SISTEMES DE FOTOPROTECCIÓ A *Quercus ilex* L. I APLICACIÓ DE LA NIRS (ESPECTROSCÒPIA DE REFLECTÀNCIA A L'INFRAROIG PROPER) COM A TÈCNICA ECOFISIOLÒGICA PER LA DETECCIÓ D'ESTRÈS OXIDATIU

MARTA PINTÓ I MARIJUAN

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Memòria de la tesi presentada per

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Doctorand **Vistiplau de la directora**

MARTA PINTÓ I MARIJUAN **Dra. ISABEL FLECK BOU**

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TREE HUGGER

The flower said, "I wish I was a tree"
The tree said, "I wish I could be
A different kind of tree,
The cat wished that it was a bee,
The turtle wished that it could fly
Really high into the sky,
Over rooftops and then dive
Deep into the sea.

And in the sea there is a fish,
A fish that has a secret wish,
A wish to be a big cactus
With a pink flower on it.
And in the sea there is a fish,
A fish that has a secret wish,
A wish to be a big cactus
With a pink flower on it.

And the flower
Would be its offering
Of love to the desert.
And the desert,
So dry and lonely,
That the creatures all
Appreciate the effort.

And the rattlesnake said,
"I wish I had hands so
I could hug you like a man."
And then the cactus said,
"Don't you understand,
My skin is covered with sharp spikes
That'll stab you like a thousand knives.
A hug would be nice,
But hug my flower with your eyes."

The flower said, "I wish I was a tree"
The tree said, "I wish I could be
A different kind of tree,
The cat wished that it was a bee,
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Kimya Dawson

ÍNDEX

INTRODUCCIÓ

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1. L'alzina (*Quercus ilex* L.) en el bosc Mediterrani

L'alzina és una espècie monòica i perennifòlia que pertany a la família de les Fagàcies. Morfològicament té la capçada densa i l'escorça del tronc i branques primes; les fulles tenen de 3 a 7 cm, són el·líptiques o oblongues, subenteres o amb dents poc espinoses, coriàcies i amb un pecíol de 3 a 4 cm de llargada (Bolòs *et al*. 1990). Es caracteritza per ésser una espècie tolerant a l'ombra i de creixement lent (Gràcia *et al*. 1999), però amb una vigorosa capacitat de rebrot (Retana *et al*. 1992). En quant a les condicions per a l'establiment de les noves plàntules necessita una elevada humitat i una baixa intensitat lumínica (Espelta *et al*. 1995).

L'espècie *Quercus ilex* L. ocupa més de 120.000 ha de la superfície forestal de Catalunya i és una de les espècies forestals més importants en les regions boreals-Mediterrànies (Terradas i Savé 1992). En quant a l'hàbitat requereix climes relativament humits i d'influència marítima. Generalment es troba en zones amb estius no molt llargs i hiverns que poden ser freds (en alguns alzinars de la Península Ibèrica s'han registrat temperatures mínimes absolutes de -25ºC).

L'alzina té un elevat valor de protecció dels ecosistemes Mediterranis ja que contribueix a la formació de sòls ben estructurats. Els alzinars afavoreixen la maduració dels sòls gràcies a la gran densitat de coberta (i conseqüent aportació de materia orgànica) generant un microclima molt especial al sotabosc, menys àrid que el clima general (Bolòs i Bolòs 1950), i també gràcies a la potència del seu sistema radicular i a la gran capacitat reguladora d'escorrenties.

A aquests valors s'hi hauria d'afegir l'estètic, ja que l'alzinar forma part del paisatge natural Mediterrani i promou una base econòmica fruit de l'aprofitament de la seva llenya, dels seus fruits i dels tanins de la seva escorça (Terradas i Savé 1992).

L'alzinar representa el tipus de vegetació dominant de la zona de transició entre els boscos de clima moderat, la qual es caracteritza per un doble estrès: hivern fred i estiu sec i càlid. Aquestes condicions climàtiques en determinen les respostes fisiològiques i morfològiques. Així doncs, degut a les seves característiques de resistència a la sequedat amb fulles perennes i escleròfil·les *Quercus ilex* es considera la típica planta mediterrània.

1.1. L'alzina i els homes

Degut a la seva importància per als humans des de temps prehistòrics, molts arbres de fulla ampla han sigut el subjecte de llegendes, mitologia i religió. Alguns s'han associat a la saviesa, la fortalesa i la fiabilitat (Ciesla 2002).

Els *Quercus* spp., amb la seva imponent alçada i longevitat s'han descrit com a sagrats a múltiples cultures. Les espècies perennes com *Quercus ilex* L. i *Quercus suber* L. foren especialment venerades i adorades en societats humanes primerenques, tal i com adoraven altres meravelles de la natura que ells no podien entendre (Mirov i Hasbrouck 1976). Foren els arbres sagrats pels hebreus i àmpliament introduïts a la Bíblia amb més de 60 referències, així com pels primers gals acollint-los com símbol del seu Déu suprem. Per als druides foren considerats com l'arbre sagrat celestial i present en la majoria de cerimònies celtes-druides (Lust 1990).

