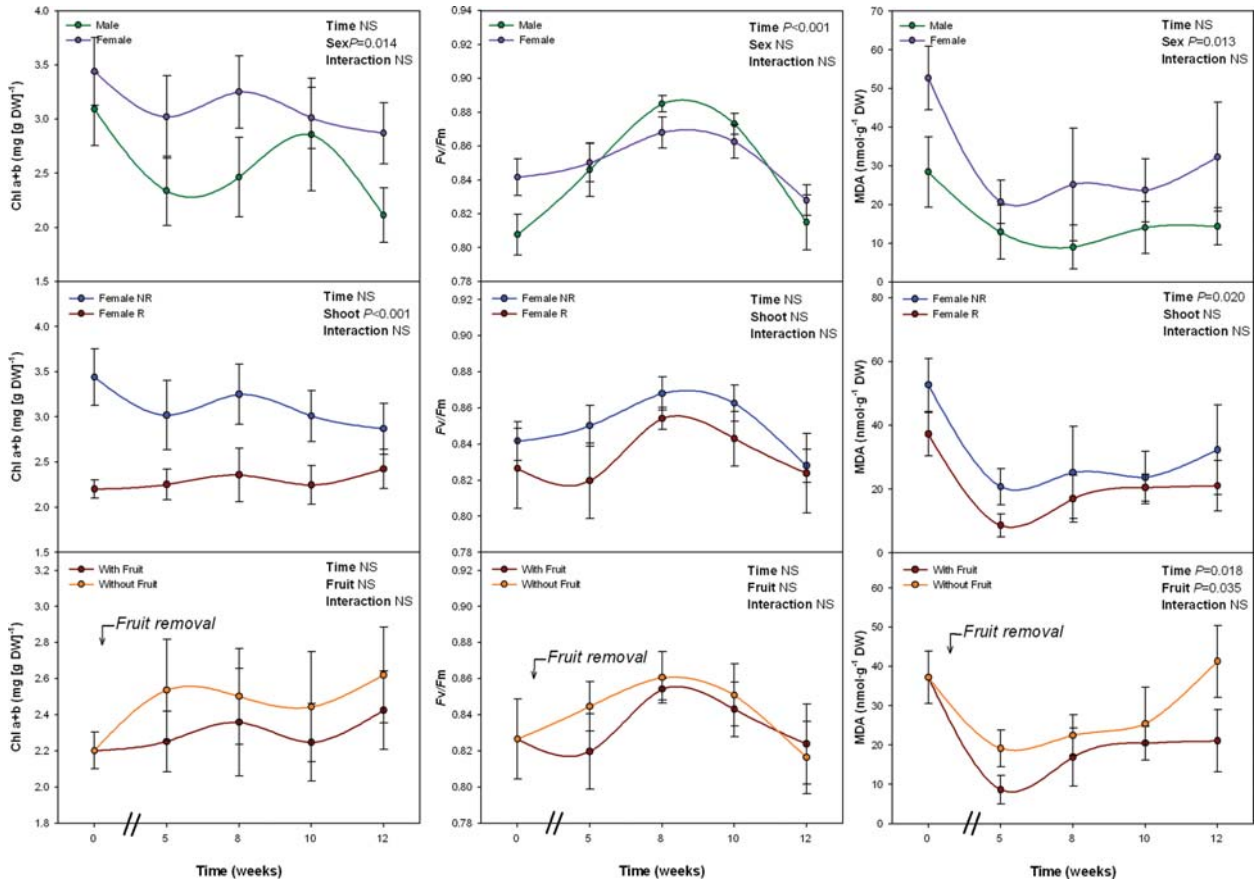

Fig. 1. Sex-related differences during winter in the maximum efficiency of PSII photochemistry (F_v/F_m), and levels of chlorophyll (Chl) a+b and malondialdehyde (MDA) in *Pistacia lentiscus*. Data represent mean ± SE of six individuals. Significant differences between groups were tested by two-way factorial analyses of variance (ANOVA) with time and plant sex (females vs. males), shoot (R, reproductive; NR, nonreproductive), or fruit (shoots with and without fruits) as factors. NS, not significant (this figure is available in colour at *JXB* online).

Measurements started after acclimating leaves at the highest light intensity (2500 µmol quanta m^{-2} s^{-1}), followed by measurements at decreasing light intensities at intervals of 500 µmol quanta m^{-2} s^{-1} until final acclimation to darkness. Net CO_2 assimilation (A) and stomatal conductance (g_s) rates were calculated on steady state photosynthesis as described by von Caemmerer and Farquhar (1981). Chl fluorescence was determined concomitantly at each point of gas exchange measurement. From the fluorescence measurements, the relative efficiency of the photosystem II (PSII) photochemistry (Φ_{PSII}) and NPQ (= $(F_m - F_m')/F_m'$) were determined according to van Kooten and Snel (1990). The instantaneous water use efficiency (WUE) was calculated as A/g_s, where A is the net CO_2 assimilation rate and g_s is the stomatal conductance.

Lipoxygenase activity and hormone levels

To further evaluate the causes of increased lipid peroxidation in females at predawn, which occurred irrespective of sex-related differences in photoprotection, this work examined lipoxygenase activity and hormone levels. Hormone analyses included determination of cytokinins as an indicator of sink strength and ABA and JA, which are known regulators of lipoxygenase activity (Melan *et al.*, 1993; Fischer *et al.*, 1999).

Lipoxygenase activity was assayed as described by Fukuchi-Mizutani *et al.* (2000). In short, samples were extracted for 30 min with 0.01 M potassium phosphate buffer, pH 7.2, using ultrasonication. After centrifugation, the pellet was re-extracted with the same solvent. Supernatants were pooled and an aliquot was added to the reaction mixture (1 ml) at 37 °C. The reaction mixture consisted of 0.1 M sodium acetate buffer, pH 5, 0.0025% Tween 20, and 0.2 mM linoleic acid and activity was estimated spectrophotometrically at 234 nm.

The levels of cytokinins, including zeatin, zeatin riboside, isopentenyladenosine (IPA) and 2-isopentenyladenine (2-iP), ABA, and JA, were determined by UPLC-MS/MS as described by Müller and Munné-Bosch (2011). Internal standards were used for estimating recovery rates for quantification.

Statistical analyses

In the first experiment, statistical differences between time through the year and sex were tested by two-way factorial analysis of variance (ANOVA), using time and sex as factors. The second experiment assessed (1) the sex effect, comparing NR shoots from both males and females; (2) the shoot effect, comparing R and NR shoots in females; and finally (3) the fruit effect, comparing R shoots with and without fruits. Fruits were removed in the latter. Statistical differences between time and sex, shoot, or fruit were tested by two-way factorial analysis of variance (ANOVA), using time and sex, shoot, or fruit as factors. Additionally, Student's t-tests were used to specifically evaluate differences between sexes at a given time point. In all cases, differences were considered significant at a probability level of $P < 0.05$.

Results

Sex-related seasonal variations in lipid peroxidation and photoprotection

Climatological conditions during the experiment were typical of the Mediterranean climate, with a summer drought (total

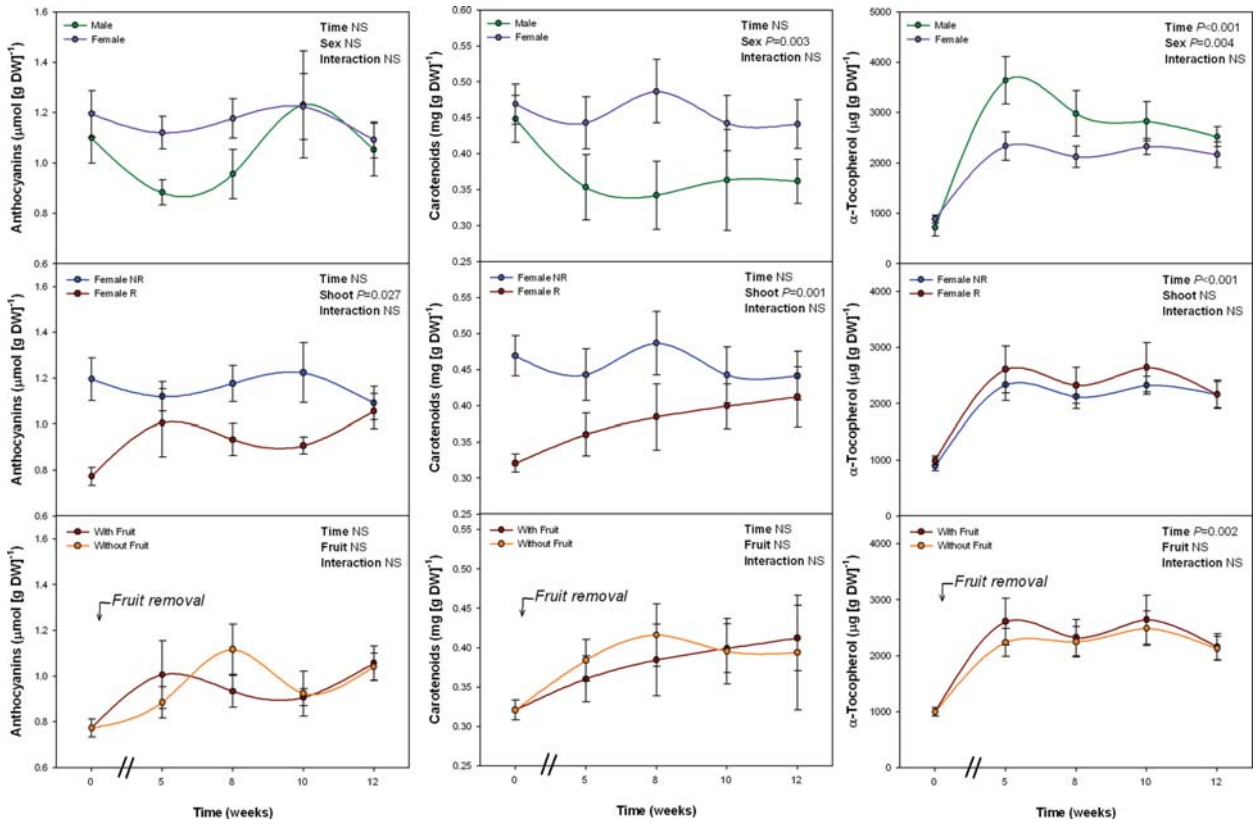

Fig. 2. Sex-related differences during winter in the levels of total anthocyanins (Ant), carotenoids (Car) and α-tocopherol in *Pistacia lentiscus*. Data represent mean ± SE of six individuals. Significant differences between groups were tested by two-way factorial analyses of variance (ANOVA) with time and plant sex (females vs. males), shoot (R, reproductive; NR, nonreproductive), or fruit (shoots with and without fruits) as factors. NS, not significant (this figure is available in colour at *JXB* online).

rainfall between June and August was below 60 mm) followed by a rainy autumn (more than 100 mm during October) and lower temperatures during winter (below 10 °C at midday, Supplementary Fig. S1, available at *JXB* online). During samplings, midday temperatures and PPFDs ranged between 10 °C and 32 °C, and between 876 and 1940 µmol m^{-2} s^{-1} (during winter and summer, respectively, Supplementary Table S1). Despite severe summer drought, both males and females plants maintained RWC values above 75% and F_v/F_m above 0.80 throughout the experiment (Supplementary Fig. S2). Chl a+b contents were however strongly reduced during spring and summer, although no sex-related differences were observed. Neither seasonal nor sex-related differences were observed in lipid peroxidation, estimated as MDA levels, although females showed higher levels than males both during autumn (38%) and winter (46%, $P < 0.05$, Student's t-test, Supplementary Fig. S2). Total anthocyanin, carotenoid and α-tocopherol levels, either per dry weight or per Chl unit, showed seasonal variations but no sex-related differences. However, anthocyanin and α-tocopherol levels per unit of Chl were 25% lower in females than in males during winter (Supplementary Fig. S3).

Sex-related differences in lipid peroxidation and photoprotection during winter

To better understand such sex-related differences in lipid peroxidation and photoprotection during winter a second experiment was conducted. Sex-, shoot- and fruit-related differences were tested. First, NR shoots of both males and females were selected to evaluate sex-related differences. Fruits were developing in R shoots of females during samplings, while inflorescences had abscised several months ago in males; therefore, although sampled shoots had not reproduced, gender-related differences in reproductive effort were maximal at the whole-plant level during winter. Second, R and NR shoots of females were compared to evaluate shoot-related differences in females. Third, female R shoots with and without fruits (in which fruits were manually eliminated at the beginning of the experiment) were compared to evaluate effects specifically caused by fruit development. During samplings, plants were exposed to suboptimal low temperatures (ranging between 10 °C and 15.3 °C at midday, Table 1). RWC values kept always above 80% in all cases (Supplementary Fig. S4). The F_v/F_m did not differ between males and females, but Chl levels and MDA levels were significantly higher in females than in males (Fig. 1). However, neither F_v/F_m nor MDA levels differed between R and NR of females, while Chl levels were lower in the former. Fruit removal in female R shoots did not alter Chl levels or F_v/F_m, but increased MDA levels. R shoots without fruits behaved as NR shoots in terms of MDA accumulation (Fig. 1).

Sex-related differences in α-tocopherol levels followed exactly the opposite trend than MDA levels. α-Tocopherol levels were lower in females than in males, both on a dry weight (Fig. 2) and on a Chl basis (Supplementary Fig. S5). Anthocyanin did not differ between males and females, but carotenoid levels were higher in the latter (Fig. 2). However, neither anthocyanin nor carotenoid levels per Chl unit differed between sexes (Supplementary Fig. S5). Anthocyanin

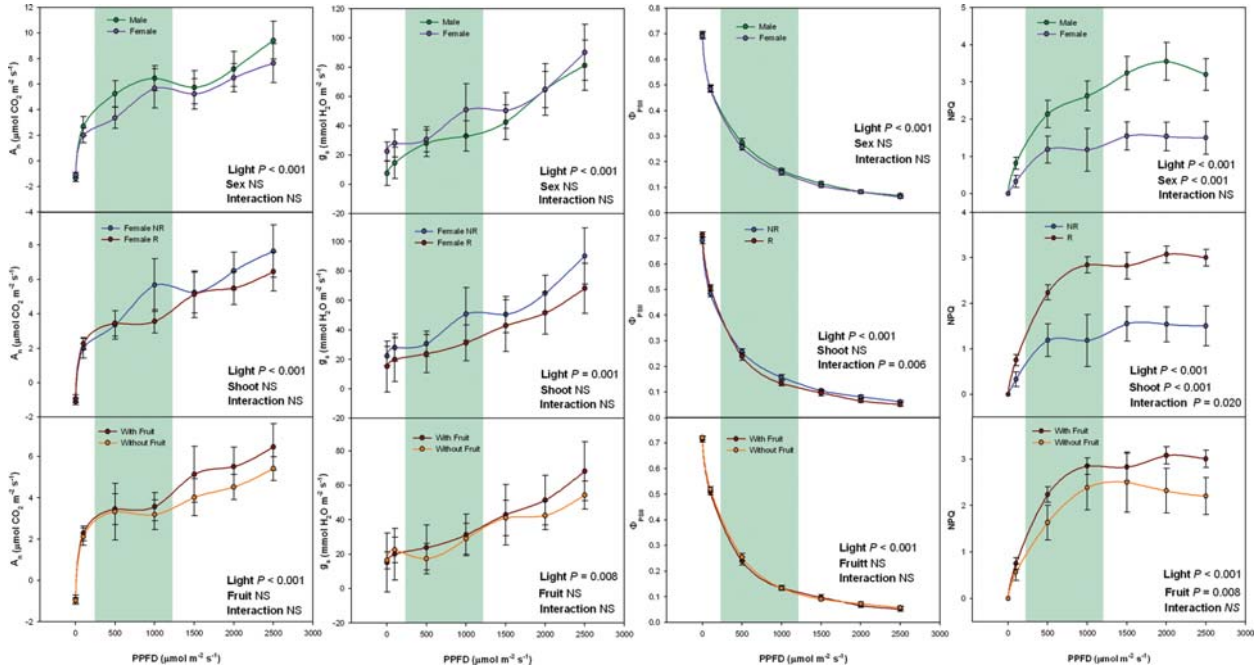

Fig. 3. Sex-related differences during winter in the CO_2 assimilation rate (A), stomatal conductance (g_s), photochemical PSII efficiency (Φ_{PSII}), and nonphotochemical quenching (NPQ) response curves to photosynthetically active photon flux density (PPFD) in *Pistacia lentiscus*. Data represent mean ± SE of six individuals. Significant differences between groups were tested by two-way factorial analyses of variance (ANOVA) with time and plant sex (females vs. males), shoot (R, reproductive; NR, nonreproductive), or fruit (shoots with and without fruits) as factors. NS, not significant. Green shading indicates midday sampling photosynthetically active photon flux density range during winter (this figure is available in colour at *JXB* online).

levels were lower in R than in NR shoots of females (Fig. 2), but differences disappeared when expressed on a Chl basis (Supplementary Fig. S5). Female R shoots not only showed lower Chl levels than NR shoots (Fig. 1), but also higher carotenoid and α-tocopherol levels per unit of Chl (Supplementary Fig. S5). Fruit removal did not cause any effect on anthocyanin, carotenoid, or tocopherol levels (Fig. 2 and Supplementary Fig. S5).

Light intensity-dependent curves of net CO_2 assimilation (A) and stomatal conductance (g_s) rates, as well as the quantum yield of PSII photochemistry (Φ_{PSII}) and NPQ were additionally measured during winter (Fig. 3). While Φ_{PSII}, A, and g_s did not differ between sexes, NPQ values indicated lower dissipation of excess energy by heat in females than in males. Furthermore, NPQ was much (up to 2-fold) higher in R than in NR of females, and fruit removal reduced the extent of NPQ, particularly at the highest light intensities (Fig. 3).

Diurnal sex-related differences in lipid peroxidation and possible causes

The extent of lipid peroxidation, together with the pigment and tocopherol levels (including its oxidation state), were examined on a diurnal basis during winter. Chl levels were not altered by daytime, sex, shoot, or fruit removal (Fig. 4). MDA levels were neither affected by any of these factors, except for time of day in females. The extent of lipid peroxidation decreased both in R and NR of females during the afternoon, while this diurnal pattern was not observed in males. In other words, females showed higher MDA levels than males at predawn and midday ($P < 0.05$, Student's t-test), but not during the evening (Fig. 4), which is indeed consistent with the differences observed during winter at midday (Fig. 1).

Such diurnal variations occurred in parallel with sex-related differences in the oxidation state of α-tocopherol

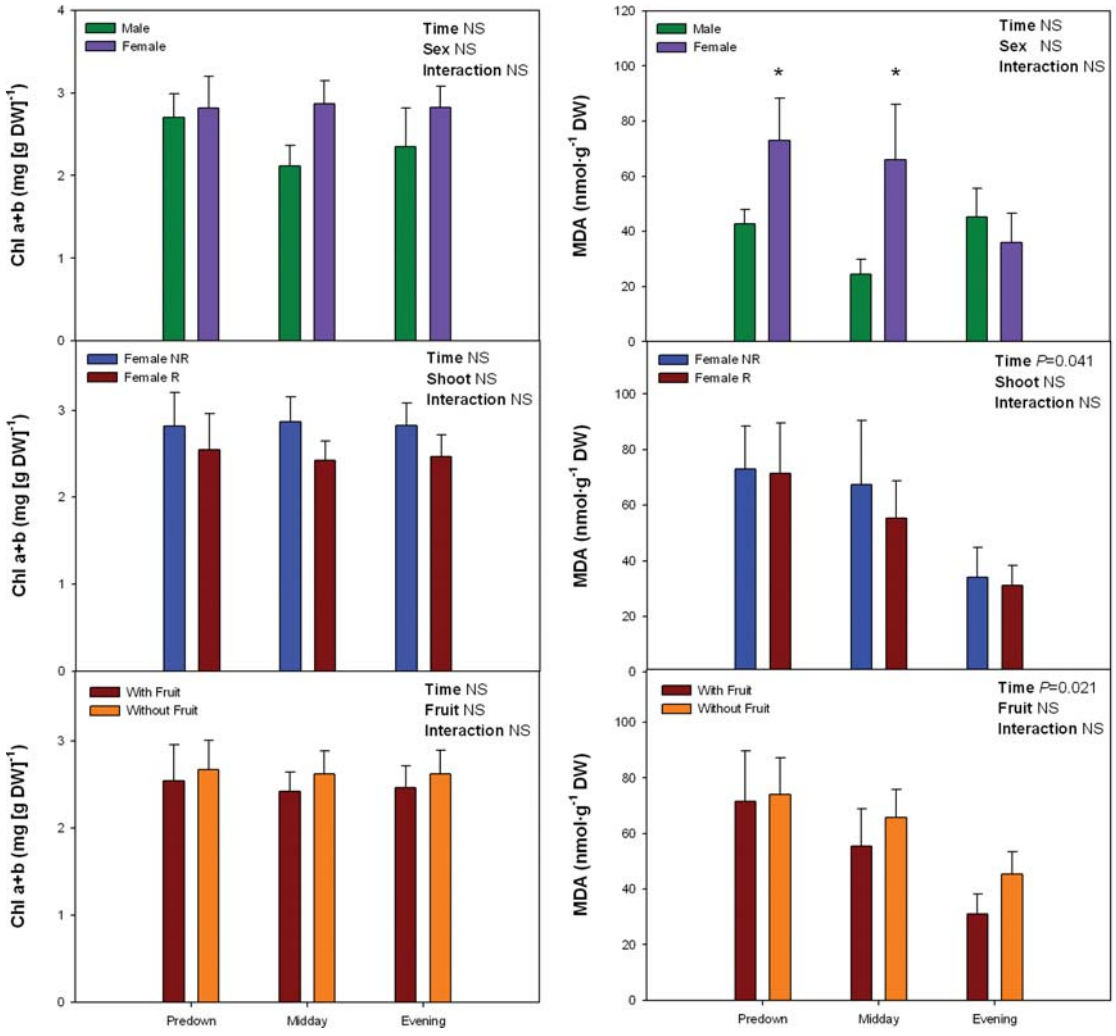

Fig. 4. Sex-related differences in the diurnal variations in levels of chlorophyll (Chl) a+b and malondialdehyde (MDA) in *Pistacia lentiscus* during winter (25 January). Data represent mean ± SE of six individuals. Significant differences between groups were tested by two-way factorial analyses of variance (ANOVA) with time and plant sex (females vs. males), shoot (R, reproductive; NR, nonreproductive), or fruit (shoots with and without fruits) as factors. Asterisks indicate significant differences between males and females, R and NR shoots, or shoots with and without fruits at a given time point (Student's t-test, $P < 0.05$). NS, not significant (this figure is available in colour at *JXB* online).