Un dels aspectes més intrigants de l'alzina i el roure com a arbres sagrats és la associació generalitzada amb els Déus del tro a múltiples cultures europees. Probablement és degut al fet que els *Quercus* spp. semblen atraure els llamps més que cap altre arbre dels boscos. Per als nord-Europeus fou l'arbre sagrat del Déu del tro, *Thor*. Fou també sagrada per al principal Déu grec, *Zeus*, amb el seu llampec, així com per la seva contrapart romana, *Júpiter*. L'oracle de *Zeus* i *Dodana*, mencionat per Homer, succeí en una alzinar sagrat, i les prediccions foren fetes interpretant el xiuxiueig de les fulles de les alzines. Els països eslaus tingueren també les seves pròpies versions del Déu del tro associat al roure. William Shakespeare a *El Rei Lear* feu referència a "oak cleaving thunderbolts" (Walker 1990).

A Grècia i Roma, l'alzina i el til·ler, *Tilia cordata* Mill., foren associats a la mítica història de *Baucis* i *Philemon*, una parella de joves que mostraren amabilitat i hospitalitat als Déus *Zeus* i *Hermes*, mentre que tots els seus veïns, amb molta més riquesa, refusaren acollir als Déus. Com a càstig per a tots els veïns insolidaris, els dos Déus, cobriren totes les cases de l'àrea amb un llac, excepte el pujol ocupat per *Baucis* i *Philemon*, el qual fou transformat en un preciós temple i els garantiren una mort simultània permetent-los romandre sempre junts. Després de la mort, els Déus els transformaren en dos arbres que creixeren sempre de costat. *Baucus* esdevingué un til·ler, símbol de l'amor conjugal, i *Philemon* una alzina, símbol de l'hospitalitat (Lust 1990).

A Anglaterra, el nom "d'arbre de l'Evangeli" fa referència a els temps quan les pregàries i les *Gospel truths o* veritats sagrades es deien a l'ombra d'un

Quercus spp. on es llegien els passatges de l'Evangeli i es pregaven benediccions per la gent (Grieve 1931).

1.2. El canvi climàtic a l'àrea Mediterrània i l'alzinar

La vida a la Terra és possible gràcies a l'energia solar que arriba principalment en forma de llum visible. Aproximadament el 30% de la radiació solar torna a l'espai per l'acció de l'atmosfera exterior, però la resta arriba a la superfície terrestre, que la reflecteix en forma de radiació infraroja.

A l'atmosfera que embolcalla el nostre planeta hi ha una sèrie de gasos (sobretot el vapor d'aigua i el diòxid de carboni) que tenen un efecte d'hivernacle, és a dir, absorbeixen i reemeten la radiació infraroja. D'aquesta manera, impedeixen que part d'aquesta radiació escapi de la terra i mantenen el planeta 30ºC més calent que si aquesta capa no existís, contribuint a que la temperatura mitjana de l'aire superficial del planeta sigui apta per a la vida. L'efecte d'hivernacle és, per tant, un fenomen natural de l'atmosfera.

El problema actual és que la quantitat d'aquests gasos naturals amb efecte d'hivernacle a l'atmosfera ha augmentat i que s'hi han abocat, a més, gasos amb efecte d'hivernacle provinents de l'activitat humana no presents de forma natural a l'atmosfera alterant-ne la composició i l'efecte, que se suma a la variabilitat natural del clima, observada durant períodes de temps comparables. Aquest canvi s'admet que posa en perill la composició, la capacitat de recuperació i la productivitat dels ecosistemes naturals i el mateix desenvolupament econòmic i social, la salut i el benestar de la humanitat (http://mediambient.gencat.net/cat/el_medi/C_climatic/).

Conseqüències del canvi climàtic

Els models climàtics prediuen (Christensen *et al*. 2007), entre d'altres: un augment de la temperatura mitjana d'1.4ºC a 5.8ºC durant aquest segle, la desertificació de certes zones del planeta, les pluges de caràcter torrencial en altres zones, la pujada del nivell del mar entre 9 i 88 cm per a l'any 2100, que inundaria zones avui densament poblades i la difusió de certes malalties de tipus tropical en zones avui de clima temperat. Els efectes seran un major risc per a la salut, una reducció de la generació hidroelèctrica, una disminució de la producció agrícola i un increment dels incendis forestals a nivell mundial. A les zones Mediterrànies, un augment de la temperatura juntament amb una disminució de

les precipitacions (Houghton *et al*. 2001) incrementaran, per tant, del potencial d'evapotranspiració, reduint la disponibilitat d'aigua i augmentant el risc de sequera unit a freqüència i intensitat dels incendis (Mouillot *et al*. 2002). Igualment, aquests canvis poden afectar la capacitat de rebrot després d'incendi i el seu desenvolupament (Reich *et al*. 1990, Kruger i Reich 1997), i per tant, pot tenir un gran impacte sobre les àrees Mediterrànies (Chaves *et al*. 2003) amb efectes en la fisiologia de les espècies i comunitats vegetals de la zona. Segons Brasier (1996) és possible que la disminució dels alzinars Mediterranis pugui ser un símptoma del sobreescalfament global.

El CO_2 és, sens dubte, el principal responsable del canvi climàtic. En aquest sentit considerem interessant l'ampliació en el coneixement de les respostes fisiològiques dels alzinars (com a poblacions predominants en l'àrea Mediterrània) sota concentracions de CO_2 elevades, i més concretament l'aprofundiment en el paper dels sistemes fotoprotectors en aquestes condicions canviants. Així mateix, també s'aprofundirà en l'estudi de les diferents respostes sota estrès per elevades temperatures combinades amb altes concentracions de CO_2, simulant la tendència global originada per les emissions andròmenes.

1.3. Els incendis a l'àrea Mediterrània i la capacitat de rebrot de les alzines després d'una pertorbació

Les cinc regions de clima Mediterrani (conca Mediterrània i zones litorals de Chile, Califòrnia, Austràlia i Sudàfrica indicades a la **Figura 1**) ocupen menys del 5% de la superfície total de la Terra, però és on s'hi troben aproximadament el 20% de les espècies de plantes vasculars descrites (Cowling *et al*. 1996). Aquest fet provoca que siguin regions d'elevat interès, no tan sols a nivell florístic, sinó també a nivell ecològic.

El foc és un dels factors de pertorbació de la vegetació més freqüent tant a la conca Mediterrània com a les altres regions Mediterrànies de la Terra (Trabaud 1981, Moreno i Oechel 1994) ja que hi ha devastat importants extensions. Des del punt de vista ecològic, els incendis es consideren un factor de destrucció amb grans pèrdues econòmiques, que contribueixen a la degradació i eliminació de la vegetació natural. No obstant, s'ha de destacar la força selectiva dels incendis forestals per la promoció evolutiva de diferents formes de regeneració i com a procés clau en la convergència estructural i funcional de les diferents comunitats vegetals Mediterrànies, fet que fa

inapropiat considerar el foc com a agent de destrucció natural, si més no a petita escala (Trabaud 1998).

La majoria dels incendis forestals a les regions de clima Mediterrani tenen lloc durant l'estiu (Kruger i Bigalke 1984, Keeley 1986, Millán *et al*. 1998); les elevades temperatures i escasses precipitacions fan baixar el contingut hídric relatiu de les plantes i per tant n'augmenten la inflamabilitat (Elvira-Martín i Hernando-Lara 1989).

Figura 1 | Principals regions amb clima Mediterrani: conca Mediterrània i zones litorals de Chile, Califòrnia, Austràlia i Sudàfrica.

Les espècies Mediterrànies disposen d'una sèrie de mecanismes per minimitzar l'impacte del foc, com són, entre altres: la protecció de les llavors o d'altres òrgans de la planta, la disminució de l'activitat fisiològica durant els períodes crítics en els quals el foc pot tenir una major incidència i l'estimulació, després de l'incendi, de la germinació de les llavors o de la capacitat de rebrotar (Sabaté i Gràcia 1996). La regeneració per rebrot, característica d'espècies com *Quercus ilex, Quercus suber* i *Quercus rubra* (Canadell *et al*. 1991, Retana *et al*. 1992, Kruger i Reich 1993, 1997) és d'important rellevància per la recuperació de les poblacions vegetals en regions mediterrànies (Hastings *et al*. 1989, Reich *et al*. 1990). El ràpid desenvolupament dels rebrots gràcies a les reserves del sistema radicular suposa un avantatge important respecte a les plantes que es reprodueixen únicament per llavors (Trabaud i Méthy 1988; Lloret *et al*. 1996).

El procés de rebrot de les plantes llenyoses és probablement una característica primitiva d'adaptació que permet la supervivència després de la pertorbació causada pel foc (Malanson i Trabaud 1988), mentre que el recobriment que afavoreix el foc es considera com una adaptació derivada (Wells 1969). En els ecosistemes Mediterranis arbustius s'ha observat un ràpid creixement de la vegetació de rebrot després d'incendis (Saruwatari i Davis

1989, Fleck *et al*. 1995, 1998) així com després de tales (Castell 1992, Sabaté 1993, Peña-Rojas *et al*. 2005), però aquesta regeneració necessita una important remobilització dels recursos emmagatzemats en òrgans subterranis per la planta abans de la pertorbació (Bowen i Pate 1993). El carboni i/o nitrogen poden remobilitzar-se a partir d'un teixit i utilitzar-se posteriorment pel creixement o manteniment d'un altre teixit (Millard 1988; Millard i Proe 1993) siguent aquest procés característic de plantes perennes.