(Fig. 5), but not with levels of anthocyanins, carotenoids, or α-tocopherol neither on a DW nor on a Chl basis (Supplementary Figs. S5 and S6). Reductions of MDA levels during the day coincided with the decrease of α-TQ levels on a DW basis and the oxidation state of α-tocopherol, given as α-TQ/α-Tt, where α-Tt is total α-tocopherol (reduced plus oxidized, Fig. 5).

Furthermore, higher MDA levels and oxidation state of α-tocopherol in females than in males at predawn also coincided with increased lipoxygenase activity in the former (Fig. 6). Lipoxygenase activity was 85% higher in females than in males during predawn ($P < 0.05$, Student's t-test), but values did not differ between sexes at midday or evening. Similar diurnal differences were observed between R and NR shoots in females, and fruit removal increased lipoxygenase activity in R shoots at predawn (and to a lower extent at evening, $P < 0.05$, Student's t-test,), but not at midday (Fig. 6).

Diurnal variations in hormonal levels were determined in males and females to further examine the sex-related differences in the extent of lipid peroxidation at predawn, which could not be related to sex-related differences in photoprotection. Increased MDA levels in females compared to males at predawn (Fig. 6) were associated with a 75% reduction of IPA levels at predawn in the former ($P < 0.05$, Student's t-test, Fig. 7). Zeatin riboside levels were instead lower in females than in males at midday ($P < 0.05$, Student's t-test), and 2-iP levels were lower in females than in males at midday and evening ($P < 0.05$, Student's t-test, Supplementary Fig. S8). Neither ABA nor JA differed between sexes at predawn (Fig. 7). In contrast, ABA levels were reduced by 72% in females compared to males at midday ($P < 0.05$, Student's t-test, Fig. 7), which was associated with a reduction by 20–40% in the WUE at and above 1500 µmol quanta m^{-2} s^{-1} in females compared to males ($P < 0.05$, Student's t-test, Supplementary Fig. S9).

Discussion

In Mediterranean-type ecosystems, dioecious plants are representative of ecologically relevant species. Both in coastal and more dry, continental areas, dominant trees, such as the dioecious *Pistacia lentiscus* and *Juniperus thurifera*, are

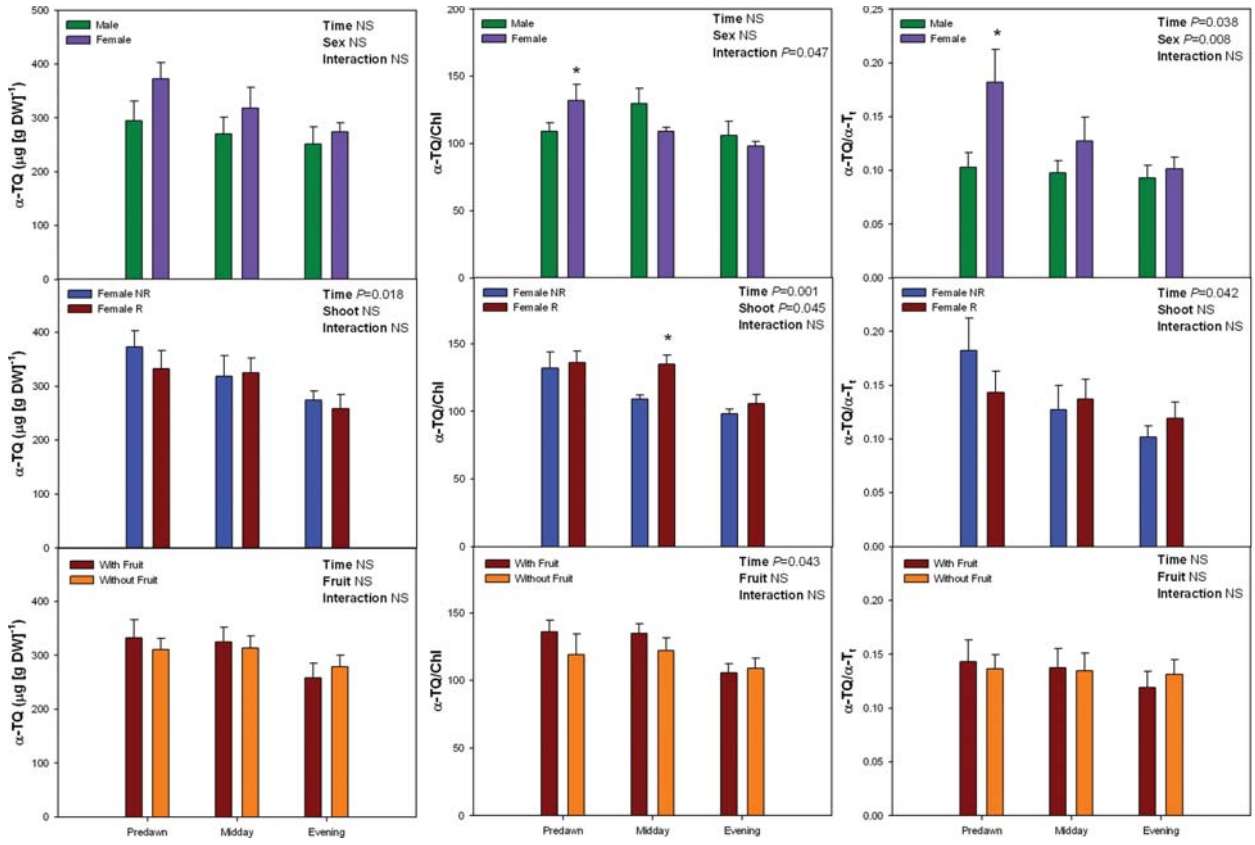

Fig. 5. Sex-related differences in the diurnal variations in levels of α-tocopherol quinone (α-TQ), given both per g of dry weight (DW) and per unit of chlorophyll (Chl) a+b, and the oxidation state of α-tocopherol (estimated as $\alpha\text{-TQ}/\alpha\text{-T}_t$, where $\alpha\text{-T}_t = \alpha\text{-T} + \alpha\text{-TQ}$) in *Pistacia lentiscus* during winter (25 January) (this figure is available in colour at *JXB* online).Data represent mean ± SE of six individuals. Significant differences between groups were tested by two-way factorial analyses of variance (ANOVA) with time and plant sex (females vs. males), shoot (R, reproductive; NR, nonreproductive), or fruit (shoots with and without fruits) as factors. Asterisks indicate significant differences between males and females, R and NR shoots, or shoots with and without fruits at a given time point (Student's t-test, $P < 0.05$). NS, not significant (this figure is available in colour at *JXB* online).

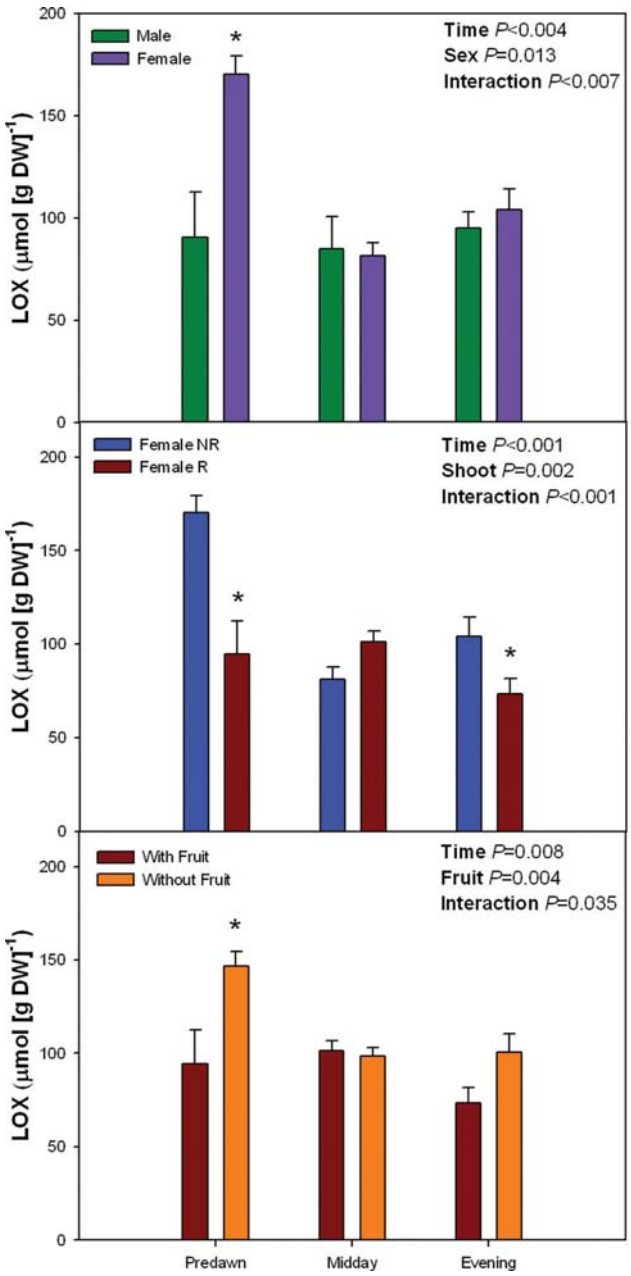

Fig. 6. Sex-related differences in the diurnal variations in lipoxygenase (LOX) activity in *Pistacia lentiscus* during winter (25 January). Data represent mean ± SE of six individuals. Significant differences between groups were tested by two-way factorial analyses of variance (ANOVA) with time and plant sex (females vs. males), shoot (R, reproductive; NR, nonreproductive), or fruit (shoots with and without fruits) as factors. Asterisks indicate significant differences between males and females, R and NR shoots, or shoots with and without fruits at a given time point (Student's t-test, $P < 0.05$). NS, not significant (this figure is available in colour at *JXB* online).

typically found at (or close) the seashore and the mountain regions, respectively, of the western Mediterranean basin. Despite the dominant role of these plant species in their respective habitats, little is known about gender-specific effects on their physiology, except some studies showing higher drought sensitivity for females in both species in terms of gas exchange and growth (Correia *et al.*, 1992; Jonasson *et al.*, 1997; Barradas and Correia, 1999; Rozas *et al.*, 2009). These studies show that females are more sensitive than males to drought stress in terms of productivity and leaf gas exchange, females of *Pistacia lentiscus* showing reduced CO_2 assimilation rates and PSII efficiency compared to males. The present study hypothesized that this reduced PSII efficiency may be associated with an increased photo-oxidative stress in females. Interestingly, increased lipid peroxidation associated with lower photoprotection was observed in females, but only during the winter. Seasonal variations in lipid peroxidation did not reveal sex-related differences during spring or summer, when plants are exposed to excess light energy, but during winter only. During the winter, plants were exposed to suboptimal temperatures (between 10 °C and 15.3 °C at midday), and the sex-related differences in reproductive effort were the highest, females developing fruits while males not reproducing. This is in agreement with the fact that in dioecious species the cost of reproduction involves the prioritization of resources in fruit development rather than in vegetative growth or protection in females. This major investment in reproduction has been generally associated with a disadvantage in terms of leaf gas exchange and productivity, leading even in some cases to cause increased oxidative stress and cellular injuries, particularly under adverse conditions (Xu *et al.*, 2008; Chen *et al.*, 2010; Zhang *et al.*, 2010, 2011). The current work additionally shows that this increased sensitivity of females to lipid peroxidation is particularly observed in NR shoots. Indeed, when females R and NR shoots were compared, R shoots resulted to be more photoprotected, not only by an enhanced excess energy dissipation by heat (as indicated by a higher NPQ), but also by an improved antioxidant protection (as indicated by higher carotenoid and tocopherol levels per Chl unit).

Photoprotection capacity differed between male and female plants. Despite PSII efficiency and CO_2 assimilation rates were identical between both sexes during winter; NPQ values were lower in females than males. Furthermore, tocopherol levels were lower in females than in males, thus indicating that enhanced lipid peroxidation is associated with a lower photoprotection capacity in females. Indeed, α-tocopherol oxidation was higher in females than in males, thus suggesting a greater accumulation of ROS in the former. This is particularly interesting since it allows linking reduced photoprotection with increased lipid peroxidation in females. This is in agreement with previous studies suggesting that both LOX upregulation and downregulation of photoprotection mechanisms may be involved in the production of lipid-peroxide-derived signals (reviewed by Demmig-Adams *et al.*, 2013).

Females appeared to be able to reduce the extent of lipid peroxidation as time elapsed during the day. A decrease in MDA levels along the day was observed in females. MDA levels were higher in females than in males at midday, but the extent of lipid peroxidation was even higher at predawn. Furthermore, this occurred in parallel with an increased oxidation of α-tocopherol, and this antioxidant cannot be

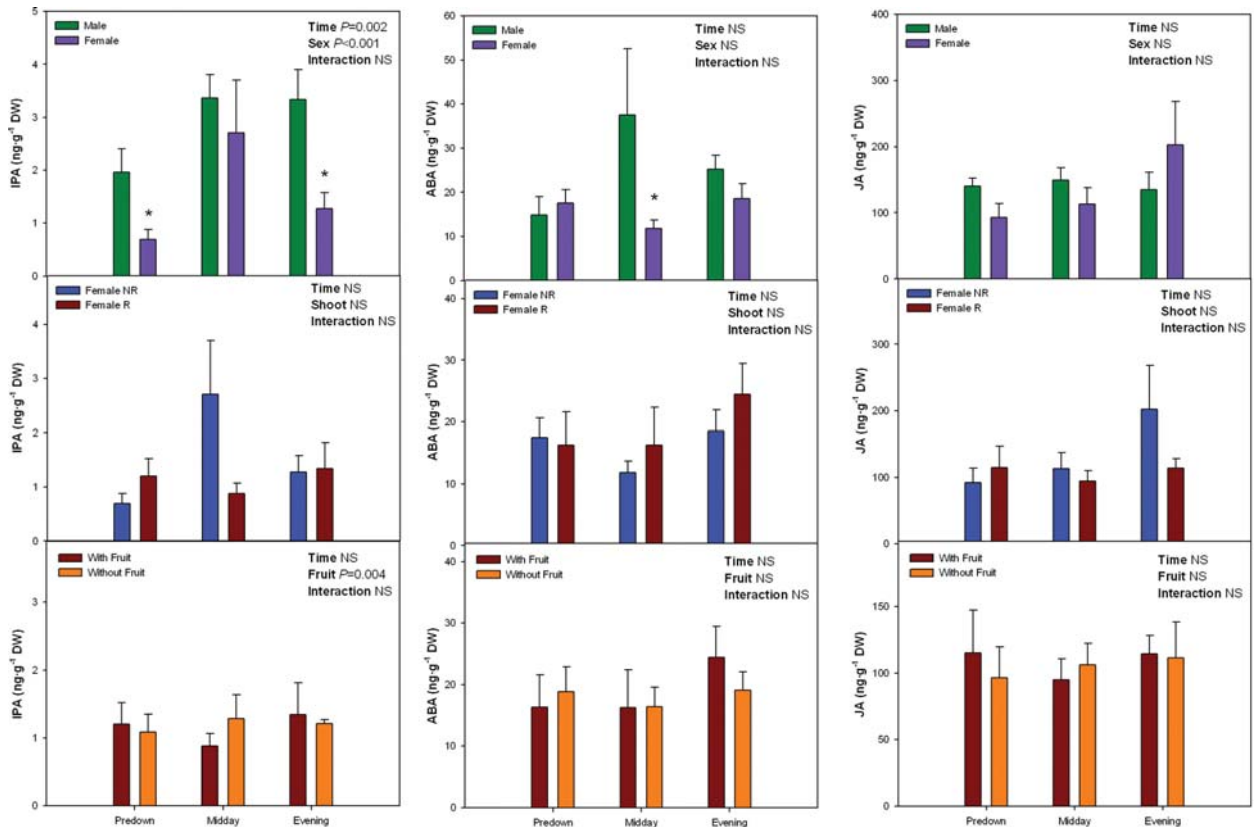

Fig. 7. Sex-related differences in the diurnal variations in isopentenyladenosine (IPA), abscisic acid (ABA) and jasmonic acid (JA) levels in *Pistacia lentiscus* during winter (25 January). Data represent mean ± SE of six individuals. Significant differences between groups were tested by two-way factorial analyses of variance (ANOVA) with time and plant sex (females vs. males), shoot (R, reproductive; NR, nonreproductive), or fruit (shoots with and without fruits) as factors. Asterisks indicate significant differences between males and females, R and NR shoots, or shoots with and without fruits at a given time point (Student's t-test, $P < 0.05$). NS, not significant (this figure is available in colour at *JXB* online).

oxidized by singlet oxygen at predawn (in darkness). Higher lipoxygenase activity in females than in males (at predawn only) was the cause of higher MDA levels in the former. Furthermore, co-oxidation of fatty acids and α-tocopherol has been shown to occur *in vitro* (Hakansson and Jagerstad, 1990), which suggests that both the higher oxidation of α-tocopherol and increased lipid peroxidation in females compared to males at predawn was due to increased lipoxygenase activity. In turn, this was associated with reduced IPA levels in females compared to males at predawn. IPA is one of the most active cytokinins in *Pistacia lentiscus* leaves modulating leaf growth (Juvany *et al.*, 2013). Since cytokinin levels determine the capacity for cell division and the capacity of the organ to act as a sink for photoassimilates (reviewed by Roitsch and Ehness, 2000), results suggest that reductions in IPA in females at predawn may be associated with a sink limitation, which is, in turn, known to induce lipoxygenase activity (Fischer *et al.*, 1999). It appears therefore that increased lipid peroxidation in females compared to males at midday occurred irrespective of an increased lipoxygenase activity and was mainly due to reduced photoprotection; while a sink limitation at predawn resulted in increased lipoxygenase activity and accumulation of lipid-peroxide-derived signals. These results highlight the importance of considering the complexity of mechanisms leading to lipid peroxidation at the whole-plant level, as it has recently been pointed out for photoinhibition (Adams *et al.*, 2013). Furthermore, levels of other cytokinins and ABA were reduced at midday in females compared to males, which may be linked to a reduced vigour and defence capacity, which is in agreement with the parallel observation of reduced photoprotection capacity in females. It is still to be determined whether or not reduced ABA and cytokinin levels could transiently affect hydraulic conductivity or cell division, respectively, in females at midday, two aspects that warrant further investigations.

In conclusion, these results show that females are more sensitive to lipid peroxidation than males, but only during winter (when sex-related differences in reproductive effort over the year are the highest). Reduced photoprotection led to enhanced lipid peroxidation in females at midday, while increased lipoxygenase activity, probably mediated by a sink limitation-reduced cytokinin levels, was responsible for the increased lipid peroxidation at predawn. Finally, results showed that photoprotection capacity was higher in R relative to NR shoots in females, thus suggesting that females prioritized protection to fruit-bearing shoots.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Table S1. Climatological conditions at midday during Experiment 1

Supplementary Fig. S1. Seasonal variations in climatological conditions from March 2012 to February 2013

Supplementary Fig. S2. Sex-related differences in the seasonal variations in the relative leaf water content, maximum efficiency of PSII photochemistry, and levels of chlorophyll a+b and malondialdehyde in *Pistacia lentiscus*

Supplementary Fig. S3. Sex-related differences in the seasonal variations in levels of total anthocyanins, carotenoids, and α-tocopherol in *Pistacia lentiscus*

Supplementary Fig. S4. Sex-related differences during winter in the relative leaf water content of *Pistacia lentiscus*

Supplementary Fig. S5. Sex-related variations during winter in the levels of total anthocyanins, carotenoids, and α-tocopherol in *Pistacia lentiscus*

Supplementary Fig. S6. Sex-related differences in the diurnal variations in levels of total anthocyanins, carotenoids, and α-tocopherol in *Pistacia lentiscus* during winter

Supplementary Fig. S7. Sex-related differences in the diurnal variations in levels of total anthocyanins, carotenoids, and α-tocopherol in *Pistacia lentiscus* during winter (expressed per unit of chlorophyll)

Supplementary Fig. S8. Sex-related differences in the diurnal variations in levels of zeatin, zeatin riboside, and 2-isopentenyladenine in *Pistacia lentiscus* during winter

Supplementary Fig. S9. Sex-related differences during winter in the instantaneous water use efficiency response curves to photosynthetically active photon flux density in *Pistacia lentiscus*

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Supporting Information

Sex-related differences in lipid peroxidation and photoprotection in *Pistacia lentiscus*

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Table S1 Climatological conditions at midday (photosynthetically-active photon flux density [PPFD], air temperature [T_{air}] and relative humidity [RH]) during the measurements of Experiment 1.

Season	Date	PPFD ($\mu mol\ m^{-2}\ s^{-1}$)	T_{air} (ºC)	RH (%)
Spring	28 March	1442	23.0	25
Summer	18 July	1940	32.0	38
Autumn	29 October	1152	14.5	36
Winter	24 January	876	10.0	52

Fig. S1 Seasonal variations in climatological conditions (monthly average air temperature [Tair], monthly average relative humidity and monthly precipitation) from March 2012 to February 2013.