A nivell fisiològic, els rebrots disposen d'algunes particularitats respecte la vegetació original (control). S'han estudiat múltiples motius pels quals la vegetació de rebrot experimenta unes taxes de bescanvi de gasos superiors a la vegetació control. Aquest fet pot ser degut a múltiples variables com: la disponibilitat d'aigua (Saruwatari i Davis 1989) i nutrients (Oechel i Hastings 1983), la qual és superior en els rebrots degut a que, abans de la pertorbació, el sistema radicular es trobava associat a una biomassa aèria molt més gran (De Souza *et al*. 1986, Kruger i Reich 1993), mentre que després de l'incendi, la pèrdua de cobertura vegetal i reducció de l'índex d'àrea foliar (LAI) és evident. Aquest fet, afavoreix la disponibilitat dels recursos emmagatzemats a la rel així com de l'aigua del sòl. Igualment, la disponibilitat lumínica a les zones pertorbades es veu fortament incrementada per la major exposició per reducció del LAI, permetent, també, una major taxa fotosintètica, malgrat un excés de radiació solar que pot provocar fotoinhibició.

1.4. Les respostes fisiològiques de les alzines sota condicions ambientals d'estrès

Les espècies Mediterrànies entre elles el *Q. ilex*, es troben freqüentment exposades a condicions de forta sequera, provocant una disminució de l'estat hídric de les fulles (expressat com a contingut hídric relatiu: RWC o potencial hídric: Ψ) (Quick *et al*. 1992, Savé *et al*. 1999, Larcher 2003). L'alzina té un ús conservador de l'aigua (Tognetti *et al*. 1998, Fotelli *et al*. 2000), induint el tancament estomàtic en front a l'estrès hídric (Gulías *et al*. 2002, Medrano *et al*. 2002) a fi de mantenir un RWC operatiu. A més, presenten baixes taxes de transpiració cuticular i una gran capacitat per l'ajust osmòtic (Terradas i Savé 1992, Sala *et al*. 1994). La sequera, la elevada radiació incident i temperatura a l'estiu, així com les baixes temperatures a l'hivern (Baker 1994) influeixen marcadament l'activitat enzimàtica de les plantes Mediterrànies limitant la fixació de CO_2 (Filella *et al*. 1998, Savé *et al*. 1999, Llorens *et al*. 2002).

En qualsevol de les dues estacions, l'energia originada per la llum absorbida pot sobrepassar l'energia necessària per l'assimilació del CO_2, i per tant l'absorció d'energia lluminosa pot esdevenir excessiva, fet que pot induir danys als fotosistemes i provocar la fotoinhibició de l'aparell fotosintètic (Méthy *et al*. 1996). A *Q. ilex* s'han descrit diversos sistemes de protecció de l'aparell fotosintètic, sota condicions d'excés d'irradiància com la dissipació en forma de calor duta a terme pel cicle de les xantofil·les (VAZ) (Fleck *et al*. 1998, Peñuelas *et al*. 1998) o pel de la luteïna epòxid (Llorens *et al*. 2002, García-Plazaola *et al*. 2003).

Aquests sistemes de fotoprotecció, juntament amb altres marcadors d'estrès tenen un elevat interès com a indicadors de la necessitat de fotoprotecció per part de la planta en front d'un determinat estrès. En aquest treball es vol ampliar el coneixement de les variacions d'aquests compostos en funció de diferents estressos així com la seva funció en les fulles de *Quercus ilex*.

2. Sistemes de fotoprotecció

L'aparell fotosintètic en les plantes és el responsable de la funció essencial de convertir l'energia lluminosa en energia química utilitzada per la fixació de CO_2, però aquesta maquinària complexa és susceptible de sofrir danys induïts per l'excés de llum (Niyogi 2000).

Sovint l'energia radiant que arriba a la superfície de les fulles és superior a la seva capacitat d'assimilació fotosintètica, fet que provoca un excés d'estat de reducció dels transportadors de la cadena d'electrons i una acumulació d'energia d'excitació als centres de reacció. Això afavoreix la fotoinhibició (reducció de l'activitat fotosintètica deguda, bàsicament, a una reducció en l'eficiència fotoquímica del fotosistema II) i la formació d'espècies reactives de l'oxigen (ROS) que indueixen la peroxidació de lípids i l'oxidació de proteïnes que poden finalment destruir l'aparell fotosintètic. En aquest sentit, els pigments cloroplàstics, tenen una funció fotoprotectora, canalitzant l'excés d'energia des de la clorofil·la als carotenoides per evitar que aquesta energia passi a l'oxigen i es formin les ROS (Ma *et al*. 2003).

A fi d'evitar la fotoinhibició, la primera estratègia de les plantes en front de l'excés lumínic és la **disminució de la interceptació** de llum mitjançant moviments de les pròpies fulles o estratègies de modificacions estructurals amb la finalitat d'absorbir la mínima radiació en els complexes antena (Königer *et al*. 2008, Takagi 2003).

No obstant, una vegada la llum és interceptada existeixen tres mecanismes que competeixen entre ells per dissipar l'excés d'energia d'excitació absorbida. El primer és la dissipació metabòlica, on s'utilitza l'energia captada per realitzar **processos fotosintètics o fotorespiratoris (2.1)**. El segon sistema consisteix en la **dissipació per emissió de fluorescència (2.2)** que porten a terme directament les molècules de clorofil·la. L'últim mecanisme és el la **dissipació tèrmica (2.3)**, el qual mitjançant determinats carotenoides, elimina l'energia excedent en forma de calor.

Simultàniament als processos de dissipació d'energia citats s'activen un altre grup de mecanismes de fotoprotecció, els quals inclouen **compostos antioxidants** (Niyogi 1999) i **processos de reparació (2.4)** de la peroxidació lipídica (Baier i Dietz 1999), dels centres de reacció dels fotosistemes (Melis 1999) i inclús dels senyals sistèmics i dels processos d'aclimatació (Karpinski *et al*. 1999).

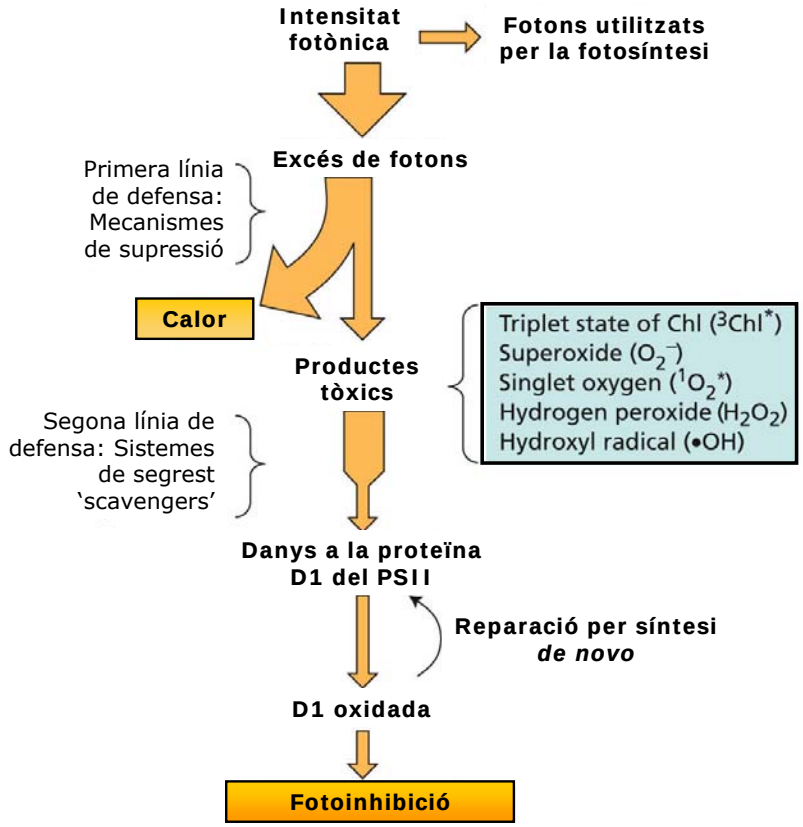

Figura 2 | Regulació de la captació de fotons i de la protecció i dels processos de reparació del fotodany. Adaptat de Taiz i Zeiger 2006.

Si finalment l'energia rebuda segueix sent superior a la dissipada i/o segrestada per els 'scavengers' (**Figura 2**) s'inicien els processos d'oxidació de proteïnes arribant a la fotoinhibició crònica i fotooxidació.

El nostre grup d'investigació ha descrit diversos sistemes de protecció de l'aparell fotosintètic sota condicions d'excés d'irradiància a *Q. ilex*. Entre aquests s'hi inclouen estudis sobre la dissipació d'energia per calor tant a través del cicle de les xantofil·les (Fleck *et al*. 1998) com a través del cicle de la luteïna epòxid (Llorens *et al*. 2002), la contribució de sistemes antioxidants (El Omari *et al*. 2003a) i l'expressió de les proteïnes de shock de calor de baix pes molecular (heat-shock proteins: sHsps) (Verdaguer *et al*. 2003). En aquest nou treball vol ampliar-se aquest coneixement des d'una perspectiva més bioquímica, aprofundint en l'estudi de diverses molècules reconegudes com a marcadors d'estrès (pigments fotoprotectors, sistemes antioxidants i poliamines).

2.1. Processos fotorespiratoris

La fotorespiració és una via metabòlica alternativa que implica la participació de tres orgànuls cel·lulars: el cloroplast, el peroxisoma i el mitocondri (**Figura 3**). L'enzim encarregat del primer pas d'aquest procés és la mateixa ribulosa 1,5-bifosfat carboxilasa-oxigenasa (Rubisco) en la seva funció oxigenasa, la qual s'uneix a dos O_2. En una segona etapa, ja dins el peroxisoma, s'utilitzen dues molècules més d'O_2 per a generar l'H_2O_2 que serà reduïda per la catalasa a H_2O i O_2. En la fase mitocondrial s'allibera CO_2, permetent el tancament del cícle passant pel peroxisoma fins al cloroplast. Així doncs, durant la fotorespiració no es produeix assimilació, però es consumeix poder reductor i ATP acumulats (generats al tilacoide) (Azcón-Bieto i Talón 2008).