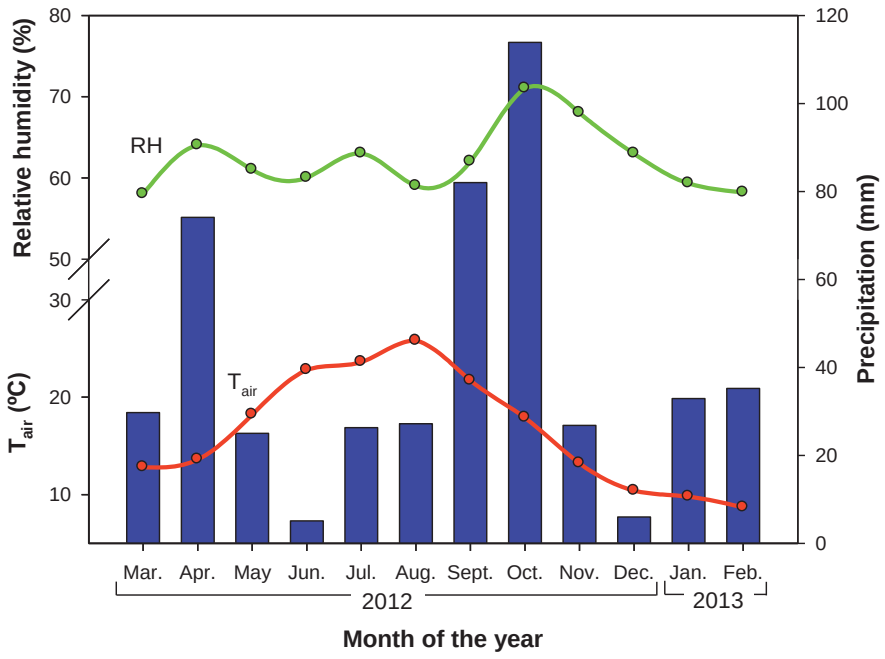


Fig. S2 Sex-related differences in the seasonal variations in the relative leaf water content (RWC), maximum efficiency of PSII photochemistry (F_v/F_m ratio), and levels of chlorophyll (Chl) a+b and malondialdehyde (MDA) in *P. lentiscus*. Data represent the mean ± SE of n=6 individuals. Significant differences between groups were tested by two-way factorial analyses of variance (ANOVA) with time and plant sex as factors. An asterisk indicates significant differences between males and females at a given time point (Student's t-test, $P<0.05$). NS, not significant.

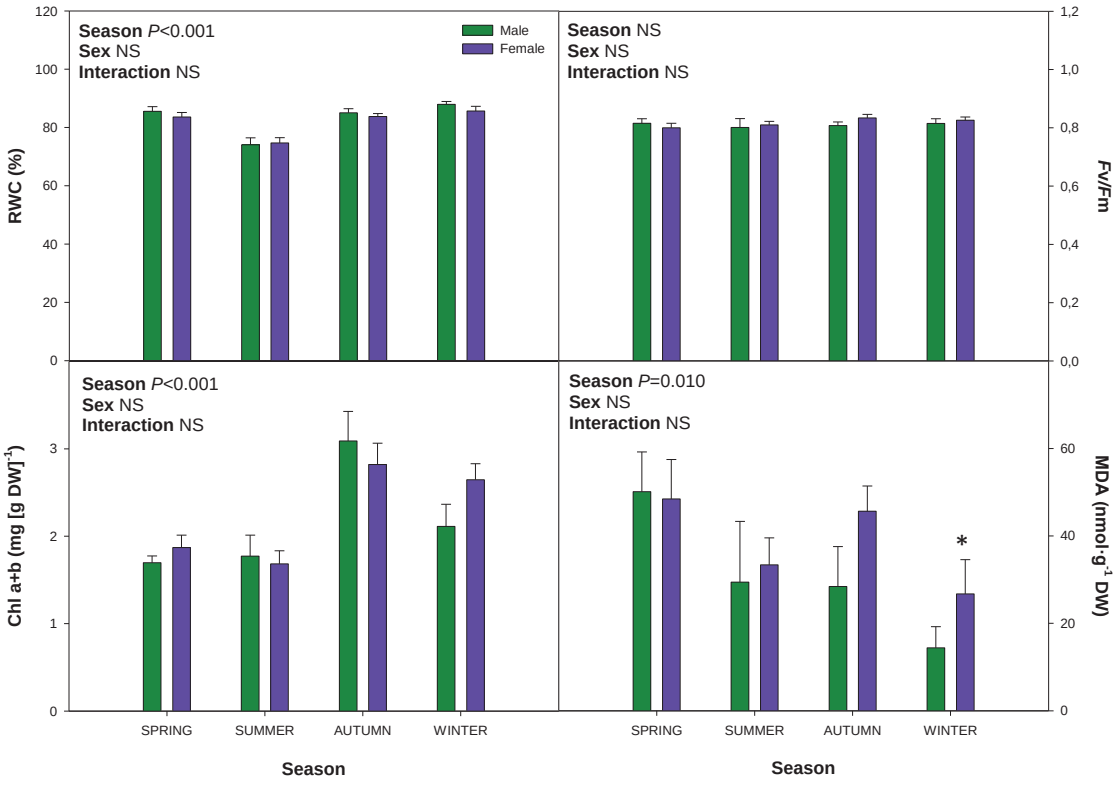


Fig. S3 Sex-related differences in the seasonal variations in levels of total anthocyanins (Ant), carotenoids (Car) and α-tocopherol (α-Toc), expressed per g of dry weight (DW) and per unit of chlorophyll (Chl) a+b in *P. lentiscus*. Data represent the mean ± SE of n=6 individuals. Significant differences between groups were tested by two-way factorial analyses of variance (ANOVA) with time and plant sex as factors. An asterisk indicates significant differences between males and females at a given time point (Student's t-test, $P<0.05$). NS, not significant.

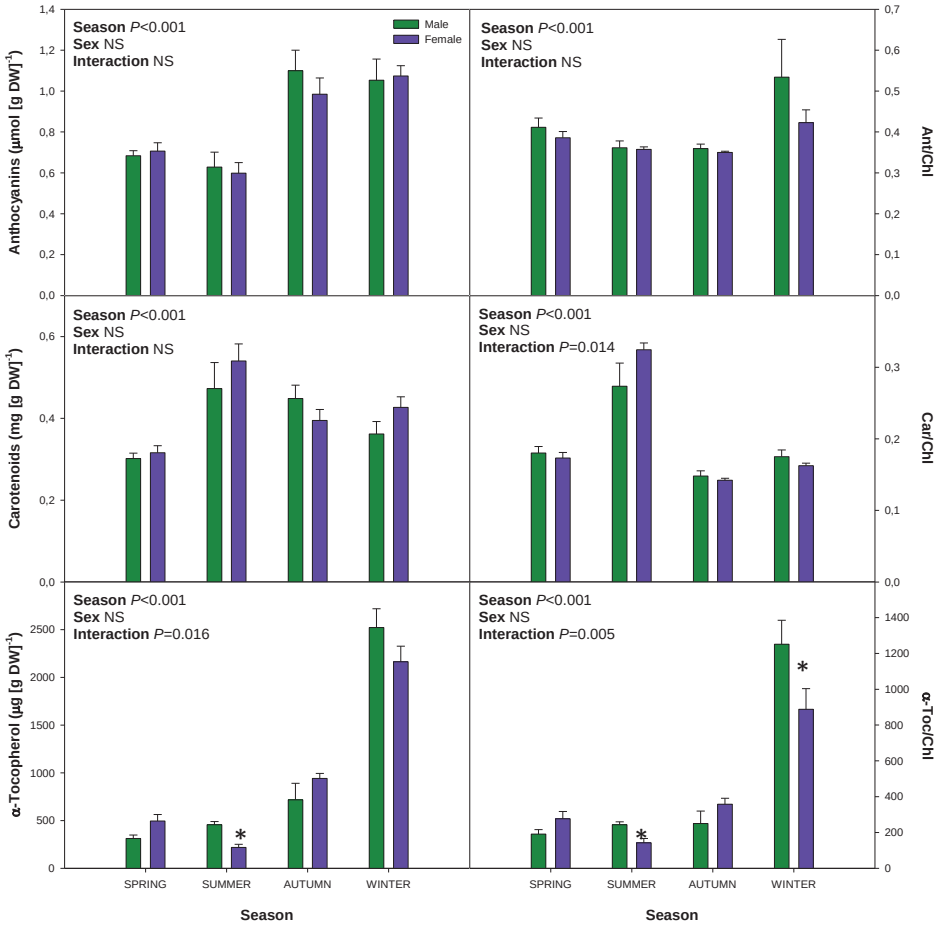


Fig. S4 Sex-related differences during winter in the relative leaf water content (RWC) of *P. lentiscus*. Data represent the mean ± SE of n=6 individuals. Significant differences between groups were tested by two-way factorial analyses of variance (ANOVA) with time and plant sex (females *vs.* males), shoot (reproductive -R- *vs.* non-reproductive –NR-) or fruit (shoots with and without fruits) as factors. NS, not significant.

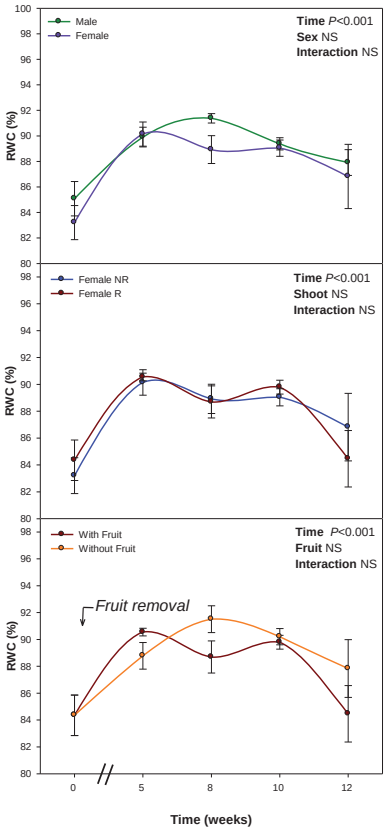


Fig. S5 Sex-related variations during winter in the levels of total anthocyanins (Ant), carotenoids (Car) and α-tocopherol (α-Toc),expressed per unit of chlorophyll (Chl) in *P. lentiscus*. Data represent the mean ± SE of n=6 individuals. Significant differences between groups were tested by two-way factorial analyses of variance (ANOVA) with time and plant sex (females *vs.* males), shoot (reproductive -R- *vs.* non-reproductive –NR-) or fruit (shoots with and without fruits) as factors. NS, not significant.

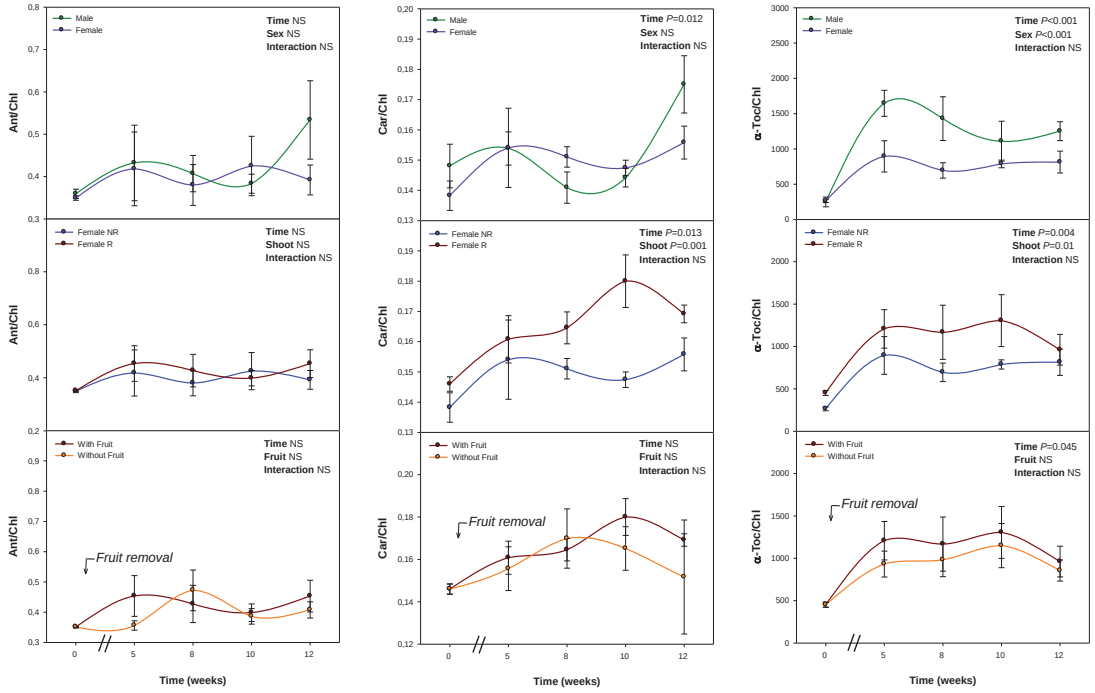


Fig. S6 Sex-related differences in the diurnal variations in levels of total anthocyanins (Ant), carotenoids (Car) and α-tocopherol (α-Toc) in *P. lentiscus* during winter (25 January). Data represent the mean ± SE of n=6 individuals. Significant differences between groups were tested by two-way factorial analyses of variance (ANOVA) with time and plant sex (females *vs.* males), shoot (reproductive -R- *vs.* non-reproductive –NR-) or fruit (shoots with and without fruits) as factors. An asterisk indicates significant differences between males and females, R and NR shoots, or shoots with and without fruits at a given time point (Student's t-test, $P<0.05$). NS, not significant.

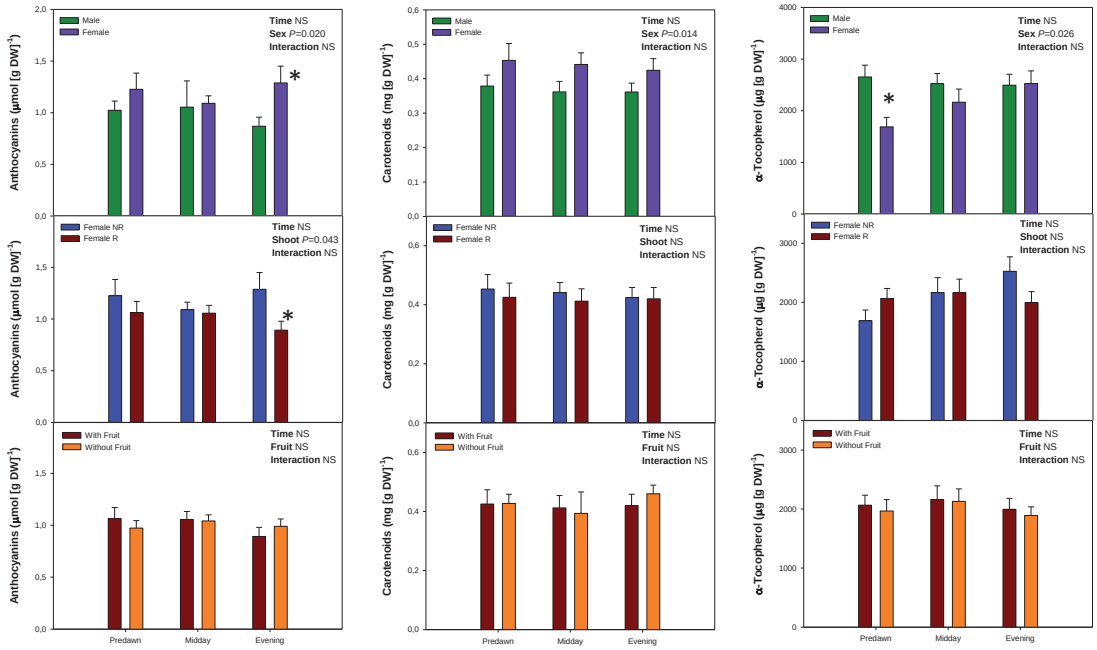


Fig. S7 Sex-related differences in the diurnal variations in levels of total anthocyanins (Ant), carotenoids (Car) and α-tocopherol (α-Toc) expressed per unit of chlorophyll (Chl) in *P. lentiscus* during winter (25 January). Data represent the mean ± SE of n=6 individuals. Significant differences between groups were tested by two-way factorial analyses of variance (ANOVA) with time and plant sex (females *vs.* males), shoot (reproductive -R- *vs.* non-reproductive –NR-) or fruit (shoots with or without fruits) as factors. An asterisk indicates significant differences between males and females, R and NR shoots, or shoots with and without fruits at a given time point (Student's t-test, $P<0.05$). NS, not significant.

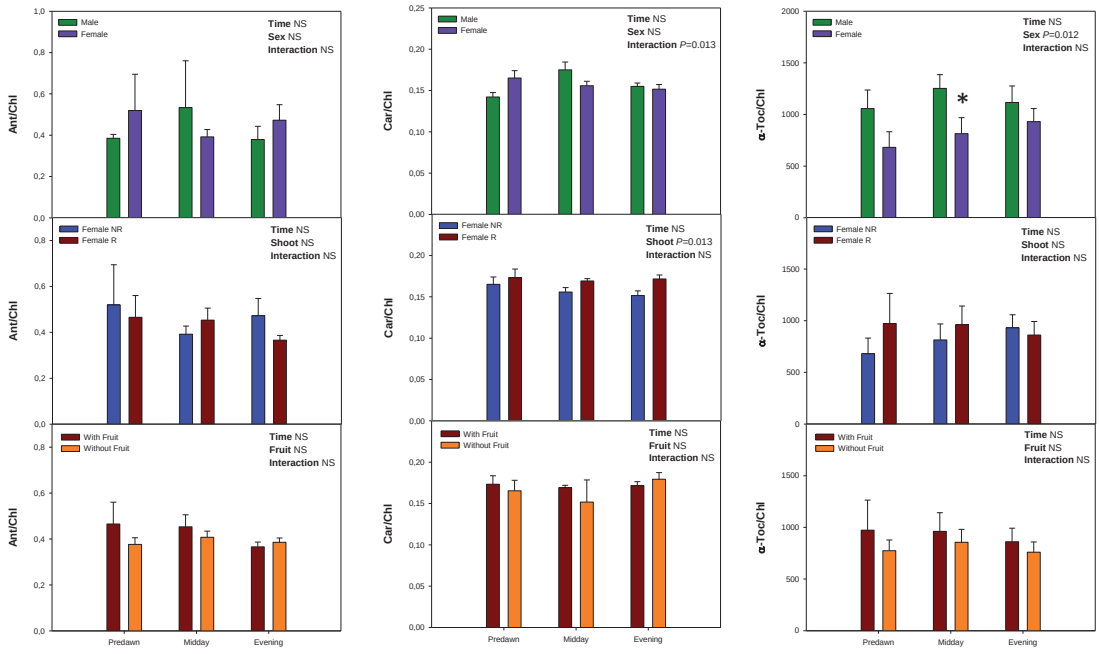


Fig. S8 Sex-related differences in the diurnal variations in levels zeatin (Z), zeatin riboside (ZR) and 2-isopentenyladenine (2iP) in *P. lentiscus* during winter (25 January). Data represent the mean ± SE of n=6 individuals. Significant differences between groups were tested by two-way factorial analyses of variance (ANOVA) with time and plant sex (females *vs.* males), shoot (reproductive -R- *vs.* non-reproductive –NR-) or fruit (shoots with or without fruits) as factors. An asterisk indicates significant differences between males and females at a given time point (Student's t-test, $P<0.05$). NS, not significant.

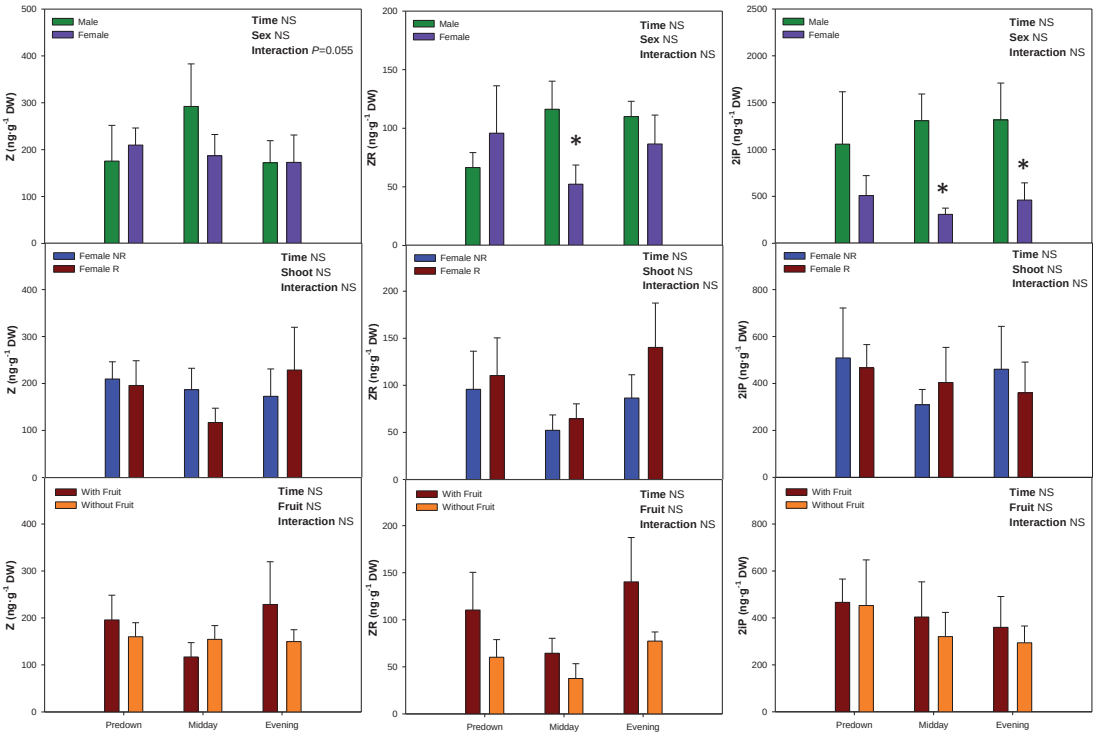
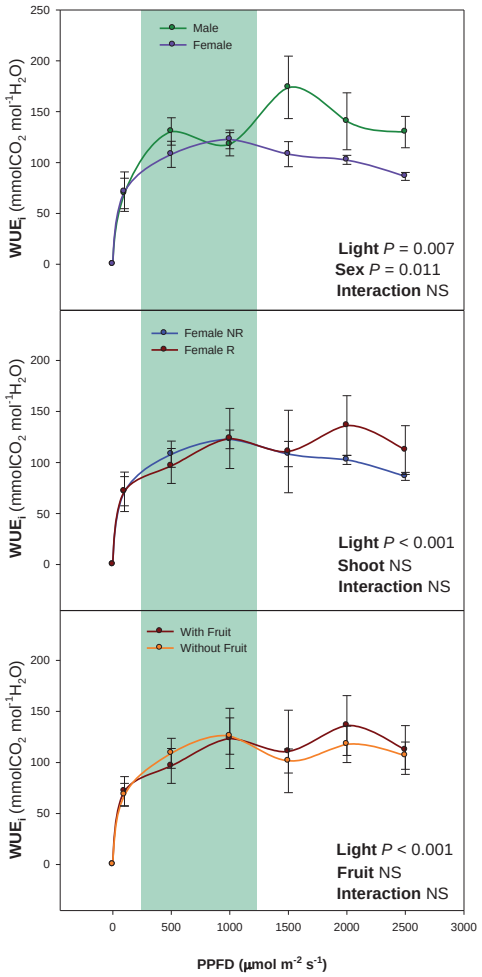


Fig. S9 Sex-related differences during winter in the instantaneous water use efficiency (WUE) response curves to photosynthetically-active photon flux density (PPFD) in *P. lentiscus*. Data represent the mean ± SE of n=6 individuals. Significant differences between groups were tested by two-way factorial analyses of variance (ANOVA) with time and plant sex (females *vs.* males), shoot (reproductive -R- *vs.* non-reproductive –NR-) or fruit (shoots with and without fruits) as factors. NS, not significant. Green shading indicates midday sampling PPFD range during winter.