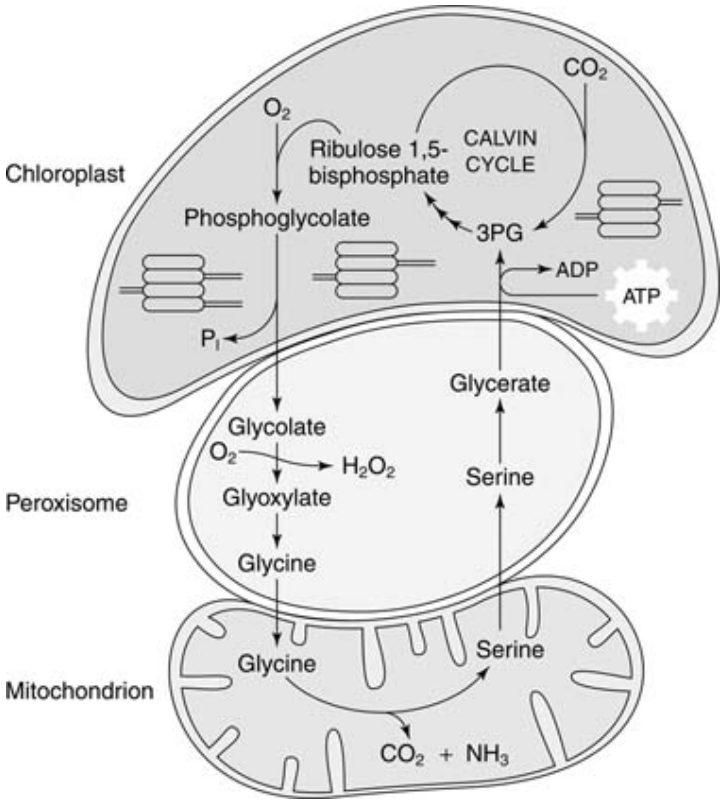

Figura 3 | Cicle de la fotorespiració acoblat a la fotosíntesi (Cicle de Calvin). http://www.cliffsnotes.com/WileyCDA/CliffsReviewTopic/topicArticleId-24594,articleId-24522.html.

Totes les situacions d'estrès que afecten, directa o indirectament l'equilibri hídric de la planta indueixen el tancament estomàtic per reduir la transpiració. No obstant, aquest tancament també redueix la concentració de CO_2 intracel·lular. El descens en la relació CO_2/O_2 del cloroplast promou

l'activitat oxigenasa de la Rubisco i augmenta la taxa de fotorespiració. Tanmateix, els estressos que provoquen el tancament estomàtic també indueixen a l'estrès per llum, i és aleshores quan la fotorespiració contribueix, en condicions d'alta il·luminació i baixa disponibilitat de CO_2, prevenint els possibles danys a l'aparell fotosintètic causats per l'excés de llum evitant la saturació de la cadena de transport d'electrons per acumulació de NADPH i ATP, i per tant facilitant la dissipació d'electrons èxtres (Osmond i Grace 1995).

2.2. Emissió de fluorescència per les clorofil·les

L'estructura molecular de la clorofil·la és un anell de porfirina (tetrapirrol) coordinat en un àtom central de magnesi i una cua hidrocarbonada amb residus metil ($^{-}CH_3$) (o fitol) amb la funció d'anclatge a la zona lipòfila de la membrana fotosintètica. El tetrapirrol conté dobles enllaços que permeten les transicions d'electrons i les reaccions de reducció-oxidació. Varies clorofil·les difereixen únicament per el patró de dobles enllaços o per els radicals substituents al voltant de l'anell. L'energia absorbida per les clorofil·les, un cop excitades, només pot passar a tres processos alternatius que competeixen entre ells (**Figura 4**): (**1**) l'emissió de fluorescència vermella de les clorofil·les, (**2**) el treball fotoquímic cap a la cadena de transport d'electrons (qP) o (**3**) la dissipació d'energia tèrmica (NPQ: non photochemical quenching). Mesurant les variacions en la fluorescència, s'obté la informació sobre dels canvis en la eficiència dels altres processos.

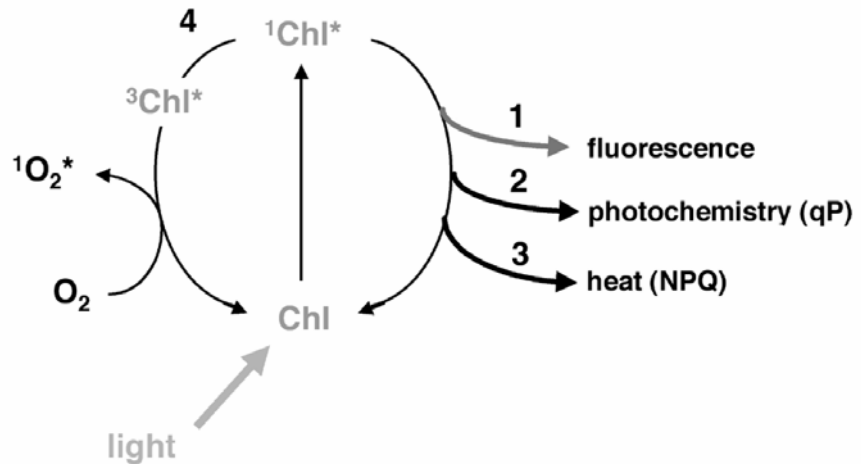

Figura 4 | Possibles destins de les molècules de clorofil·la excitada (Chl^*). Adaptat de Müller *et al*. 2001.

Tot i que la quantitat d'energia en forma de fluorescència que emeten les molècules de clorofil·la excitades és molt baix (entre l'1 i el 2% del total d'energia absorbida), la seva quantificació és molt senzilla. L'espectre de l'energia emesa per fluorescència és diferent al de la llum absorbida (**Figura 5**) ja que emet a una longitud d'ona superior (més propera als 700nm) a la de l'enegia que absorbeixen les clorofil·les (vermella i blava). Per tant, el rendiment de la fluorescència es pot quantificar exposant a una fulla a una longitud d'ona coneguda i mesurant mitjançant un fluorímetre modulat la quantitat de llum re-emesa a longituds d'ona més llargues (Maxwell i Johnson 2000).

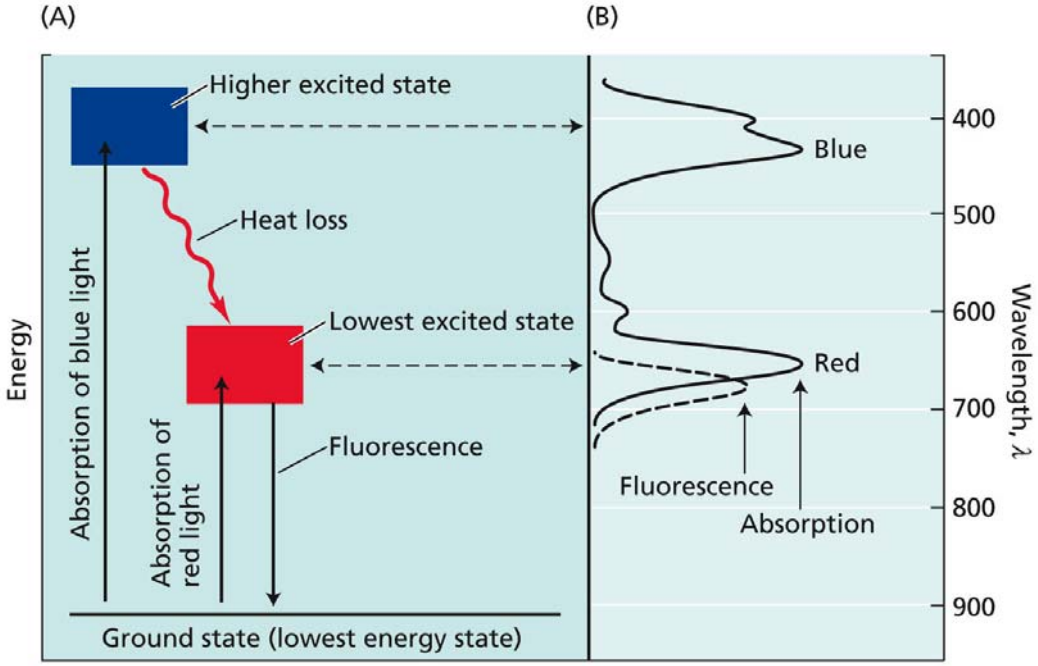

Figura 5 | Graficació de l'absorció i l'emissió de llum per la clorofil·la. **A |** Diagrama del nivell d'energia. L'absorció o emissió de la llum s'indica amb les fletxes verticals que connecten el nivell basal amb l'estat d'energia més baix (*Ground sate (lowest energy state)*) amb els estats dels electrons excitats representats per rectangles (el més alt en blau i el més baix en vermell). L'absorció de les clorofil·les a les longituds d'ona del blau i del vermell corresponen a les fletxes ascendents (*Absorption of light*). Cada qualitat de llum absorbida provoca a la molècula de clorofil·la un canvi de l'estat basal a l'estat excitat. La fletxa descendent indica la fluorescència (*Fluorescence*), on la molècula, mentre emet energia en forma de fotó, baixa de l'estat d'excitació més baix fins al nivell basal (estat d'energia més baix). **B |** Espectre d'absorció i fluorescència. L'absorció de la longitud d'ona més llarga (vermell) de les clorofil·les correspon a la llum que té l'energia necessària per provocar la transició des del nivell basal fins el primer estat d'excitació. L'absorció de la longitud d'ona més curta (blau) correspon a la llum que provoca la transició fins l'estat d'excitació més alt. Adaptat de Taiz i Zeiger 2006.

Aquesta mesura permet conèixer l'estat fisiològic del sistema fotoquímic i se n'extreu informació sobre la taxa de transport d'electrons, el rendiment quàntic del PSII i si es dona fotoinhibició de la fotosíntesi. En condicions d'irradiància òptimes per la fotosíntesi, l'energia que es perd per fluorescència (**Figura 4.1**) o per calor (**Figura 4.3**) és baixa, mentre que si les condicions

impliquen un estrès per a l'aparell fotosintètic, aquest serà menys eficient i l'energia que no passarà a treball fotoquímic serà superior.