Discussió general

DISCUSSIÓ GENERAL

1. L'estrès oxidatiu com a marcador fisiològic

L'estrès oxidatiu ha estat utilitzat per determinar l'estat fisiològic de les plantes tant en condicions d'estrès (abiòtic i biòtic) com durant altres processos intrínsecs al desenvolupament de la planta, com ara la senescència. L'aspecte més important a l'hora de mesurar l'estrès oxidatiu és comptar amb vàries mesures que ens ajudin a visualitzar de manera més amplia el seu efecte en la planta (Pintó-Marijuan & Munné-Bosch, 2014).

Per tal de determinar l'efecte de l'estrès oxidatiu aquest pot ser mesurat de manera directa mitjançant els nivells de ROS endògens com el $^1O_2$ (Hideg *et al.*, 2002; Fischer *et al.*, 2013) i el H_2O_2 (Queval *et al.*, 2008). L'estrès també el podem mesurar de manera indirecta a través de l'anàlisi dels factors implicats en l'eliminació de les ROS, com els antioxidants enzimàtics (Noctor & Foyer, 1998) i els no enzimàtics (Jahns & Holzwarth, 2012; Munné-Bosch & Alegre, 2002a), ja que l'estrès oxidatiu sol estar acompanyat d'un augment d'aquests factors com a conseqüència d'un mecanisme de la planta per mantenir l'homeòstasi redox. La quantificació dels productes generats per l'oxidació de les ROS, com l'abast de la peroxidació lipídica (Mueller *et al.*, 2006), pot ser una altra manera de mesurar l'efecte de l'estrès oxidatiu indirectament, així com la fluorescència de les clorofil·les, que ha resultat ser una eina molt útil per a l'estudi de diferents aspectes de la fotosíntesi i la detecció d'estrès en plantes (Krause & Weis, 1991).

En aquesta tesi es varen utilitzar diversos marcadors d'estrès oxidatiu com l'eficiència màxima del PSII (F_v/F_m), els nivells de pigments fotosintètics i d'antioxidants no enzimàtics com els antocians i l'α-tocoferol, però centrant-nos sobretot en el paper dual del MDA com a marcador tant d'estrès oxidatiu com de senyalització. Aquesta dualitat es va fer palesa en els resultats del **capítol 1**, en el qual l'increment de MDA en fulles més joves podria estar implicat en un possible rol en senyalització, mentre que

en les etapes més avançades de la senescència ens indica el procés de degradació que estan experimentant les fulles. En el **capítol 2**, les mesures de MDA, entre d'altres marcadors, ens serveixen per a caracteritzar l'estrès oxidatiu en condicions d'estrès ambiental i per a determinar l'edat fisiològica de les plantes.

La formació de MDA es dóna a través de l'oxidació dels PUFAs tant per la via enzimàtica (LOX) com per la no enzimàtica (ROS) (**Figura 7**).

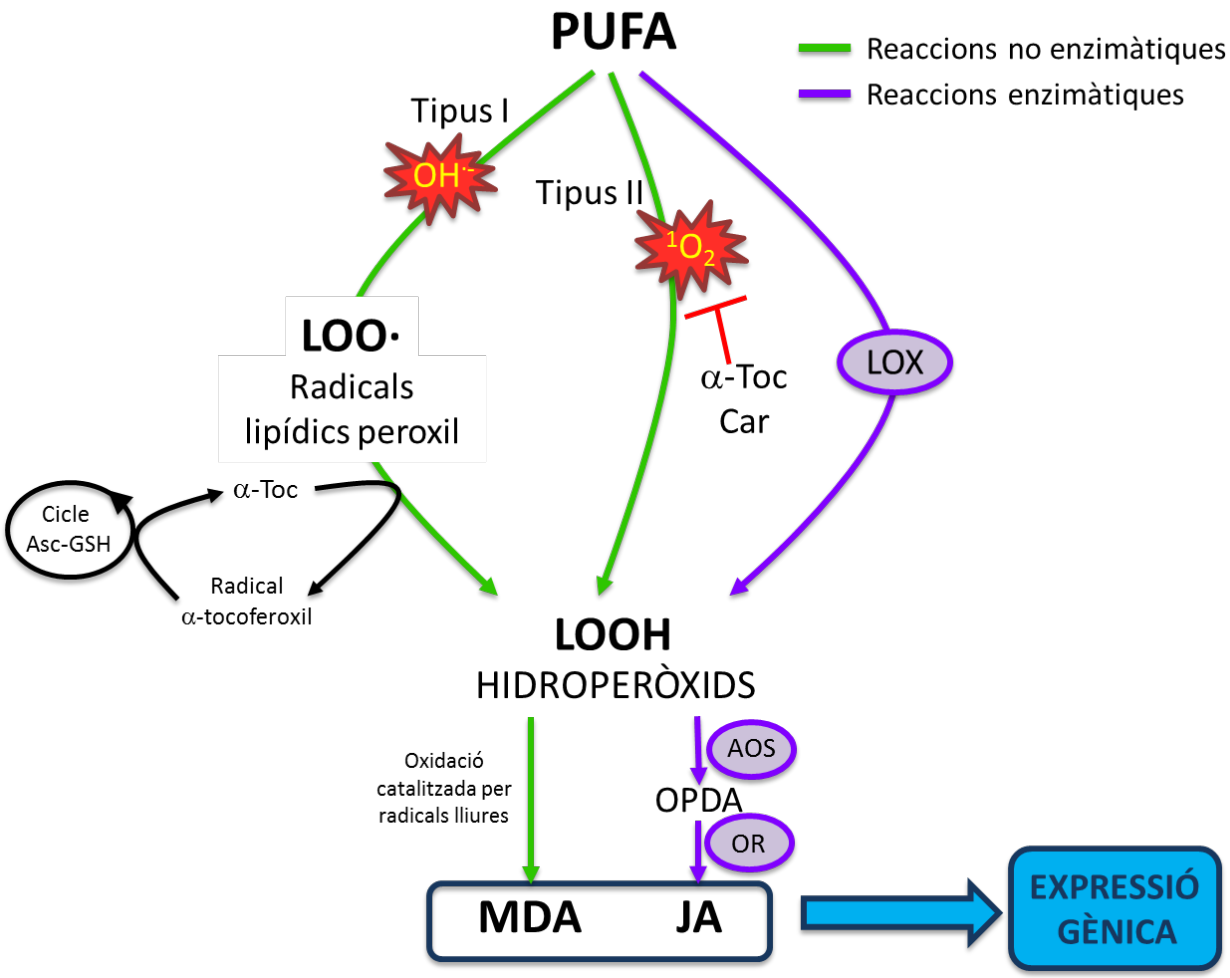


Figura 7: Esquema representatiu de la formació d'àcid malondialdehid (MDA) i d'àcid jasmònic (JA) com a resultat de la peroxidació lipídica en plantes. Tant la peroxidació lipídica enzimàtica (formació de JA a través de l'acció d'enzims), com la no enzimàtica (formació de MDA a través de la fragmentació de lípids mitjançant l'oxidació catalitzada per radicals lliures) es donen de manera simultània en condicions d'estrès. $^1O_2$, singlet d'oxigen; $OH^{\cdot}$, radical hidroxil; α-Toc, α-tocoferol; AOS, *allene oxide* sintasa; Asc, ascorbat; Car, carotenoides; GSH, glutatió; LOO·, radicals lipídics peroxil; LOOH, hidroperòxids; LOX, lipoxigenasa; OPDA, àcid oxofitodienoic; OR, OPDA reductasa; PUFA: àcids grassos poliinsaturats.

Per tant, l'increment dels nivells de MDA no només mostra un efecte del possible estrès oxidatiu que la planta pugui estar patint, sinó que també es pot relacionar amb un procés de senyalització interna d'aquesta. S'ha demostrat que el MDA és capaç d'induir l'expressió de gens, entre ells gens de resposta a estressos abiòtics (Weber *et al.*, 2004), i, per altra banda, també és un indicador de la peroxidació lipídica, la qual pot portar a la formació d'oxilipines, entre elles el JA, implicades també en senyalització cel·lular (Demmig-Adams *et al.*, 2013). L'anàlisi dels nivells de MDA en el **capítol 3** suggereixen la seva implicació en senyalització mitjançant l'observació d'un increment paral·lel dels nivells de JA. Aquesta hipòtesi torna a proposar-se en el **capítol 4**, en el qual es descriu una major peroxidació lipídica a partir de la via enzimàtica (LOX) abans de l'alba. Tot i que en aquest últim cas no es va traduir en un augment visible dels nivells de JA, sí que es va poder relacionar amb una interacció amb altres hormones.

El conjunt de mesures per a determinar l'estrès oxidatiu en plantes de diferents edats varen aportar informació sobre l'edat fisiològica de les plantes. L'estudi a nivell de fulla realitzat en el **capítol 1** revela la utilitat de l'efecte complementari de la determinació conjunta entre l'edat cronològica i fisiològica a l'hora d'identificar les etapes del seu desenvolupament. A nivell de planta sencera però, els resultats obtinguts en els **capítols 2** (en el qual es comparen plantes juvenils de diferents edats cronològiques) i **3** (comparació entre arbres amb edats cronològiques i fisiològiques diferents) mostren clarament un avantatge de la consideració conjunta dels nivells d'estrès oxidatiu i l'edat cronològica de la planta per a poder determinar-ne l'edat fisiològica.

Per últim, remarcar que l'anàlisi de l'estrès oxidatiu va contribuir a una millor caracterització dels processos estudiats, com el desenvolupament foliar (**capítol 1**) i l'esforç reproductiu (**capítol 4**) en *P. lentiscus* i el trencament de la dormició en gemmes de faig (**capítol 3**).

2. L'estrès oxidatiu en la determinació de l'edat de la planta

2.1. Estudi a nivell de fulla

Al llarg de la seva vida, les fulles experimenten una sèrie de processos durant el seu desenvolupament, altament controlats a nivell molecular, bioquímic i fisiològic, que culminen en la senescència i mort de la fulla. La senescència foliar n'és l'etapa final i està lligada a l'edat de la fulla, tot i que engloba la complexa regulació dels processos corresponents a les etapes anteriors de la vida de la fulla, així com diversos factors ambientals i endògens, per tal d'ajustar-ne l'inici, la progressió i la finalització d'aquesta (Buchanan-Wollaston *et al.*, 2005; Lim *et al.*, 2007; Woo *et al.*, 2013). La interacció de tots ells implica que una visió global dels canvis fisiològics al llarg de la vida de la fulla pot ser determinant per entendre'n el seu desenvolupament. En el **capítol 1** es va utilitzar l'estudi a nivell de fulla com a model per comparar l'edat cronològica i la fisiològica, ja que ens proporciona una història de vida fàcilment reproduïble i controlable. A l'inici de l'experiment es varen marcar les fulles joves de llentiscle acabades d'emergir (en procés de creixement i expansió) per tal de poder seguir els canvis fisiològics al llarg de tot el seu desenvolupament fins a la senescència i saber-ne l'edat cronològica en cada moment.

Les fulles de *Pistacia lentiscus* presentaven símptomes d'estrès oxidatiu en els dos extrems del seu desenvolupament. Les mesures del rendiment quàntic màxim del PSII (relació F_v/F_m), tant en les fulles emergents com en les senescents, mostraven uns nivells per sota de 0.75, valor indicatiu de dany en el PSII causat per fotoinhibició (Björkman & Demmig, 1987). Així mateix, aquests valors coincidien amb una acumulació dels nivells de MDA. Atès que les fulles joves es troben en etapes de creixement, els baixos nivells de F_v/F_m podrien ser causats per un aparell fotosintètic immadur, associat a un cloroplast que encara no s'ha desenvolupat del tot. En conseqüència, les fulles emergents poden ser molt sensibles a qualsevol

estrès ambiental i, per tant, susceptibles de patir una disminució de l'eficiència del PSII o, fins i tot, una acumulació temporal de ROS. Concordant amb els resultats obtinguts en aquest primer capítol, altres estudis també mostren que les fulles en expansió presenten estrès oxidatiu transitori caracteritzat pels baixos nivells de F_v/F_m, així com per un increment dels nivells d'antioxidants (Jiang *et al.*, 2005; Maayan *et al.*, 2008; Lepeduš *et al.*, 2011). En el cas de l'estudi de Jiang *et al.* (2005) en fulles de soja, l'activitat dels antioxidants enzimàtics era elevada durant les primeres etapes del desenvolupament foliar, així com en el cas dels antioxidants no enzimàtics (com els carotenoides i l'α-tocoferol) en el treball dut a terme per Lepeduš *et al.* (2011). L'increment d'antioxidants podria considerar-se com un mecanisme compensatori per a combatre l'estrès oxidatiu al qual podrien estar sotmeses aquestes fulles.

La intensa coloració vermella i els elevats nivells d'antocians en el cas de les fulles en expansió de llentiscle en el **capítol 1** feien pensar en una possible acumulació transitòria d'antocians, juntament amb la de carotenoides, com un possible mecanisme fotoprotector (Steyn *et al.*, 2002), per a contrarestar la quantitat de llum absorbida per les clorofil·les en unes fulles on l'aparell fotosintètic es troba en desenvolupament. No obstant, les concentracions d'antocians i de carotenoides, així com d'α-tocoferol, per unitat de clorofil·la en les fulles emergents eren més baixes en relació a les madures. La pèrdua de clorofil·les és una conseqüència de la seva degradació en estressos com l'alta intensitat de llum o la sequera; no obstant, també ha estat considerada com un mecanisme de fotoprotecció en plantes estressades. La reducció del contingut de clorofil·les redueix la quantitat de llum captada per aquestes, causant un augment de la capacitat fotoprotectora i antioxidant de les fulles, ja que la quantitat d'antioxidant per llum absorbida augmenta (Kyparissis *et al.* 1995, 2000; Munné-Bosch & Alegre, 2000; Balaguer *et al.* 2002). Aquest fet podria explicar l'estrès oxidatiu que mostraven les fulles més joves, atès que, tot i els nivells elevats d'antioxidants, presentaven els nivells més alts de clorofil·les en

comparació a les altres fulles en etapes de desenvolupament més avançat i, per tant, valors d'antioxidants per clorofil·la baixos.

L'estrès oxidatiu en fulles en expansió de llentiscle és transitori atès que, a mesura que avança el desenvolupament, aquest va disminuint fins a tornar a augmentar en fulles senescents. En els primers estadis de la senescència, un cop aquesta ja ha estat induïda, l'increment dels nivells d'antioxidants ajuda a mantenir funcional la fulla fins a acomplir la seva tasca de remobilització de nutrients (Hoch *et al.*, 2001; García-Plazaola *et al.*, 2003). Passat aquest punt, en les etapes més avançades del procés de senescència, es produeix una disminució dels antioxidants, com ara l'α-tocoferol i els antocians en les fulles de llentiscle, juntament amb un increment de l'estrès oxidatiu, símptomes característics de l'etapa final de degradació de la senescència (Munné-Bosch & Peñuelas, 2003; Lim *et al.*, 2007).

Les fulles pateixen estrès oxidatiu en les primeres etapes del seu creixement, quan els cloroplasts reben grans quantitats de llum però els mecanismes de fotoprotecció i antioxidants encara no estan del tot desenvolupats. Conseqüentment, aquest excés d'energia d'excitació no pot ser dissipat del tot, provocant l'augment de l'estrès oxidatiu. Malgrat això, aquest estrès és ràpidament esmorteït pel desenvolupament eficient de la maquinària antioxidant alhora que la fulla s'expandeix, sense arribar a provocar un dany irreversible. Així doncs, l'estrès oxidatiu transitori en les fulles joves de *P. lentiscus* suggereix una possible funció de senyalització en protecció.

Semblant als canvis que pateixen les fulles emergents, els mecanismes de fotoprotecció i antioxidants s'activen en les fulles senescents durant el procés de remobilització de nutrients. No obstant, a mesura que avança la senescència aquests mecanismes minven, provocant a la fulla un dany oxidatiu irreversible que li pot arribar a comportar la mort (Lim *et al.*, 2007; Procházková & Wilhelmova, 2007) (**Figura 8**).

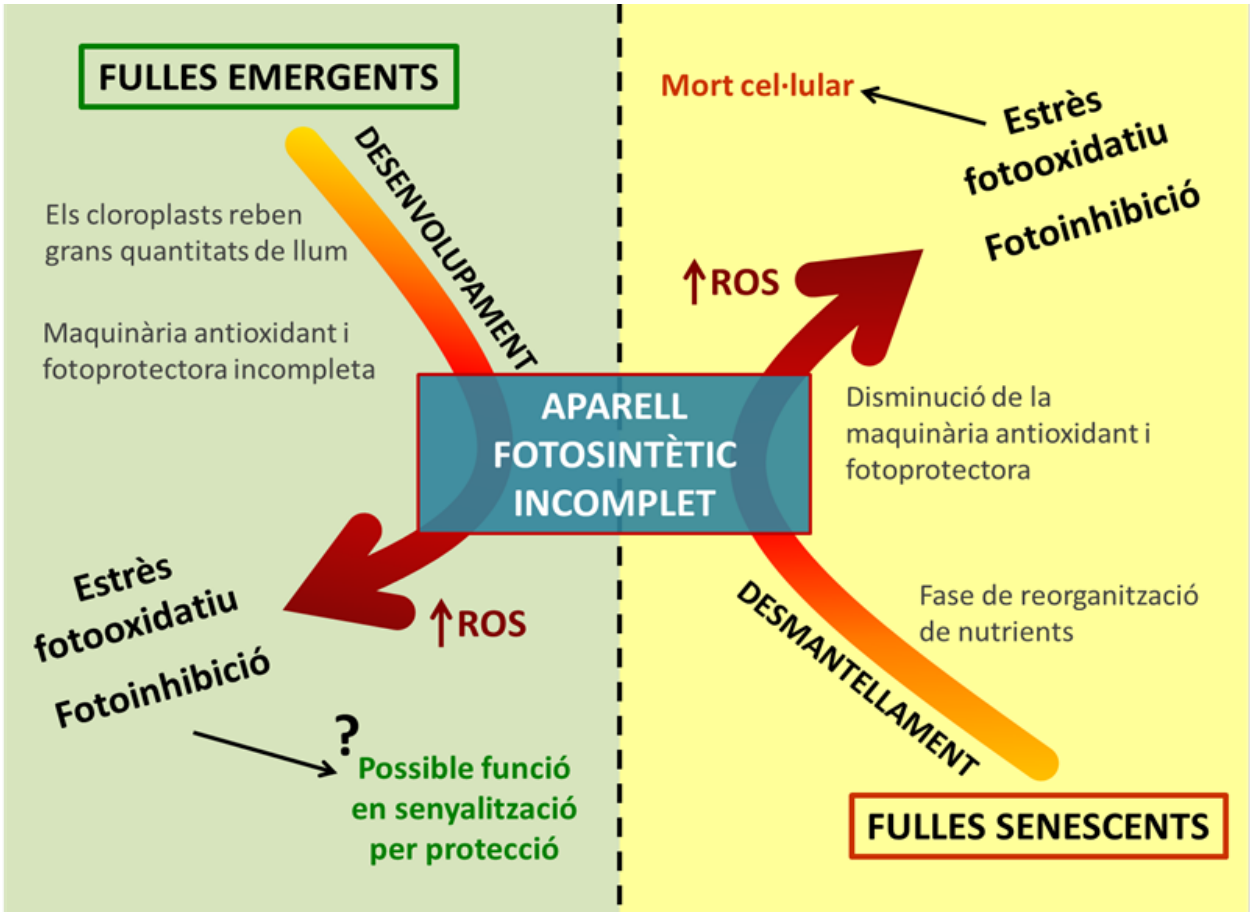


Figura 8: Esquema resum de la fotoinhibició en fulles emergents i senescents. La fotoinhibició assoleix el seu màxim en els dos extrems de la vida de la fulla, ja que presenten el mateix problema: un aparell fotosintètic incomplet. Les causes difereixen entre els dos tipus de fulles: mentre les fulles emergents estan desenvolupant la maquinària fotosintètica, les senescents l'estan desmantellant. Per aquesta raó ambdues són sensibles a patir estrès fotooxidatiu i fotoinhibició. El resultat final també és completament diferent en ambdós casos: l'estrès oxidatiu en fulles emergents juga un paper possiblement de senyalització en protecció (ja que les fulles són capaces de seguir desenvolupant-se), mentre que en estadis avançats de senescència arriba a causar dany i finalment la mort. ROS, espècies reactives de l'oxigen.

L'edat cronològica de la fulla influeix inevitablement en el seu desenvolupament. Les fulles inicïen la seva vida com a primordi foliar i durant el seu desenvolupament i creixement esdevenen fotosintèticament competents i capaces d'acumular nutrients. Arribat a un punt s'indueix el procés de senescència, seguit per la mort i abscisió foliar. La senescència implica un procés de desgast fisiològic al llarg de la vida de la fulla, no tan sols en les etapes finals. En el cas de la planta model *Arabidopsis*, s'ha observat que en absència de qualsevol estímul extern que pugui accelerar el procés, l'edat de la fulla en resulta ser el factor més influent en la iniciació

de la senescència (Gan & Amasino, 1997). Així mateix, l'augment de l'estrès oxidatiu ha estat proposat com un canvi fisiològic crucial en la inducció de l'envelliment i senescència dels organismes (Harman, 1956; Sohal & Weindruch, 1996), i encara que en plantes no ha estat demostrada aquesta teoria a nivell d'organisme, si que s'ha pogut observar un increment de ROS al llarg de la vida foliar (Woo *et al.*, 2013).

Els resultats adquirits ens indiquen que l'estrès oxidatiu pot ser un bon marcador de l'edat cronològica de la fulla, però no inequívoc. En el **capítol 1** s'observa un increment de l'estrès oxidatiu en les fulles d'edat més avançada en relació a les madures, concordant amb la teoria dels radicals lliures; per contra, les fulles emergents també presentaven alts nivells d'estrès oxidatiu sense necessitat d'estar relacionat amb un deteriorament fisiològic. En conseqüència, en aquest estudi no es va poder relacionar l'increment de l'estrès oxidatiu i l'edat de la fulla, ja que aquesta relació tant sols es va complir per les etapes més avançades.

Cal fer menció al fet que, al tractar-se d'un estudi en condicions naturals, els canvis observats en els paràmetres fisiològics no varen ser causats únicament per l'edat de la fulla sinó que foren resultat de la interacció conjunta amb les condicions climàtiques a les que varen estar exposades les plantes.

2.2. Estudi a nivell de planta sencera

L'extensió de la vida d'una planta està influenciada per la interacció de diferents factors com el creixement, la mida i l'edat, juntament amb els estressos ambientals als que ha estat sotmesa amb el pas del temps (Roach, 2012). A causa de la seva constant interacció amb el medi, les plantes han desenvolupat mecanismes per tal de combatre'n els possibles efectes perjudicials. La plasticitat de les plantes és una característica gràcies a la qual aquestes posseeixen la capacitat de presentar diferents fenotips a partir d'un sol genotip en diferents situacions de creixement (Guyomarc'h *et al.*, 2005). De tal manera que, al ser exposades a diferents estressos, les

plantes són capaces de dur a terme una sèrie d'ajustos fisiològics per tal de combatre'ls. L'efecte acumulatiu dels canvis en l'ambient, no tant sols en el present sinó també en l'ambient al que ha estat exposat l'individu en el transcurs de la seva vida (Monaghan, 2008) influencien l'esperança de vida i la supervivència d'aquest (Tuljapurkar & Horvitz, 2006).

Atès que molts altres factors repercuteixen en el desenvolupament de la planta a part de l'edat, en aquesta tesi es va proposar l'estrès oxidatiu com a marcador de l'edat fisiològica en comparació a l'edat cronològica, com a mesura més acurada de l'estat fisiològic i del possible grau d'envelliment de la planta sencera. Amb aquest propòsit en el **capítol 2** es varen comparar plantes de *P. lentiscus* en etapa juvenil que diferien en edat cronològica. Una diferència de 3 anys va ser suficient perquè presentessin una desigualtat notable respecte a la seva mida i biomassa, ja que les plantes d'edat més avançada presentaven 10 vegades més biomassa total en comparació amb les plantes de dimensions menors. S'ha demostrat que la mida de la planta és un factor rellevant en molts aspectes del desenvolupament, influenciant les taxes de creixement foliar en plantes madures i produint un efecte deleteri en processos fisiològics com la fotosíntesi i la distribució del carboni (Mencuccini, 2003; Mencuccini *et al.*, 2005), no només en perennes llenyoses (Vanderklein *et al.*, 2007; Oñate & Munné-Bosch, 2008) sinó també en perennes herbàcies (Zotz *et al.*, 2001, 2002). Tot i les anteriors evidències, ni l'efecte de la l'edat ni de la mida associada a aquesta van afectar les plantes de llentiscle al ser exposades a un estrès ambiental com la sequera durant l'estiu mediterrani. No es varen observar diferències a nivell d'estrès oxidatiu entre les plantes de diferents edats. Els nivells d'antioxidants (antocians, carotenoides i α-tocoferol), de F_v/F_m i de peroxidació lipídica no diferien entre les plantes de *P. lentiscus* (**Figura 9**). Val a dir però, que tot i que les condicions climàtiques durant els mesos d'estiu varen ser les típiques del clima mediterrani, amb temperatures altes i precipitacions escasses, les plantes de llentiscle no varen presentar símptomes d'estrès sever. En totes les plantes tant sols es

va poder observar una lleugera disminució de la relació F_v/F_m, però el contingut hídric relatiu no va variar entre maig i juliol, així com tampoc es va detectar un increment de l'abast de peroxidació lipídica. La disminució d'antioxidants, com els carotenoides i els antocians, es podria veure compensada per un augment d'α-tocoferol al juliol, antioxidant clau en la defensa contra la fotoinhibició (Murata *et al.*, 2012). Cal destacar però, que l'absència de diferències entre les plantes de diferents edats podria ser deguda a que l'estrès al que varen estar sotmeses no va ser suficient.

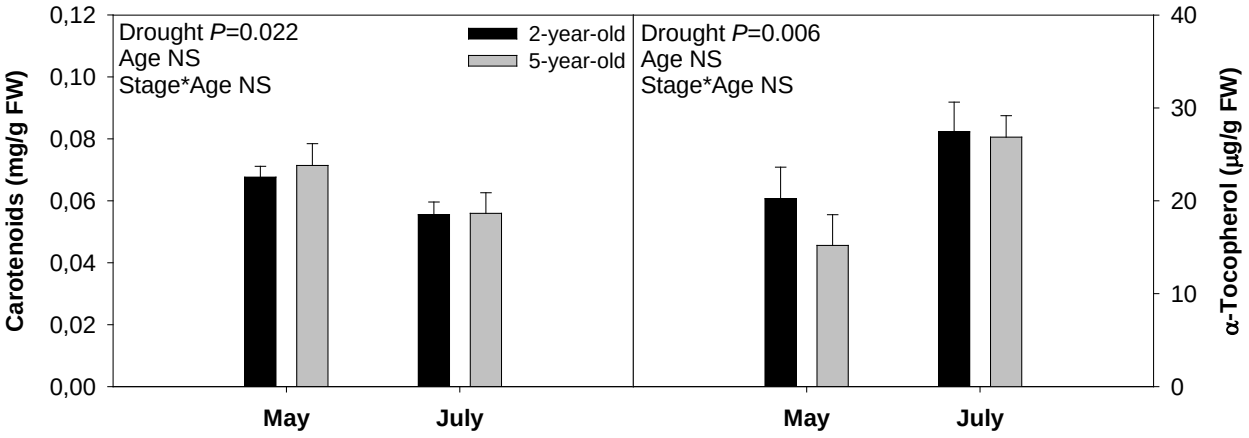


Figura 9: Nivells endògens de carotenoides i α-tocoferol en plantes de llentiscle de 2 i 5 anys abans (Maig, considerat com a control) i durant la sequera estival (Juliol, plantes estressades). Els valors corresponen a la mitjana ± ES d'una n=6 individus. Els resultats estadístics indiquen diferències al llarg del temps (efecte de la sequera entre maig i juliol) i entre les plantes de diferents edats mitjançant un anàlisi de variància ANOVA de dos factors.

Per altra banda, es varen observar diferències significatives en els nivells de zeatina (Z) lligades a l'edat, fenomen que podria estar relacionat amb la maduració del cloroplast. Les citocinines són hormones que juguen un paper important en el creixement de la planta implicades en la divisió i diferenciació cel·lular (Mok & Mok, 2001). A més, també estan involucrades en el control de la biogènesi i la funció del cloroplasts. Les citocinines afecten l'estructura del cloroplast, les activitats enzimàtiques, l'acumulació de pigments i la taxa fotosintètica (Zubo *et al.*, 2008; Okazaki *et al.*, 2009), i també s'ha observat que modulen l'expressió de gens que codifiquen per la proteïna d'unió del complex captador de llum clorofil·la a/b (de la Serve *et*

al., 1985). En el nostre estudi, les plantes de major edat, presentaven reduccions significatives en els nivells de Z, associats a un lleuger però significatiu increment de la relació clorofil·la a/b. Atès que en plantes de tabac Wilhelmova & Kutik (1995) van observar que l'aplicació de citocinines disminuïa la relació clorofil·la a/b en el cloroplast, els nostres resultats suggereixen que els canvis relacionats amb l'edat en les plantes joves de *P. lentiscus* impliquen diferències en la maduració del cloroplast i l'acumulació de pigments fotosintètics. Els anàlisis de correlacions posteriors ens varen ajudar a corroborar que efectivament un augment de Z correlacionava negativament amb els nivells de clorofil·la a/b.

Un augment de clorofil·la a respecte a clorofil·la b suposa un increment dels centres de reacció en comparació a les pantalles col·lectores de llum, aquest efecte s'ha descrit en varis estudis com una característica adaptativa a condicions climàtiques adverses mitjançant la reducció de la quantitat de llum captada per les fulles, evitant així un possible dany per fotoinhibició (Kyparissis *et al.* 1995, 2000; Munné-Bosch & Alegre, 2000; Balaguer *et al.* 2002). Així mateix, es va observar una correlació positiva entre la relació clorofil·la a/b i els nivells d'antocians, resultats que suggerien que les fulles de plantes de més edat de llentiscle estaven més predisposades a patir estrès fotooxidatiu. Altrament, no s'observaren diferències entre les plantes de diferents edats en termes d'estrès oxidatiu durant el creixement foliar (**Figura 10**).

Les mesures d'estrès oxidatiu en aquest estudi ens indiquen que les plantes de diferents edats cronològiques presenten una edat fisiològica similar. L'edat fisiològica no està afectada tant sols per l'edat cronològica sinó que també hi influeixen les condicions climàtiques adverses a les que han estat sotmeses les plantes al llarg de la seva vida. No obstant això, les plantes juvenils de llentiscle, tot i tenir edats cronològiques diferents varen estar trasplantades als camps experimentals al mateix moment, i tant sols va passar un any abans de dur a terme l'experiment, temps segurament insuficient per arribar a desenvolupar i acumular diferències degudes a

l'efecte del clima entre elles. Les respostes diferencial a les condicions climàtiques adverses dependents de l'edat han estat atribuïdes principalment a dos mecanismes fisiològics diferents. Les dues hipòtesis, ja sigui proposant que un augment en mida i alçada de la planta suposen per una banda, una major resistència hidràulica (Carrer & Urbinati, 2004); i per altra, el retard del moviment basipètal d'hormones de creixement (Rossi *et al.*, 2008), estan relacionades amb l'increment de la grandària més que amb l'edat.

De tota manera, un factor important a tenir en compte va ser l'elevada taxa de mortalitat entre els individus més joves (11.43%) respecte als altres en que no va morir cap planta. Si tenim en compte que la relació brot/arrel disminuïa amb l'edat, juntament amb el fet que les plantes de 2 anys

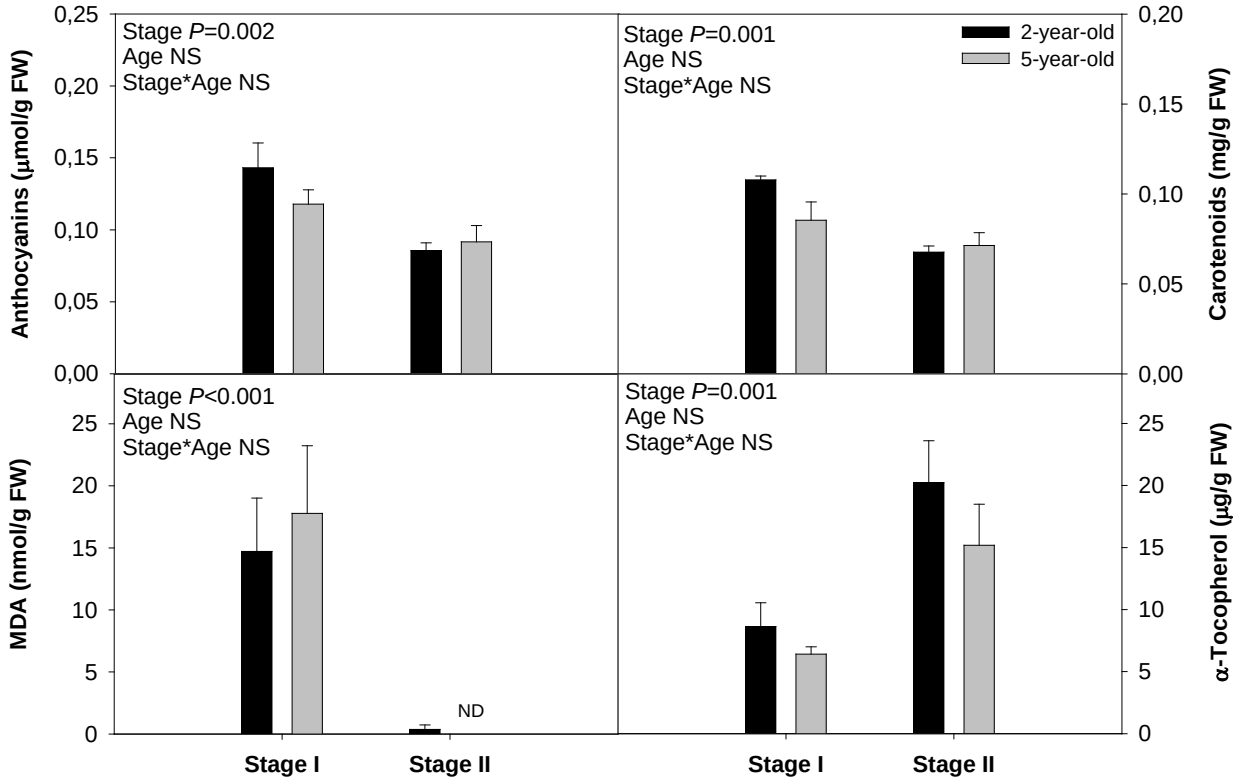


Figura 10: Nivells d'antocians, àcid malondialdehid (MDA), carotenoides i α-tocoferol en plantes de llentiscle de 2 i 5 anys abans i durant les primeres fases del desenvolupament foliar des de gener (estadi I, inici) fins a maig (estadi II, màxim creixement). Els valors corresponen a la mitjana ± ES d'una n=6 individus. Els resultats estadístics indiquen diferències al llarg del temps (entre els estadis) i entre les plantes de diferents edats mitjançant un anàlisi de variància ANOVA de dos factors.

presentaven una biomassa d'arrels molt inferior, això ens indueix a pensar que aquestes plantes encara es trobaven en fase d'establiment en el sòl. Altres estudis en que s'ha testat la capacitat de diferents espècies d'arbusts mediterranis per la restauració en zones afectades per la desertització, també han observat una elevada mortalitat en el cas de la *P. lentiscus,* generalment associada a l'aridesa que pateixen durant l'estiu (de Dato *et al.*, 2009). S'ha demostrat que l'escassetat d'aigua és un dels obstacles principals per al creixement de plantes en climes mediterranis (Vallejo *et al.*, 2006) el que pot arribar a provocar un increment de la mortalitat d'aquestes després de períodes de sequera (Martínez, 2003; Castro *et al.*, 2004). L'efecte de la sequera juntament amb la presència d'altres plantes pot limitar la supervivència d'aquestes a causa de la competició per als nutrients i la humitat limitada del sòl (Rey Benayas & Camacho-Cruz, 2004; Navarro Cerrillo *et al.*, 2005; Midoko-Iponga *et al.*, 2005). Aquest fet indueix a pensar que en plantes juvenils l'efecte de l'edat podria resultar ser un factor positiu, donat que les més joves segurament requereixen d'un esforç superior per a acomplir un establiment exitós en el sòl i, per tant, això es veuria reflectit en un estrès oxidatiu superior a les plantes més petites.

En el **capítol 3** es varen utilitzar arbres de faig en etapa madura amb clares diferències no tant sols en l'edat cronològica sinó també en l'edat fisiològica entre ells. Els arbres moribunds es van escollir com a exemple d'arbre amb una edat fisiològica avançada atès que havien perdut la dominància apical, fet que ha estat relacionat amb condicions d'estrès (Gruntman & Novoplansky, 2011).

Els arbres moribunds presentaven branques epicòrmiques al llarg de tot el seu tronc. La seva formació es dóna a partir d'una gemma dorment, també anomenada gemma epicòrmica, que s'activa sota condicions d'estrès ambiental. Canvis bruscs en el clima com la sequera, el fred o l'atac d'insectes, poden induir la seva formació (Bégin & Filion, 1999; Cooper-Ellis *et al.*, 1999; Nicolini *et al.*, 2001; Deal *et al.*, 2003; Burrows, 2008;

Mujuri & Demchik, 2009; Meier *et al.*, 2012). La majoria d'aquests estressos provoquen una reducció de l'àrea foliar de manera que la inducció de branques epicòrmiques pot ser una estratègia per incrementar la superfície foliar exposada a la llum evitant pèrdues en l'aportació de biomassa en la planta (Meier *et al.*, 2012).

Atès que les branques epicòrmiques en els arbres moribunds es formen generalment a causa d'un dany en l'arbre, en aquest capítol vàrem hipotetitzar que possiblement les gemmes foliars derivades d'aquestes branques podrien ser un reflex del deteriorament fisiològic dels arbres. Per aquest motiu en vam estudiar el vigor de les gemmes així com la peroxidació lipídica. El vigor de les gemmes va ser un dels símptomes més clars del possible deteriorament fisiològic en arbres moribunds en comparació als arbres sans, pel fet que el nombre de gemmes que arribaven a desenvolupar-se al llarg dels anys era significativament més baix.

El trencament de la dormició és un procés regulat per factors ambientals, fisiològics i del desenvolupament. S'indueix principalment per temperatures fredes i dies llargs, ajudant a la planta a sincronitzar el seu creixement amb unes condicions ambientals favorables. Un cop iniciat, els factors interns en regulen el procés. L'ABA promou la dormició de la gemma, mentre que les GA i les citocinines són hormones necessàries per al trencament de la dormició i posterior creixement de la gemma vegetativa (Horvath *et al.*, 2003; Cooke *et al.*, 2012). Les auxines també tenen un paper molt important en el trencament de la dormició, ja que el transport basipetal d'auxines a través del tronc principal inhibeix el creixement de les gemmes axil·lars (Cline, 2000). Així mateix, s'ha observat que l'aplicació exògena de cianamida d'hidrogen (H_2CN_2) en arbres fruiters i vinya indueix el trencament de la dormició (Pérez & Burgos, 2004; Pérez *et al.*, 2009). Per tant, l'estrès oxidatiu ha estat proposat com un factor que podria estar implicat en el trencament de la dormició (Mazzitelli *et al.*, 2007; Leida *et al.*, 2010).

En el cas del faig, els factors ambientals com el fotoperíode i la temperatura han estat descrits com a factors clau en el trencament de la dormició (Vitasse & Basler, 2013). Els resultats de l'estudi amb gemmes de faig ens van mostrat que, així com passa amb altres espècies, les hormones inductores d'aquest procés fisiològic en aquesta espècie són les GA, auxines i citocinines, ja que els seus nivells varen augmentar al trencar-se la dormició. Els dos tipus d'arbres només presentaven diferències respecte als nivells d'auxines. Mentre els arbres sans mostraven nivells més elevats en gemmes dormides i tancades respecte els moribunds, aquests últims mostraven un augment d'auxines al llarg de tot el procés, el que podria ser un indicador per al restabliment del procés de creixement en les branques epicòrmiques.

El trencament de la dormició de gemmes vegetatives en arbres de faig va incrementar l'abast de la peroxidació lipídica. Els nivells de MDA varen augmentar significativament en gemmes ja obertes, paral·lelament als nivells de JA, tant en arbres sans com en arbres moribunds. Així, aquesta observació podria indicar un increment de peroxidació lipídica produït tant per la via no enzimàtica com per l'enzimàtica durant el trencament de la dormició (Müller *et al.*, 2008). Així mateix, els arbres moribunds presentaven uns nivells de peroxidació lipídica majors que els arbres sans, el que podria ser interpretat com a símptoma d'un deteriorament fisiològic major. Per tant, en les branques epicòrmiques dels arbres moribunds, no només un nombre menor de gemmes aconseguien arribar a brotar, sinó que aquelles que n'eren capaces, presentaven una peroxidació lipídica major.

Cal tenir en compte però, que l'augment de la peroxidació lipídica i la producció de senyals dels derivats lipídics podrien tenir un possible rol en defensa. Tant el JA com el MDA en fulles tenen un paper en protecció modulant gens relacionats amb estressos tant biòtics com abiòtics (Weber *et al.*, 2004; Müller *et al.*, 2008). En els últims anys, gràcies als estudis amb mutants, s'ha pogut establir una connexió entre la síntesi d'oxilipines derivades de la peroxidació lipídica i la seva possible funció en

fotoprotecció (Demmig-Adams *et al.*, 2013), el que podria explicar l'augment dels nivells de MDA i JA principalment en gemmes obertes. Una major exposició a la llum en aquestes gemmes en les quals els comencen a sortir les fulles joves en desenvolupament podria induir un augment de MDA i JA com a mecanisme de fotoprotecció. Amb referència a aquest fet, també s'ha de considerar que no només els senyals derivats de la peroxidació lipídica incrementen en arbres moribunds, sinó que també ho fan els nivells de l'àcid 1-aminociclopropà-1-carboxílic (ACC), el precursor de l'ET. Aquests resultats mostren que els senyals procedents de la peroxidació lipídica podrien actuar juntament amb l'ET per protegir les branques epicòrmiques dels arbres moribunds.

Els resultats del **capítol 3** suggereixen que els arbres moribunds presenten una edat fisiològica diferent, més avançada si considerem que presenten major estrès oxidatiu, i que podrien ser més sensibles als estressos que els arbres sans. Aquesta edat fisiològica en els arbres moribunds no va lligada a un increment del dany sinó a l'activació de vàries respostes de defensa a nivell bioquímic que poden ajudar a les branques epicòrmiques a que les fulles desenvolupades siguin del tot funcionals. La mesura de l'estrès oxidatiu ens mostra que aquests arbres són capaços de desenvolupar mecanismes per evitar el possible deteriorament fisiològic, contemplant els canvis associats a l'edat com un possible fenomen associat a la plasticitat fenotípica del món vegetal i no necessàriament com un dany o degeneració.

3. Estrès oxidatiu i sexe

El major efecte negatiu en les femelles de plantes dioiques en ser exposades a estressos ambientals, causat per un major esforç reproductiu, està extensament acceptat. En varis estudis s'ha observat que sota condicions d'estrès els mascles tendeixen a ser més resistents i presentar un ús de l'aigua més conservador que les femelles (Dawson & Ehleringer, 1993; Gehring & Monson, 1994; Ward *et al.*, 2002; Dawson *et al.*, 2004; Rozas *et al.*, 2009). No obstant, l'existència d'altres estudis en que es mostren resultats oposats, posen en dubte aquesta afirmació àmpliament assumida. Correia & Barradas (2000), a l'investigar plantes de *Pistacia lentiscus* en condicions d'estrès durant l'estiu, varen observar una major eficiència en l'ús de l'aigua en les femelles; així mateix, Montesinos *et al.* (2012) no varen advertir diferències degudes al gènere a nivell d'intercanvi de gasos en plantes de *Juniperus thurifera*. Els anàlisis fets per Dudley (2006) i Dudley & Galen (2007) en *Salix glauca*, tot i presentar una lleugera diferència entre sexes, no aportaren resultats clarament significatius. Per altra banda, també s'ha considerat que els costos de reproducció es reflecteixen en una menor supervivència de les femelles en hàbitats sotmesos a condicions adverses reflectint un biaix entre sexes (Dawson & Ehleringer, 1993; Groen *et al.*, 2010). Ara bé, existeixen casos en els quals aquest biaix no s'han trobat (Verdú & García-Fayos, 1998; Soldaat *et al.*, 2000) o fins i tot en els que són les femelles les que hi predominen (Barradas & Correia, 1999). En el cas de l'estudi amb l'espècie *P. lentiscus* dut a terme en el **capítol 4**, no es varen observar diferències significatives degudes al sexe al llarg de l'any pel que fa a l'eficiència fotosintètica, a els nivells de pigments fotosintètics o al seu contingut hídric que ens permetessin afirmar amb contundència un efecte negatiu de l'esforç reproductiu en femelles. En definitiva, és difícil generalitzar sobre els costos de reproducció i el seu efecte en plantes femenines a causa de la gran diversitat de resultats, no només entre plantes herbàcies i llenyoses (Obeso, 2002), sinó també entre espècies.

Sota condicions ambientals adverses les plantes experimenten un augment de l'estrès oxidatiu. Tot i així, la majoria d'estudis en que s'investiguen les diferències entre sexes en circumstàncies d'estrès no utilitzen mesures d'estrès oxidatiu, sinó que es centren en mesures d'intercanvi de gasos i relacions hídriques. Altrament, el grup del Dr. Li de Nanjing (Xina) ha aportat un gran nombre de publicacions durant els últims anys referents a diferències en estrès oxidatiu degudes al sexe en situacions d'estrès per sequera (Zhang *et al.*, 2010, 2012a; Han *et al.*, 2013), baixes temperatures (Zhang *et al.*, 2011, 2012b), excés de manganès i zinc (Chen *et al.*, 2013; Jiang *et al.*, 2013), CO_2 elevat (Li *et al.*, 2013); radiació per llum ultraviolada-B (Xu *et al.*, 2010), salinitat (Chen *et al.*, 2010a), fins i tot combinació d'estressos (Chen *et al.*, 2010b). Tots ells conclouen que les femelles són menys resistents que els mascles i presenten un major estrès oxidatiu quan es troben en condicions ambientals desfavorables. De tota manera, és important remarcar que la homogeneïtat de resultats en tots aquests estudis podria ser deguda tant sols a l'efecte del gènere, ja que tots els experiments s'han realitzat en plantes de *Populus*.

En el **capítol 4** es varen analitzar diferents mesures d'estrès oxidatiu en les plantes de *P. lentiscus* per observar si diferien segons el sexe al llarg de l'any. Les variacions estacionals en la peroxidació lipídica no varen revelar diferències entre sexes durant els mesos de primavera o estiu, època caracteritzada en el clima mediterrani per una exposició a un excés d'intensitat lumínica i escasses precipitacions. Només es varen observar diferències en els mesos d'hivern, quan les plantes varen estar sotmeses a temperatures subòptimes i les diferències degudes a l'esforç reproductiu eren majors, ja que les femelles estaven en època de desenvolupament dels fruits mentre que els mascles ja havien perdut totes les seves inflorescències (Milla *et al.*, 2005). Els resultats concorden amb altres estudis en que s'ha demostrat prèviament que aquesta espècie és molt resistent a la sequera però, per contra, sembla ser força sensible a l'estrès per fred (Palacio *et al.*, 2005; Varone & Gratani, 2007) i, per tant, no és

d'estranyar que les majors diferències entre sexes s'observin durant les condicions climàtiques més adverses per la planta.

Un estudi més detallat durant els mesos d'hivern va determinar que l'increment de la peroxidació lipídica en femelles estava relacionat amb uns nivells més baixos d'α-tocoferol, però no d'altres antioxidants com els antocians o els carotenoides. El tocoferol és un antioxidant clau en evitar la propagació de la peroxidació lipídica i en l'eliminació química del $^1O_2$ mitjançant la seva pròpia oxidació (Munné-Bosch & Alegre, 2002a; Rastogi *et al.*, 2014). Així mateix, els nivells d'oxidació de l'α-tocoferol majors en femelles suggerien una major acumulació de ROS en aquestes. A més a més, els valors de NPQ (de l'anglès "*non-photochemical quenching*") indicaven una menor taxa de dissipació tèrmica de l'excés d'energia en les femelles en comparació als mascles. Tots aquests resultats apuntaven a una capacitat de fotoprotecció menor en femelles. Per contra, això no va afectar negativament l'eficiència màxima del PSII, que presentava valors per sobre de 0.75 en ambdós sexes (Björkman & Demmig, 1987), ni tampoc es varen observar diferències en les taxes d'assimilació de CO_2 ni en el contingut hídric respecte els mascles. Així doncs, les diferències relacionades al sexes entre plantes de llentiscle no eren degudes a un major dany en les femelles sinó possiblement a l'ús de mecanismes de fotoprotecció diferents a els dels mascles.

Mesures durant el dia dels nivells de MDA varen indicar que la peroxidació lipídica en femelles no tant sols era major que la dels mascles al migdia sinó també abans de l'alba, coincidint amb uns nivells d'oxidació d'α-tocoferol més alts. L'oxidació d'aquest antioxidant durant la nit no podia estar causada pel $^1O_2$, ja que aquesta ROS es genera en condicions de fotoinhibició. Per contra, en condicions *in vitro* s'ha observat que l'α-tocoferol pot cooxidar-se amb els àcids grassos mitjançant l'activitat de la LOX (Hakansson & Jagerstad, 1990). L'anàlisi dels nivells de LOX en les plantes de *P. lentiscus* va suggerir que tant els nivells d'oxidació del

tocoferol com de peroxidació lipídica majors en femelles respecte mascles abans de l'alba podien ser causats per un augment de l'activitat de la LOX. En estudiar els nivells endògens d'hormones es va advertir que els nivells de LOX més alts en femelles estaven associats a uns nivells d'isopentenil adenosina (IPA) més baixos. Les citocinines, l'IPA entre elles, són hormones que regulen la divisió cel·lular i la capacitat de l'òrgan per actuar com a embornal per als fotoassimilats (Roitsch & Ehneβ, 2000). Per aquest motiu els resultats suggereixen que la reducció d'IPA en femelles abans de l'alba podria estar associat a una limitació de la seva capacitat com a embornal, el que al seu torn s'ha demostrat que pot induir l'acció de la LOX (Fischer *et al.*, 1999).

Per tant, sembla ser que els majors nivells de peroxidació en femelles en comparació amb els mascles al migdia es dóna independentment de l'activitat LOX i, per tant, deu estar causat per una reducció en fotoprotecció. Per altra banda, sembla que una limitació de la capacitat d'embornal deguda a una reducció de citocinines abans de l'alba es tradueix en un increment de l'activitat LOX i l'acumulació de senyals derivats de lípids. Mitjançant aquesta senyalització les femelles de llentiscle són capaces d'utilitzar mètodes de compensació per evitar el dany en l'exposició a estressos ambientals. En aquesta espècie ja s'han descrit altres mecanismes de compensació en les femelles com l'increment de la longevitat foliar (Jonasson *et al.*, 1997) o l'avortament de les seves estructures reproductives depenent de les condicions ambientals (Jordano, 1988, 1989).

Les diferències entre sexes de plantes dioiques en aspectes fisiològics causades per un possible esforç reproductiu major per part de les femelles, generalment s'interpreta com un desavantatge per aquestes. En aquest estudi però, es demostra que l'increment de l'estrès oxidatiu en femelles pot estar induït tant per fotoinhibició com a nivell enzimàtic, i que aquest no es tradueix en un dany en cap cas, sinó en la utilització de mecanismes diferents als dels mascles mitjançant una regulació interna de la planta.

Atès que els resultats en el **capítol 4** no demostren que les femelles estiguin desfavorides, sinó que tant sols utilitzen mecanismes de protecció diferent, aquests resultats estan en contraposició a tot el descrit fins ara, principalment pel grup del Dr. Li respecte a l'estrès oxidatiu. Així doncs, en aquesta tesi es posa de manifest que l'espècie podria ser un factor determinant en els mecanismes de compensació entre mascles i femelles i que, per tant, són necessaris els estudis amb altres espècies vegetals per tal de ser capaços d'obtenir-ne una resposta més contundent.

Tot i que l'espècie sembla ésser un factor molt important en l'efecte de l'esforç reproductiu entre mascles i femelles com s'ha pogut observar, val a dir que, en el cas dels estudis realitzats amb *P. lentiscus* també s'han obtingut resultats discrepants entre ells. Altres estudis realitzats també han determinat que les plantes d'aquesta espècie són propenses a la fotoinhibició durant els mesos d'hivern (Flexas *et al.*, 2001; Said *et al.*, 2013), però les diferències entre mascles i femelles es varen observar durant la primavera (Said *et al.*, 2013). Retuerto *et al.* (2000) van arribar a la conclusió que les diferències entre sexes en *Ilex aquifolium* depenien del context ambiental en el qual havien crescut les plantes, el que podria explicar les discrepàncies entre els resultats de llentiscle. Delph (1999) va assenyalar que el dimorfisme sexual sovint no s'expressa fins després que les plantes hagin experimentat diversos episodis reproductius. Atès que les plantes utilitzades en el **capítol 4** només s'havien reproduït un cop abans de l'experiment, aquest fet podria explicar perquè les diferències entre sexes només s'observaven a nivell de regulació interna sense afectar les femelles negativament com s'ha vist en altres estudis.

Alguns autors han considerat la importància del fet que les plantes estiguin formades per un conjunt de subunitats repetitives i parcialment autònomes anomenades mòduls (Vuorisalo & Tuomi, 1986; Peñuelas & Munné-Bosch, 2010). Per tant, en estudiar organismes modulars és important considerar els diferents mòduls a l'hora d'analitzar els costos de reproducció, ja que probablement els brots que sustenten les estructures

reproductives es comportin diferent als que no (Henriksson & Ruohomäki, 2000; Hasegawa & Takeda, 2001; Obeso, 2002; Oñate & Munné-Bosch, 2009). En l'estudi a nivell modular de llentiscle es varen comparar brots en els que s'hi havien format les inflorescències i posteriorment els fruits (reproductius) amb els brots que tants sols presentaven estructures vegetatives (no reproductius).

Els brots reproductius varen resultar estar més fotoprotegits que els no reproductius, no només mitjançant l'increment de dissipació d'energia (NPQ), sinó també al presentar nivells d'antioxidants com ara carotenoides i α-tocoferol per clorofil·la majors que els brots no reproductius. Aquest estudi posa de manifest la importància en l'elecció de la mostra a l'hora d'avaluar l'efecte del sexe, ja que els diferents brots es comporten diferent i, per tant, els resultats obtinguts es poden veure influenciats.

4. Les plantes perennes com a model

La gran majoria del coneixement en el camp de la fisiologia vegetal prové de la informació obtinguda a través del model de planta per excel·lència *Arabidopsis thaliana*. En els últims anys s'ha anat obtenint informació d'altres models vegetals que ajuden a incrementar el nostre camp de visió, ja que les notables diferències entre espècies mostren que un únic sistema no té perquè ser aplicable a tot el regne vegetal.

És innegable que *Arabidopsis* proporciona un gran ventall d'avantatges com a model d'estudi ja que es tracta d'una dicotiledònia de mida petita amb un cicle de vida ràpid capaç de produir un gran nombre de llavors. El seu genoma relativament petit la converteix en una planta fàcilment manipulable genèticament de la qual en podem obtenir una àmplia sèrie de mutants. De tota manera, no seria d'estranyar que estudis realitzats amb espècies que presenten estratègies i adaptacions diferents revelin controvèrsies amb els resultats obtinguts en *Arabidopsis* que fins a dia d'avui es consideren quasi com a absoluts. Al llarg del desenvolupament d'aquesta tesi, l'estudi de la senescència i el creixement foliar en la planta

perenne *P. lentiscus* ens va proporcionar dos casos útils a l'hora d'exemplificar aquest fenomen.

Al llarg dels anys s'ha intentat revelar i entendre la intricada regulació de la senescència foliar. Molts estudis han arribat a la conclusió que les hormones en són un factor clau i s'ha demostrat que l'ABA, el JA i el SA poden actuar com a promotors d'aquest procés, tant si està induït pel desenvolupament com si ho està per estressos ambientals (He *et al.* 2002; Buchanan-Wollaston *et al.* 2005; Abreu & Munné-Bosch, 2009; Lee *et al.* 2011). De tota manera, el nostre coneixement actual de la senescència prové majoritàriament de plantes anuals o d'altres models com el tabac o cultius com l'arròs (Lim *et al.*, 2007; Balazadeh *et al.*, 2008; Gregersen *et al.*, 2013; Zhang & Zhou, 2013), ja que en plantes perennes no ha estat gaire estudiada (Munné-Bosch, 2008). De tal manera que, al realitzar estudis amb espècies perennes, ens podem trobar amb el cas d'obtenir resultats heterogenis als predits en els models anteriors. En el **capítol 1**, l'anàlisi de les etapes finals de la senescència foliar en fulles de llentiscle va mostra que cap de les hormones descrites com a inductores del procés varen incrementar els seus nivells durant la senescència, suggerint una possible regulació per altres factors en el cas d'aquesta espècie.

Un altre exemple de disparitat entre resultats és el cas de les formes de citocinines actives en el creixement foliar. Les citocinines són derivats d'adenina que estan presents en diferents formes actives, essent les dels tipus trans-Z i isopentenil adenina les predominants en plantes. D'aquestes però, la trans-Z està generalment considerada com a la forma activa majoritària (Sakakibara, 2006; Frébort *et al.*, 2011; Hwang *et al.*, 2012). Per contra, els elevats nivells de citocinines tipus isopentenil adenina, com el 2iP i IPA, durant l'etapa de màxim creixement foliar de llentiscle en el **capítol 2** fa pensar que aquestes en són les formes més actives en aquesta espècie.

Un altre factor possiblement implicat en l'observació d'aquestes diferències són les condicions en les que es duen a terme els experiments. La majoria dels estudis amb *Arabidopsis* estan realitzats en cambres o sota condicions ambientals molt específiques, mentre que en els estudis anteriors les plantes es trobaven en condicions naturals en les quals és impossible controlar totes les variables implicades, però que alhora aporten informació per a una millor comprensió de l'ecofisiologia d'aquestes espècies. Val a dir però, que actualment els estudis d'ecofisiologia en varietats d'*Arabidopsis* està incrementant donada la seva importància (per exemple: Warner & Erwin, 2005; Flood *et al.*, 2014; Kusakina *et al.*, 2014).

Fins al dia d'avui la investigació en plantes models com *Arabidopsis* ha estat essencial en la investigació i coneixement del desenvolupament vegetal. De tota manera, altres models ens poden donar la oportunitat d'estudiar processos que no es donen en herbàcies com per exemple els canvis estacionals, la formació d'escorça, el temps de floració, etc., així com la possibilitat de comparar espècies per tal de poder arribar a entendre la complexitat fenotípica de les plantes superiors, que es faria menys evident donat un sol model. Una de les barreres més importants a l'hora de donar el pas a la utilització de nous models de plantes perennes és l'evident limitació en la realització d'anàlisis a nivell genètic i molecular en aquestes espècies, camp en el qual *Arabidopsis* n'és l'espècie per excel·lència. En els últims anys però s'han realitzat grans avenços en aquest aspecte, com ha estat per exemple el coneixement de la seqüència del genoma complet en *Populus trichocarpa* i el desenvolupament de vàries eines genètiques en aquest mateix gènere (Sterky *et al.*, 2004; Tuskan *et al.*, 2006) que ha estat proposat com nou model d'investigació en biologia vegetal (Jansson & Douglas, 2007). Un altre exemple n'és l'ús creixent d'espècies perennes com *Arabis alpina*. *A. alpina* és una espècie policàrpica de la família de les Brassicaceae de fulles perennes i de vida curta, estretament relacionada amb *Arabidopsis*, que presenta característiques favorables per al seu ús com a planta perenne model, com ara ser diploide i auto-fèrtil, amb un

genoma relativament petit i susceptible a la transformació per *Agrobacterium tumefaciens* per tal d'obtenir-ne mutants (Poncet *et al.*, 2010; Wang *et al.*, 2011; Wingler *et al.*, 2012).

Així doncs, la utilització d'espècies perennes en la recerca de la biologia vegetal suposarà l'expansió del coneixement actual a un nou camp de visió molt més ampli, donada la seva gran importància dins el món vegetal, i encara avui molt desconegut. La creixent rellevància de nous models d'investigació s'ha fet palesa en l'increment del nombre d'estudis realitzats en plantes perennes, i seguirà en augment donat que la biologia d'*Arabidopsis* està pràcticament arribant a la seva total comprensió.

Conclusions

CONCLUSIONS

- L'edat cronològica, l'edat fisiològica i l'estrès oxidatiu no sempre segueixen una correlació positiva, ja que, depenent de l'edat de la fulla o la planta, l'augment de l'estrès oxidatiu no està relacionat amb un dany oxidatiu, sinó que pot estar implicat en processos de senyalització.

- La consideració conjunta de l'edat cronològica i els nivells d'estrès oxidatiu és un bon indicador de l'edat fisiològica, tant a nivell de fulla com de planta sencera.

- Les fulles presenten estrès oxidatiu en els dos extrems del seu desenvolupament, acomplint dues funcions ben diferents. L'estrès oxidatiu en fulles emergents és transitori, amb un paper possiblement de senyalització en protecció contra estressos; mentre que en fulles en etapes avançades de senescència pot arribar a conduir a la mort cel·lular.

- Els arbres moribunds de faigs no presentaven tant sols una edat cronològica superior sinó també una edat fisiològica més avançada, com ho demostra un increment dels marcadors d'estrès oxidatiu. No obstant, l'augment d'àcid malondialdehid en les gemmes d'aquests arbres podria estar relacionat amb un possible efecte protector més que amb un procés degeneratiu.

- Una peroxidació lipídica superior en femelles respecte mascles de *P. lentiscus* durant l'hivern no afecta negativament a les femelles, sinó que reflecteixen uns mecanismes de fotoprotecció i regulació interna diferents. L'increment dels nivells de lipoxigenasa probablement

causats per una limitació de la capacitat embornal (deguda a una reducció de citocinines), paral·lels a l'augment d'àcid malondialdehid en femelles, suggereix que aquest últim pot tenir un possible rol en senyalització.

- L'estudi a nivell modular en *P. lentiscus* revela que els brots reproductius estan més fotoprotegits que els no reproductius, com indiquen els nivells d'antioxidants i la dissipació d'excés d'energia en forma de calor, emfatitzant la importància de la diferenciació entre mòduls en l'estudi de les diferències entre sexes en plantes dioiques.

ANNEX

Estrès fotooxidatiu en fulles emergents i senescents: una imatge en un mirall?

ANNEX

Photo-oxidative stress in emerging and senescing leaves: a mirror image?

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RESUM DE L'ANNEX

El cicle de vida d'una fulla pot caracteritzar-se mitjançant diferents etapes: des de l'inici del primordi foliar en el meristema apical del brot fins a la senescència. El desenvolupament foliar, des de l'inici del creixement fins a la senescència, està altament regulat pel desenvolupament de la planta i l'ambient. Aquí, per començar ens centrem en fer un recull d'evidències actuals que indiquen que l'estrès fotooxidatiu es dóna en els dos extrems de la vida foliar. Estudis recents indiquen clarament que, com succeeix en fulles senescents, les fulles emergents pateixen un estrès fotooxidatiu, el que suggereix que aquest estrès oxidatiu té un paper clau en els dos extrems del cicle de vida foliar. També es discuteixen les causes i conseqüències de patir estrès oxidatiu al llarg del desenvolupament de la fulla. Les evidències actuals mostren mecanismes que mantenen un balanç cel·lular adequat entre les espècies reactives de l'oxigen i els antioxidants. Aquest balanç és especialment important ja que permet el creixement i preveu el dany oxidatiu en les fulles joves que estan emergint, mentre que en etapes més avançades, l'estrès fotooxidatiu indueix la senescència i la mort cel·lular de les fulles. També és rellevant el fet que les reduccions en l'eficiència fotoquímica del fotosistema II no necessàriament indica estrès fotooxidatiu en fulles emergents. En aquest article de revisió es resumeix el coneixement actual en fotoinhibició, fotoprotecció i estrès fotooxidatiu en els dos extrems del cicle de vida d'una fulla: inici del creixement de la fulla i senescència foliar.

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REVIEW PAPER

Photo-oxidative stress in emerging and senescing leaves: a mirror image?

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Abstract

The life cycle of a leaf can be characterized as consisting of different stages: from primordial leaf initiation in the shoot apical meristem (SAM) to leaf senescence. Leaf development, from early leaf growth to senescence, is tightly controlled by plant development and the environment. Here, we primarily focus on summarizing current evidence indicating that photo-oxidative stress occurs at the two extremes of a leaf's lifespan. Some recent studies clearly indicate that—as happens in senescing leaves—emerging new leaves suffer from photo-oxidative stress, which suggests that oxidative stress plays a key role at both ends of the leaf life cycle. We discuss the causes and consequences of suffering from photo-oxidative stress during leaf development, paying attention to the particularities of this process at the two extremes of leaf development. Of particular importance is the current evidence showing mechanisms that maintain an adequate cellular reactive oxygen species/antioxidant (redox) balance that allows growth and prevents oxidative damage in young emerging leaves, while later on photo-oxidative stress induces cell death in senescing leaves. Also of interest is the fact that reductions in the efficiency of photosystem II photochemistry may not necessarily indicate photo-oxidative stress in emerging leaves. In this review, we summarize current knowledge of photoinhibition, photoprotection, and photo-oxidative stress at the two ends of the leaf life cycle: early leaf growth and leaf senescence.

Key words: Antioxidants, leaf growth, leaf senescence, oxidative stress, photoinhibition, photoprotection, redox regulation

Introduction

Leaf development is extremely plastic. Not only are the shape and complexity of leaves extremely varied between species, but they also are within an individual plant. Depending on the developmental stage and growth conditions, there can also be pronounced variations in leaf shape, size, and longevity. All leaves within an individual compete for light, water, and nutrients; therefore, their growth is in harmony with the architecture of the plant, which at the same time determines the life history traits and longevity of each leaf. All leaves will be exposed to a specific micro-environment and certain developmental pressures—such as interindividual and intershoot competition for resources—that will determine their development.

The life cycle of a leaf can be divided into different stages. It starts with primordial initiation in the shoot apical meristem (SAM), followed by the primary morphogenesis stage—when cell division/proliferation occurs—and a secondary morphogenesis characterized by cell expansion (Donnelly *et al.*, 1999). Leaf initiation and early leaf growth (including both cell division and expansion) are tightly controlled by endogenous factors that include hormones, reactive oxygen species (ROS), sugars, and other regulators that converge on the regulation of transcription factors and gene expression (Werner and Schmülling, 2009; Traas and Monéger, 2010). These early stages are also subject to modulation by

Abbreviations: APX, ascorbate peroxidase; ELIP, early light-induced protein; NPQ, non-photochemical quenching; PCD, programmed cell death; PS, photosystem; ROS, reactive oxygen species; SAG, senescence-associated gene, SAM, shoot apical meristem.

environmental factors such as light, water, and nutrient availability, and temperature, which allow the plant to optimize growth according to its environmental conditions (Yoshida *et al*., 2011).

During the initiation stage, leaf primordia are considered to be a heterotrophic organ as the apparatus for photosynthesis has not yet been developed and all the photoassimilates and nutrients needed for leaf development come from other organs. As its ontogenetic development proceeds, the leaf is formed and gradually becomes autotrophic. At this stage, although emerging leaves are already photosynthetically active, they still depend on imported photoassimilates for growth. Some time is needed before the leaf switches from being a carbon sink to a carbon source (Turgeon, 1989). The leaf then grows until it reaches a certain size and shape. It is generally assumed that a leaf is mature when it has expanded to its full leaf area. At this stage, the leaf has become a net carbon exporter (Fig. 1).

Senescence is considered to be the last stage in leaf development. Highly regulated changes at the molecular, cellular, biochemical, and physiological levels cause the leaf to die. Before dying, however, the leaf fulfils an essential function during senescence: nutrient remobilization, including not only mobilizing essential nutrients for the plant—such as nitrogen—but also providing substantial amounts of carbon, mainly in the form of sucrose, for other plant parts (leaf primordia, emerging new leaves, reproductive organs, or storage organs). Leaf senescence is regulated by the same endogenous and environmental factors as is early leaf growth (Fig. 2). Thus, ROS also play an essential role in the regulation of the process in concert with other endogenous regulators—such as hormones—and act as signalling molecules that translate information from environmental cues (Munné-Bosch *et al*., 2013). Senescence initiation proceeds through a series of signalling cascades that lead to changes in gene expression of the so-called senescence-associated genes (SAGs). As a result, re-organization starts and catabolic activities increase, causing the leaf to undergo a source–sink transition, whereby part of the photoassimilates and nutrients that the leaf had been accumulating during its development are remobilized and distributed to other parts of the plant. After nutrient remobilization is complete, leaf senescence eventually results in the death of the organ, which may be followed or not—depending on the species and conditions—by abscission (Lim *et al*., 2007; Fig. 2).

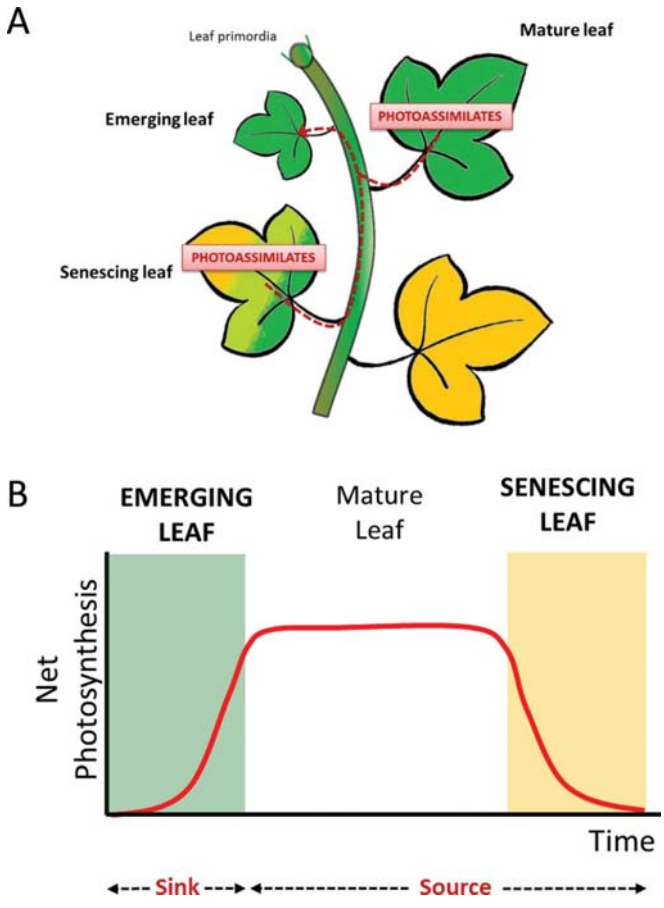


Fig. 1. Interplay between emerging and senescing leaves (A) and comparison of photosynthetic activity in emerging and senescing leaves (B).

In this review, we focus on the role of photo-oxidative stress both at early stages of leaf growth and during leaf senescence. We consider early leaf growth to be the stages at which the leaf is photosynthetically active but still depends on imported photoassimilates for net growth. During leaf senescence, we focus on the processes involved in the initiation, re-organization, and terminal phases. Furthermore, we aim at characterizing such processes beyond the information gathered in the model plant *Arabidopsis thaliana* and include recent literature on the topic obtained in perennials. Early leaf growth and senescence differ greatly between annual and perennial plants, as does the growth strategy adopted. The leaves of annual plants have a shorter lifespan and senescence is usually induced by reproduction, although environmental factors can undoubtedly modulate the process. In these plants, all the life history traits will ultimately be focused on rapidly producing seeds. The leaves of perennial plants generally live longer and their senescence is generally modulated more strongly by environmental cues, rather than by reproduction; although flower and fruit production can also play a major role. The perennial strategy is also effective at producing seeds; not by using different plant generations as in annual plants, but by the same individual producing seeds for long periods of time. Since the plant produces new shoots every spring, the energy stored in the plant parts that have survived through the winter will be successfully used for growth and reproduction. In trees, for example, structure can be built up year after year so that the tree becomes larger and capable of producing more fruit and seed than the previous year, out-competing other plants for light, water, nutrients, and space (Iwasa and Cohen, 1989). We summarize here recent studies that clearly indicate that new, emerging leaves show transient photo-oxidative stress, exactly as happens during senescence. However, oxidative damage and death occur during the terminal phase of senescence. Thus, we focus here on the particularities of photo-oxidative stress

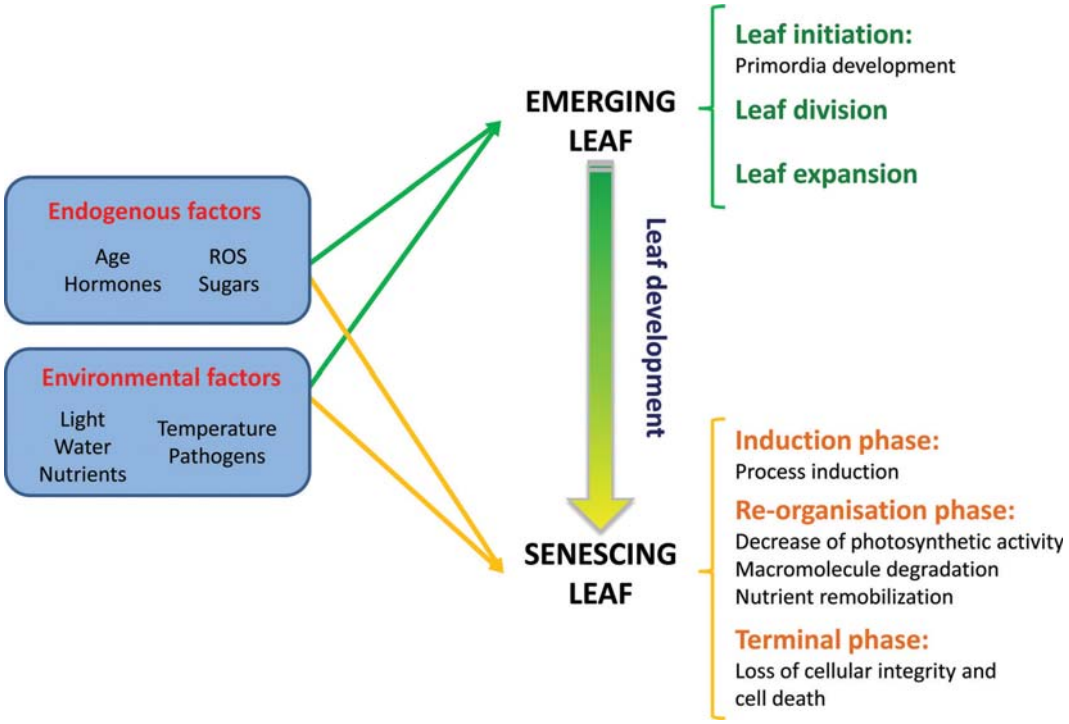


Fig. 2. Factors modulating leaf growth and senescence in plants.

at the two extremes of the life cycle of leaves, and discuss the differences that lead to different outcomes; all particularly focused on photoinhibition, photoprotection, and photo-oxidative stress.

Photoinhibition and photoprotection in emerging leaves

Light plays a dual role in the regulation of leaf growth (Fig. 3). On one hand, it plays a major role in photomorphogenesis via the induction of phytochrome function and signalling; while on the other, light energy is used for photosynthesis. Both processes (photomorphogenesis and photosynthesis) can, however, converge in the regulation of leaf growth by modulating ROS production in a complex manner. Stirnberg *et al*. (2012) have shown that *Arabidopsis* mutants with a defect in a gene encoding the transposase-related transcription factor FAR-RED ELONGATED HYPOCOTYL3 (FHY3), which is known to be involved in the far-red light-dependent regulation of seedling de-etiolation, present **oxidative stress**-related phenotypes showing retarded **leaf growth** and cell death. Interestingly, this phenotype was found to require the *AUXIN-RESISTANT1* gene, and turned out to be independent of phytochrome A (phyA). In another study, Danon *et al*. (2006) showed that cryptochrome 1 (cry1) mediates singlet oxygen-induced programmed cell death (PCD). Unexpectedly, the light-dependent release of singlet oxygen alone was not sufficient to induce PCD of the conditional fluorescent (*flu*) mutant of *Arabidopsis*, but had to act in concert with a second concurrent blue light reaction mediated by cry1. Taken together, these results indicate that photoreceptor function may be finely modulated during plant development to prevent oxidative damage and cell death during leaf initiation and early leaf growth.

Furthermore, emerging new leaves are sensitive to many environmental insults since their photosynthetic apparatus is not fully developed. During the early stages of leaf development, the photosynthetic rate gradually increases, as does stomatal conductance. The photosynthetic apparatus is under construction, so low CO_2 assimilation rates occur concomitantly with stomatal closure during early stages of leaf growth (Freeland, 1952; Choinski and Johnson, 1993; Greer and Halligan, 2001). Consequently, since chloroplasts may be exposed to excess excitation energy, photoinhibition can occur (Fig. 4). Indeed, some studies have reported that the F_v/F_m ratio, which is an indicator of the maximum efficiency of photosystem II (PSII) photochemistry, is lower in young expanding leaves than in fully expanded leaves (Jiang *et al*., 2005; Maayan *et al*., 2008; Lepedus *et al*., 2011), thereby confirming that while the photosynthetic machinery is being formed leaves are very sensitive to photoinhibition. This photoinhibition can result from immature chloroplasts and therefore photosynthetic apparatus, leading to a reduced efficiency of PSII photochemistry, or to transient ROS accumulation in chloroplasts.

It is well known that excess light can trigger the production of ROS via the photosynthetic electron transport chain (Asada, 2006). In those conditions, a superoxide anion (O^{-}_{2}) is produced as a consequence of the direct reduction of oxygen by ferredoxin in the Mehler reaction in PSI. However, the interaction between oxygen and a chlorophyll molecule in the

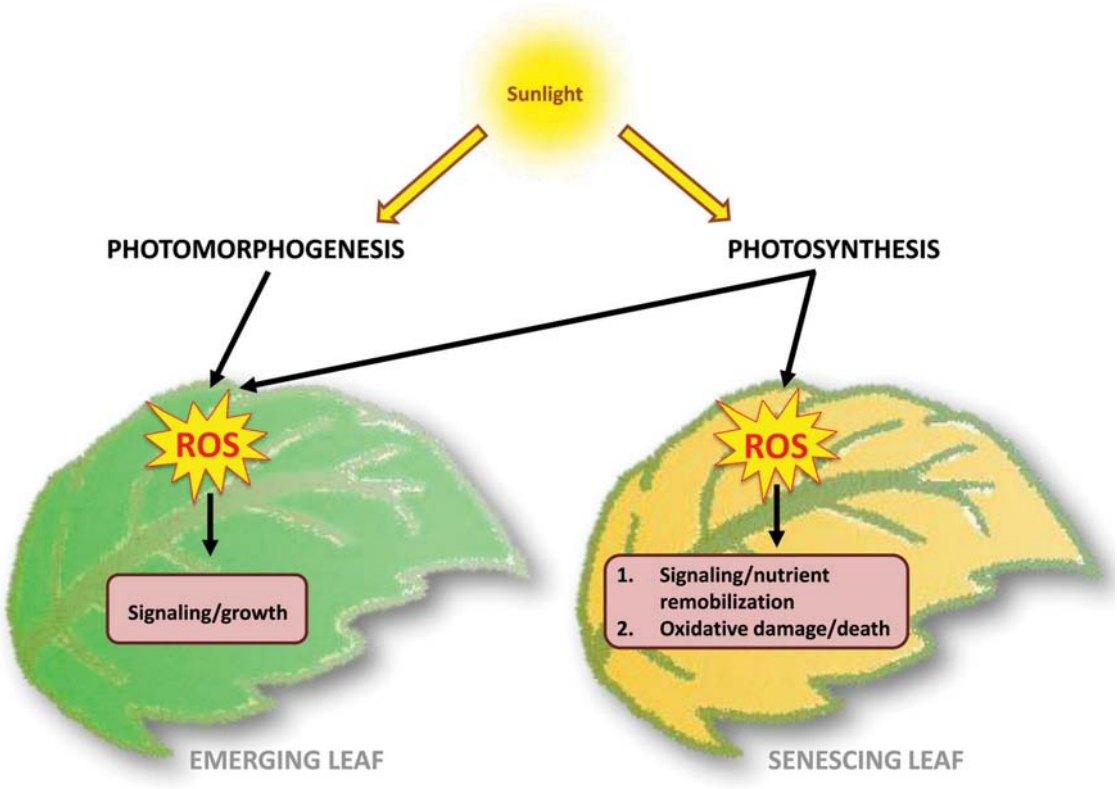
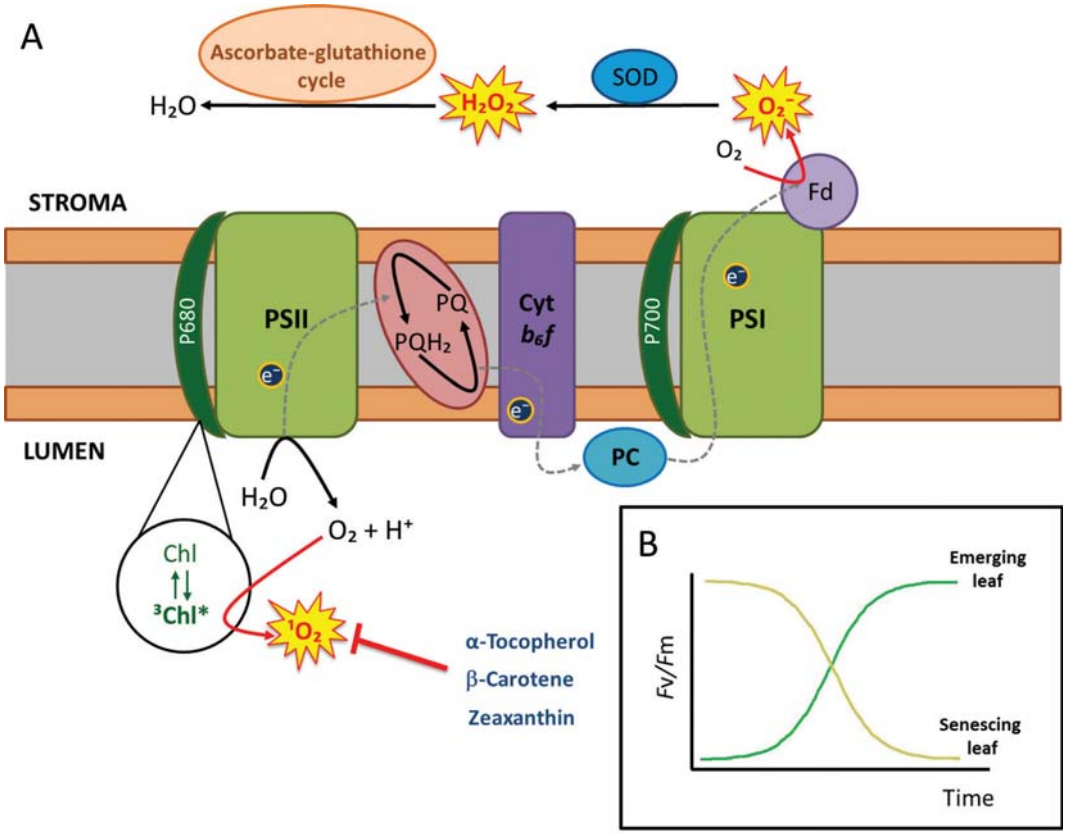


Fig. 3. Reactive oxygen species (ROS) play multiple roles depending on the developmental stage of the leaves.

triplet state (^{3}Chl*) can generate the ROS that is generally the most damaging in chloroplasts: singlet oxygen ($^1O_2$; Fig. 4). ROS generated in chloroplasts can react and oxidize a large number of molecules, including photosynthetic pigments, sugars, lipids, nucleic acids, and several proteins—such as the D_1 protein subunit of PSII, which causes photoinhibition (Takahashi and Badger, 2011). This inhibition of photosynthesis—which is reflected in reductions in the F_v/F_m ratio in young expanding leaves—will, however, not result in irreversible damage to the photosynthetic apparatus; so efficient repair of the D_1 protein is expected. D_1 protein levels measured during the first stages of leaf growth revealed a small amount of the protein, which increased while the young leaf expanded, to achieve a fully functional PSII (Maayan *et al.*, 2008; Lepedus *et al.*, 2011). Another common indicator of whether young leaves suffer photo-oxidative stress is bulk protein and lipid oxidation caused by ROS, which are measured as levels of total protein carbonyl and malondialdehyde (a lipid oxidation product), respectively. Lepedus *et al.* (2011) found that the development of the photosynthetic apparatus in expanding leaves of Norway maple (*Acer platanoides* L.) correlated negatively with the levels of protein carbonyls and positively with lipid peroxidation; that is, emerging leaves showed increased protein, but not increased lipid oxidation compared with fully expanded leaves. However, other studies have shown a negative correlation between leaf development and levels of malondialdehyde (Juvany *et al.*, 2012), thus suggesting that oxidative stress occurs in emerging leaves, but that the oxidative target may differ between species and/or growth conditions. In addition to the fact that the photosynthetic apparatus is under construction and is therefore more susceptible to photoinhibition, emerging leaves are highly susceptible to photodamage because they normally initiate at the top of a branch where they receive more direct sunlight than expanded leaves further down. Unfortunately, however, to our knowledge, no studies have been carried out to disentangle the effects of an immature photosynthetic apparatus from those of the conditions expanding leaves are exposed to. Indeed, such studies might prove difficult since different climatic conditions will in turn affect growth rates and therefore mask the results.

In any case, compelling evidence clearly indicates that it is vital that young emerging leaves have good photoprotection mechanisms. Otherwise, photodamage (understood as irreversible damage to the photosynthetic apparatus) would inhibit photosynthesis and this would cause a reduction in growth and productivity that would affect either the renewal of leaves in perennials or the chance of survival in annual plants. Emerging leaves construct the new photosynthetic apparatus progressively, thereby avoiding excess energy in newly formed chloroplasts. The levels of chlorophyll therefore increase steadily during leaf expansion (Jiang *et al.*, 2005; Maayan *et al.*, 2008; Lepedus *et al.*, 2011). Although the lack of efficient photosynthetic machinery means that emerging new leaves may be exposed to considerable photoinhibition, low amounts of chlorophyll limit light absorption and therefore potential photodamage. However, it is clear from the results discussed previously that emerging



C

Photoprotective mechanisms	EMERGING LEAF		SENESCING LEAF	
	Leaf initiation	Leaf expansion	Remobilization phase	Terminal phase
Xanthophyll cycle pool	↓	↑	↑	↓
α-Tocopherol	↓	↑	↑	↓
β-Carotene	↓	↑	↑	↓
Antioxidant enzymes (APX)	↓	↑	↑	↓

Fig. 4. Formation of reactive oxygen species (ROS) in chloroplasts (A), photoinhibition at the two extremes of the leaf lifespan (B), and summary of the photoprotection mechanisms operating in emerging and senescing leaves (C).

leaves need additional mechanisms to prevent photodamage that could be caused by increased levels of ROS production in chloroplasts. Furthermore, as young leaves are usually exposed to higher irradiance than mature leaves, they need increased photoprotective ability. One of the most important mechanisms employed against high irradiance is the xanthophyll cycle-dependent dissipation of excess energy as heat. Non-photochemical quenching (NPQ) of chlorophyll fluorescence is most extensive at the very early stages of leaf maturation (Jiang *et al.*, 2005; Lepedus *et al.*, 2011), and young, expanding leaves have relatively larger xanthophyll pools and a greater de-epoxidation state of the xanthophyll

cycle (DPS) than fully expanded leaves (Jiang *et al*., 2006), which suggests a protective role against excesses of light. A greater DPS and high NPQ values have been correlated with an increased thermal dissipation of excess energy in chloroplasts and therefore increased photoprotection capacity in several species (Demmig-Adams and Adams, 1992). Recently it has additionally been shown that xanthophylls are essential during leaf growth not only as a photoprotection mechanism but also because they are involved in PSI biogenesis (Dall'Osto *et al*., 2013). α-Tocopherol has antioxidant and photoprotective effects in leaves. It works by preventing lipid peroxidation and scavenging ROS, mainly neutralizing $^1O_2$ (Kruk *et al*., 2005). However, it does not seem to play a role in protection against excessive light in young leaves, as they present low levels of α-tocopherol (Lepedus *et al*., 2011; Juvany *et al*., 2012). Szymanska and Kruk (2008) reported high levels of γ-tocopherol in young leaves of runner bean (*Phaseolus coccineus*) that decreased with leaf age, in contrast to α-tocopherol. Their study suggests that γ-tocopherol may play a role as an antioxidant, replacing the low levels of α-tocopherol.

Furthermore, a red transitional coloration has been detected in young leaves in some species, indicating the accumulation of anthocyanins (Choinski and Wise, 1999; Juvany *et al*., 2012). Liakopoulos *et al*. (2006) found that anthocyanins in the epidermis of young grapevine (*Vitis vinifera*) leaves decreased the risk of photoinhibition. It has been suggested that anthocyanins may play a photoprotective role by modifying the quantity of incident light on chloroplasts (Steyn *et al*., 2002; Manetas *et al*., 2003; Hughes *et al*., 2005). This could indicate that some species accumulate anthocyanins in emerging new leaves as another photoprotection mechanism; although this is still controversial. Another element involved in photoprotection are the early light-induced proteins (ELIPs), which seem to be essential in light energy dissipation (Montané and Kloppstech, 2000) and in photo-oxidative stress protection (Hutin *et al*., 2003). Accumulation of these proteins has been reported under several stress conditions, such as high light stress (Zeng *et al*., 2002; Sävenstrand *et al*., 2004; Tzvetkova-Chevolleau *et al*., 2007). Maayan *et al.* (2008) and Pinto *et al*. (2011) showed that the expression of ELIPs in young leaves is correlated with low photosynthetic activity, whereas very low levels of these proteins or complete non-expression was found in mature leaves. This is consistent with an increased protective demand in emerging leaves. Finally, current ongoing research aims to identify new mechanisms of photoprotection in the chloroplasts of emerging leaves. For example, Li *et al.* (2011) have shown that ZEBRA NECROSIS, which encodes a thylakoid-bound protein of unknown function, is required to protect developing chloroplasts from ROS and excess light, especially during the assembly of thylakoid protein complexes.

At very early stages of leaf growth, low levels of ascorbate and ascorbate peroxidase (APX) activity have also been reported (Lepedus *et al*., 2011); such activity is essential for hydrogen peroxide removal (Foyer and Noctor, 2011). Low tocopherol and ascorbate levels, together with reduced APX activity suggest that there is a time window for increased ROS accumulation in emerging leaves. However, during the development of the photosynthetic apparatus in soybean (*Glycine max*), high levels of the antioxidant enzymatic protection system have also been reported, including high levels of APX (Jiang *et al*., 2005). It is possible that in this last study, the time window for reduced APX activity was not detected due to the time frame used for the sampling points. Be that as it may, it is clear that despite the multiple mechanisms involved in photoprotection, leaves suffer from photo-oxidative stress at very early stages of growth. However, this stress is rapidly buffered by the development of efficient antioxidant machinery while the leaf expands and develops.

Photoinhibition and photoprotection in senescing leaves

Leaf senescence constitutes the last developmental stage in the life of a leaf and it is characterized by highly regulated processes at the physiological, biochemical, and molecular levels. Although senescence is controlled mainly by leaf age, it can be induced by many environmental stresses, particularly those leading to excess excitation energy in chloroplasts, such as drought, excess light, heat stress, or salinity. Once senescence is induced, chloroplasts are one of the first organelles to be targeted for breakdown, while mitochondria and nuclei maintain their integrity until the terminal phase. Hence, the photosynthetic apparatus is rapidly dismantled, starting with chlorophyll degradation, a decrease in photosynthesis, and enhanced photoinhibition, which can be detected by low F_v/F_m ratios (Wingler *et al*., 2006; Abreu and Munné-Bosch, 2009).

Another sign of leaf senescence is an increase in the levels of ROS. It has been extensively reported that during the first steps of leaf senescence following chlorophyll degradation ROS increase rapidly (Smart, 1994; Buchanan *et al*., 2000). This rapid ROS production leads to pigment, protein, and lipid oxidation; oxidative processes that are needed for nutrient remobilization (Hörstensteiner and Feller, 2002). Finally, oxidative processes in combination with other mechanisms lead to leaf death (Zimmermann and Zentgraf, 2005). Therefore, oxidative stress plays multiple roles in senescing leaves; it is known to induce leaf senescence, but ROS production (such as that of singlet oxygen, one of the most potentially damaging molecules formed in chloroplasts during photo-oxidative processes; Triantaphylidès *et al*., 2008) should be controlled in both space and time in senescing chloroplasts by efficient antioxidant machinery (Munné-Bosch *et al*., 2001; Zimmerman and Zentgraf, 2005). Otherwise, the terminal phase will be induced rapidly (before nutrient remobilization occurs), and the principal function of senescence in leaves will not be fulfilled. Although senescence is usually seen as a purely degenerative process leading to death, it has a positive effect on the plant through the remobilization and recycling of nutrients to other plant parts, and may consequently be considered a survival strategy under adverse climatic conditions (Munné-Bosch and Alegre, 2004). Consequently, photoprotection of the photosynthetic apparatus plays a major

role in controlling the timing and progression of senescence; not only when senescence is initiated, but also modulating the exact period of nutrient remobilization in chloroplasts (reorganization phase) and compromising chloroplast function when these organelles are no longer needed in the terminal phase, thereby inducing cell death.

As occurs in emerging leaves, the most flexible and quantitatively important photoprotection mechanism (in terms of the amount of excess energy dissipated) in senescing leaves is the xanthophyll cycle-dependent dissipation of excess energy as heat. In senescing leaves, the chloroplasts are dismantled. This includes the degradation of the pigment antennae of PSII and PSI; therefore, the amount of photosynthetic pigments, including xanthophylls, decreases. How, then, can the xanthophyll cycle-dependent energy dissipation increase in senescing leaves? The xanthophyll cycle pool (VZA: the sum of violaxanthin, zeaxanthin, and antheraxanthin) decreases, but violaxanthin levels decrease more than those of zeaxanthin, so that the DPS (given as Z+0.5×A/VZA) increases (Munné-Bosch and Peñuelas, 2003; Duarte *et al*., 2012). The DPS, rather than zeaxanthin accumulation, has been correlated with the thermal dissipation of excess energy in chloroplasts (Demmig-Adams and Adams, 1992). Furthermore, an increase in xanthophyll cycle pigments relative to chlorophyll is generally observed in senescent leaves, thus indicating an increased photoprotection capacity relative to the amount of light absorbed by chlorophyll (Lu *et al*., 2003). It should be borne in mind that only a few molecules of zeaxanthin per reaction centre of PSII are needed, in combination with a pH gradient across the thylakoid membrane, for efficient photoprotection (Heber *et al*., 2001). Wingler *et al*. (2004) showed that the overall plant NPQ increases with leaf age in *Arabidopsis*, and NPQ remained high in the base of rosette leaves at very advanced phases of development. In that study, minimum fluorescence (F_0) temporarily increased at the tips of the inner rosette leaves from where the high F_0 spread to the base preceding cell death, thus indicating distinct spatial patterns of photoprotection in senescing leaves. Furthermore, Dai *et al*. (2004) showed that, at the beginning of senescence or under low light, wheat (*Triticum aestivum*) leaves were able to dissipate excess light energy via xanthophyll cycle-dependent NPQ; however, the xanthophyll cycle was insufficient to protect leaves against photodamage under strong light, when the leaves became severely senescent. Taken together, these studies indicate complex spatiotemporal regulation of photoprotective processes within the plant, in which NPQ increases at early stages of leaf senescence to decrease later. This suggests that NPQ is needed in periods of nutrient remobilization, while it decreases during the terminal phase when photoprotection is no longer needed.

Generally, preferential loss of chlorophyll *a* is reported in senescing leaves, leading to a decrease in the chlorophyll *a*/*b* ratio, therefore suggesting a preferential loss of chlorophyll *a*-containing proteins, which are closely associated with the reaction centres, rather than a loss of light-harvesting proteins (Rosenthal and Camm, 1997; Munné-Bosch and Peñuelas, 2003). Nonetheless, senescence does not always occur equally in all species in terms of photosynthetic pigment degradation. Krupinska *et al*. (2012) reported an increase in the chlorophyll *a*/*b* ratio at a late phase of senescence concomitantly with a decline in the level of the Lhcb1 apoprotein of the light-harvesting complex (LHC) and in the level of the corresponding transcript. Ultrastructural studies revealed the presence of chloroplasts with long, single or pairwise thylakoids that lacked large grana stacks. The authors hypothesized that the early degradation of grana thylakoids harbouring the major LHC reduced the risk of photoinhibition and might be causally related to the high yield of the barley variety studied. ELIPs, which have predominantly been found involved in photoprotection in emerging leaves (see the previous section), could also have such a role in senescing leaves. Binyamin *et al*. (2001) suggested that ELIPs may act as pigment carriers, binding free chlorophyll during senescence in the same manner as during stress conditions, and helping in maintaining chloroplast function, thus delaying senescence.

It has been suggested that anthocyanins protect the photosynthetic apparatus from excess light during senescence (Hoch *et al*., 2001). Anthocyanins, which accumulate in the leaf epidermis in most anthocyanin-bearing plant species, are thought to function as photoprotective screens, preventing overexcitation of the photosynthetic system by reducing the amount of light absorbed by mesophyll chloroplasts. However, the results to date differ between species and study conditions. In a study under field and laboratory conditions of *Cornus sanguinea* and *Parthenocissus quinquefolia*, which display considerable variation in both anthocyanin and chlorophyll concentrations during autumn, Manetas and Buschmann (2011) showed that the possible photoprotection conferred by anthocyanins may be of limited advantage, and even in the best of cases may occur only under adverse environmental conditions. Neither was conclusive evidence of enhanced photoprotection in anthocyanin-bearing leaves obtained by Merzlyak *et al.* (2008), who studied the optical properties of leaves from Norway maple (*Acer platanoides*), cotoneaster (*Cotoneaster alaunica*), hazel (*Corylus avellana*), Siberian dogwood (*Cornus alba*), and Virginia creeper (*Parthenocissus quinquefolia*), differing in pigment composition and at different stages of ontogenesis. Hughes (2011) noted that, although red leaves tend to show symptoms of shade acclimation relative to green leaves, consistent with a photoprotective function, winter-red and winter-green species often inhabit the same high-light environments, and exhibit similar photosynthetic capacities, which indicates that factors dictating interspecific winter leaf coloration remain unclear.

Apart from NPQ and anthocyanins, studies of antioxidant protection have also revealed interesting patterns of photoprotection during leaf senescence. García-Plazaola *et al*. (2003) studied photoprotection mechanisms during autumnal senescence both in leaves exposed to sunlight and in those kept in the shade of woody plants with different ecological characteristics and senescence patterns, including a shade-intolerant and early successional species (*Betula alba*), a shade-tolerant and late successional species (*Corylus avellana*), and an N-fixing tree with low N resorption efficiency (*Alnus glutinosa*). As a general senescence pattern, the authors found that nitrogen resorption preceded autumn and

started in mid-summer; furthermore, it occurred in parallel with a slight and continuous ascorbate, chlorophyll, and carotenoid degradation. Munné-Bosch and Peñuelas (2003) also found decreased ascorbate and tocopherol concentrations in mastic tree senescing leaves, which occurred concomitantly with increased lipid peroxidation. Tocopherol levels generally increase in senescing leaves (García-Plazaola *et al*., 2003), but, if sampling includes the last phases of senescence, tocopherol levels decrease (Juvany *et al*., 2012). Consistent with these results, Abbasi *et al*. (2009) found that tocopherol-deficient tobacco (*Nicotiana tabacum*) RNAi (RNA interference) transgenic lines show accelerated senescence. A considerable decrease of the ascorbate content in chloroplasts was also found during leaf senescence of pea (*Pisum sativum*) plants, with only dehydroascorbate being detectable in senescing leaves, thus specifically indicating ascorbate oxidation in chloroplasts during senescence (Palma *et al*., 2006). In contrast, glutathione levels in chloroplasts remained unaltered or increased slightly—depending on N availability—in senescing leaves, but in all cases the reduction state of glutathione increased, thus suggesting a differential antioxidant behaviour between ascorbate and glutathione in chloroplasts of senescing leaves. This latter study is of particular interest since analyses of antioxidant metabolites were performed in isolated organelles. This is particularly relevant for ascorbate and gluthatione, since these antioxidants—in contrast to tocopherols and carotenoids—are not exclusively found in chloroplasts. Therefore, ascorbate and glutathione levels in leaves do not necessarily indicate what happens in chloroplasts and are therefore not indicative of photo-oxidative stress.

In summary, it appears that a severe loss of antioxidant protection occurs in chloroplasts during the latest phases of leaf senescence, coincident with reductions in the NPQ. Therefore, antioxidant protection will be generally decreased together with the capacity to dissipate excess excitation energy in chloroplasts, consequently leading to photo-oxidative damage during the terminal phase (Fig. 4).

Photo-oxidative stress and redox signalling

As explained in the previous sections, photoinhibition can cause photo-oxidative stress in both emerging and senescing leaves, thereby eventually leading to redox signalling protective responses in the former and oxidative damage and death in the latter. ROS may trigger acclimation responses which counteract the environmental change, to maintain or increase the photosynthetic electron transport efficiency. Many such responses may involve targeted changes in nuclear gene expression and require two-way communication between the chloroplasts and nucleus. Recently, Kravchik and Bernstein (2013) showed that oxidative stress induced by salinity induces expression of genes involved in antioxidant protection in young leaves of maize (*Zea mays*). Furthermore, it has been shown that the cellular redox state can change the activity of chloroplast DNA replication (Kabeya and Miyagishima, 2013); hence, ROS play an important role during leaf growth. However, while several studies have researched the role of photo-oxidative stress and redox signalling in senescing leaves, to date none has focused on examining changes in gene expression induced by photo-oxidative stress in emerging leaves.

During the early senescing phases, efficient antioxidant machinery is needed to prevent premature death due to oxidative damage. The up-regulation of antioxidant genes during senescence answers such a need (Gepstein *et al*., 2003; Buchanan-Wollaston *et al*., 2005; van der Graaff *et al*., 2006). Oxidative stress itself could trigger the expression of genes related to its own detoxification. Some studies reported that various genes encoding antioxidant enzymes are induced by H_2O_2 (Neill *et al*., 2002). Gechev *et al*. (2002) reported that moderate doses of H_2O_2 induce antioxidant enzymes that protect tobacco from oxidative stress. Furthermore, Navabpour *et al*. (2003) showed that some of the genes involved in antioxidant protection during senescence are indeed induced by oxidative stress. One of them is the *LSC803* gene, which encodes glutathione peroxidase, an enzyme directly related to H_2O_2 detoxification (Apel and Hirt, 2004). Other antioxidant biosynthesis genes and detoxification-related genes up-regulated during senescence (Gepstein *et al*., 2003) are also induced by oxidative stress (Desikan *et al*., 2001). Some studies have also reported the up-regulation of many SAGs involved in the degradation of macromolecules, and the mobilization and transport of metabolites, suggesting that they may play a role in the mobilization of nutrients to the rest of the plant (Gepstein *et al*., 2003; Buchanan-Wollaston *et al*., 2005; van der Graaff *et al*., 2006). Transcriptomic analysis of oxidative stress-regulated genes in *Arabidopsis* (Desikan *et al*., 2001) shows that oxidative stress can also induce the expression of genes related to macromolecular degradation and recycling, thereby playing an essential role in the re-organization phase. Finally, several studies indicate that ROS, such as H_2O_2, are involved in the induction of PCD (de Pinto *et al*., 2012). Furthermore, Guo (2012) reviewed and compared genes identified in previous studies as being involved in senescence processes from different species, including *Populus*, *Arabidopsis*, and wheat (Andersson *et al*., 2004; Buchanan-Wollaston *et al*., 2005; Gregersen and Holm, 2007). The author reports that at the gene expression level, the developmental process of senescence seems to be conserved among species and not to differ much between dicots and monocots, or between annual and perennial plants. Furthermore, it has been shown by different means of retrograde signalling that photo-oxidative stress, and therefore ROS produced in chloroplasts, contributes to the regulation of nuclear genes in senescing leaves (for a review, see Pfannschimdt and Munné-Bosch, 2013). However, although several studies report on massive changes in gene expression during early leaf growth (Schmid *et al*., 2005; Efroni *et al*., 2008; Rehrauser, 2010), it is unknown to what extent photo-oxidative stress in chloroplasts contributes to ROS-regulated gene expression in emerging leaves; therefore it is not possible to establish common or differential patterns of photo-oxidative stress effects on gene expression in emerging and senescing leaves, an aspect that warrants further research. Rather, results obtained thus far on photoinhibition, photoprotection, and photo-oxidative stress reveal interesting common patterns of behaviour, but also important differences between emerging and senescing leaves.

Particularities of photo-oxidative stress in emerging and senescing leaves: a summary

Results on photoinhibition, photoprotection, and photo-oxidative stress reveal complex but characteristic spatiotemporal response patterns in emerging and senescing leaves. Here, we summarize both the common and the different responses in emerging and senescing leaves, so as to identify common strategies at the two ends of the leaf life cycle.

Young, expanding leaves are more susceptible to photoinhibition than fully expanded, mature leaves. This susceptibility to photoinhibition is particularly apparent at very early stages of growth, when chloroplasts receive large amounts of light but the photoprotection and antioxidant machinery is not yet completely built, so that excess excitation energy in the chloroplasts cannot be fully dissipated. For this short time window during early leaf development, oxidative stress occurs, but is not translated into oxidative damage, in terms of irreversible oxidative injury. Rather, it is expected that this transient oxidative stress serves a signalling function for protection.

Similarly to what occurs in emerging leaves, mechanisms of photoprotection—such as the xanthophyll cycle-dependent dissipation of excess energy—and antioxidant protection are activated in senescing leaves. In this case, however, photoprotection mechanisms are operative during the re-organization phase; when photoassimilate and nutrient remobilization is accomplished, severe photoinhibition occurs as a result of the failure of photoprotection mechanisms. Hence, the same mechanisms are operative in both leaf types, but the timing is completely different, as if reflected in a mirror: (i) photoinhibition occurs both very early in emerging leaves and very late in senescing leaves; and (ii) photoprotection is activated later in the growth phase, while it declines in advanced senescence (Fig. 4).

Photoinhibition is therefore maximal at the two extremes of the leaf life cycle because both leaf types have the same problem: incomplete photosynthetic apparatus. The causes, however, differ: emerging leaves are developing that apparatus, while senescing leaves are dismantling it; both are therefore sensitive to photoinhibition and photo-oxidative stress. The final result is completely different in the two cases: oxidative stress in emerging leaves plays a protective, presumably signalling, role, while oxidative stress at very advanced stages of senescence results in oxidative damage and death.

Conclusions and perspectives

In this review, we have shown that photoinhibition and photo-oxidative stress occur at the two extremes of the leaf life cycle, not only in senescing leaves but also at the very start of leaf expansion. It appears that this photo-oxidative stress is transiently induced both in emerging leaves and in the initial stages of senescence, but during the last stages of senescence the oxidative stress will be sustained in time and cause oxidative damage and death. Photoprotection mechanisms play a key role in the occurrence of photo-oxidative stress in both emerging and senescing leaves. Photo-oxidative stress occurs while photoprotection mechanisms are still not fully operative in emerging leaves, but its cellular signalling function is still unknown. While photo-oxidative stress is known to induce expression of SAGs and therefore to play a key role in senescence, ROS formed in chloroplasts are presumed to play a protective role in redox signalling in emerging leaves; however, evidence for this is lacking. Indeed, it is highly probable that the transient photo-oxidative stress in emerging leaves serves to make a more efficient photosynthetic apparatus, by acting as a signal to construct a functional system that will be more resistant to environmental injuries later when the leaf is fully expanded. However, it is also possible that this photo-oxidative stress plays no protective signalling role and serves as a selective pressure on emerging new leaves, so that the more susceptible leaves do not progress. This second hypothesis is more plausible for perennial plants, in which very young expanding leaves from different shoots compete for resources.

Finally, the importance of studying leaf senescence by considering the whole leaf developmental programme should be emphasized, since history traits of the same and neighbouring leaves undoubtedly affect the spatiotemporal senescence programme within the plant. A recent study has shown that repeated exposures to strong light resulted in the acclimation to high light and oxidative stress not only of the exposed leaves, but also of young emerging leaves (Gordon *et al*., 2012). Furthermore, epigenetic changes triggered by specific events encountered by leaves during their early life history—such as a specific stress imprint during the early phases of growth—are also likely to determine the timing of senescence. It remains, however, to be examined to what extent photo-oxidative stress suffered during early leaf growth affects the timing and/or progression of senescence in plants. To date, all the studies have focused on establishing how a number of abiotic stresses can affect senescence after short periods of time and not on whether a stress imprint at an early stage of leaf development can affect senescence much later in time.

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