2.3. Dissipació tèrmica pels carotenoides

Els carotenoides són pigments orgànics que es troben de forma natural a tots els organismes fotosintètics. Se'n coneix més de 700 estructuralment diferents que poden diferenciar-se en dos grans grups: els **carotens**, compostos per cadenes hidrocarbonades amb dobles enllaços conjugats i les **xantofil·les**, les quals a més contenen grups epòxi i hidroxil en els seus anells terminals. Els carotenoides més importants tant per abundància com per funció són la neoxantina, la violaxantina, la zeaxantina, l'anteraxantina, els α i β-carotè, la luteïna i la luteïna epòxid.

Els carotenoides són els **compostos fotoreceptors** que, juntament amb les clorofil·les, absorbeixen d'energia lluminosa per als processos fotoquímics; són antioxidants que poden interaccionar directament amb ROS ($^1O_2$ i O_2^-) o amb altres antioxidants com ascorbat o l'α-tocoferol (Edge *et al*. 1997). Per exemple el ß-carotè pot interaccionar directament amb el $^1O_2$ protegint les altres molècules del dany fotoxidatiu (Burton i Ingold 1984). Les xantofil·les, actuen com a estabilitzadores de membrana dels tilacoides, proporcionant rigidesa i termoestabilitat a més d'evitar la peroxidació dels lípids de membrana (Havaux 1998). Una de les funcions de les xantofil·les més estudiades en les últimes dècades és la de protecció dels complexes antena desexcitant l'estat triplet de les clorofil·les durant la dissipació tèrmica (algunes revisions: Demmig-Adams i Adams 1996a, 2006, Niyogi 2000, Niyogi *et al*. 2005, Adams *et al*. 2006).

En plantes superiors, s'han descrit dos cicles de xantofil·les implicats en la dissipació de calor: el cicle de la violaxantina (Demmig Adams i Adams 1992) i el cicle de la luteïna epòxid (García-Plazaola *et al*. 2007).

El **cicle de la violaxantina** (cicle VAZ) consisteix en interconversions de violaxantina (V) a zeaxantina (Z) a través de l'intermediari anteraxantina (A) (**Figura 6**) gràcies a l'enzim violaxantina de-epoxidasa (VDE), el qual s'activa per l'acidificació del lumen (que habitualment es dóna sota condicions de llum) (Eskling *et al*. 1997, Pfündel i Bilger 1994). Quan la llum deixa de ser excessiva, la Z s'epoxida a A i finalment a V gràcies a una epoxidasa (ZE) localitzada a

l'estroma de la membrana tilacoidal (Havaux *et al*. 2000). La seva funció fou desconeguda fins que l'any 1987, Demmig-Adams *et al*. observaren una correlació entre les concentracions de Z i la dissipació no radiant de calor per un excés de llum (expressat com a NPQ) fou estudiat extensament a partir d'aleshores amb revisions com les de: Yamamoto *et al*. (1999), Demmig-Adams (2003), Gilmore (2001) o Niyogi *et al*. (2005). Així doncs, l'acumulació d'aquests productes de-epoxidats i l'increment del gradient de pH a través de la membrana del tilacoide dóna pas a un augment de la dissipació d'energia en forma de calor. Hieber *et al*. (2004) proposaren que podia haver-hi múltiples funcions associades al cicle VAZ com una cadena de transducció de senyals per l'adaptació de les plantes a l'estrès lumínic, demostrada per Mullineaux i Karpinski (2002) i Surpin *et al*. (2002). S'ha demostrat, també, que la proteina PsbS és necessària per la dissipació tèrmica fotoprotectora (qE) de l'excés de llum absorvida en plantes (Niyogi *et al*. 2005). En *Arabidopsis*, es va demostrar un màxim NPQ en sobreexpresar-se aquesta proteïna, fet que relaciona els dos processos directament (Li *et al*. 2000).

El **cicle de la luteïna epòxid** (cicle Lx) implica la de-epoxidació de la Lx (monoepòxid) a luteïna (L) i la posterior epoxidació a Lx (**Figura 6**). La correlació entre la dissipació d'energia d'excitació per calor i el nivell de de-epoxidació del pool de Lx s'ha confirmat per múltiples autors (Niyogi *et al*. 1997, Matsubara *et al*. 2001, 2005, Llorens *et al*. 2002, García-Plazaola *et al*. 2003). La funció de la L en fotoprotecció i dissipació d'energia via NPQ segueix essent qüestionada malgrat els resultats obtinguts en mutants (Pogson *et al*. 1998, Gilmore 2001, Howitt i Pogson 2006). La diferència en la magnitud de les quantitats dels pools dels cicles de Lx i VAZ, i el fet que ambdós cicles són actius simultàniament, i probablement catalitzats pels mateixos sistemes d'enzims (VDE i ZE), són trabes importants per a l'estudi del paper específic de la de-epoxidació de la Lx en el desenvolupament del NPQ (García-Plazaola *et al*. 2007). A més, mentre que la de-epoxidació (de Lx a L) durant la llum es dóna a la mateixa velocitat que el pas de Z a V, la epoxidació de la L és molt més lenta que la de la V i la seva dependència de la llum depèn de l'espècie (Esteban *et al*. 2007, Matsubara *et al*. 2008).

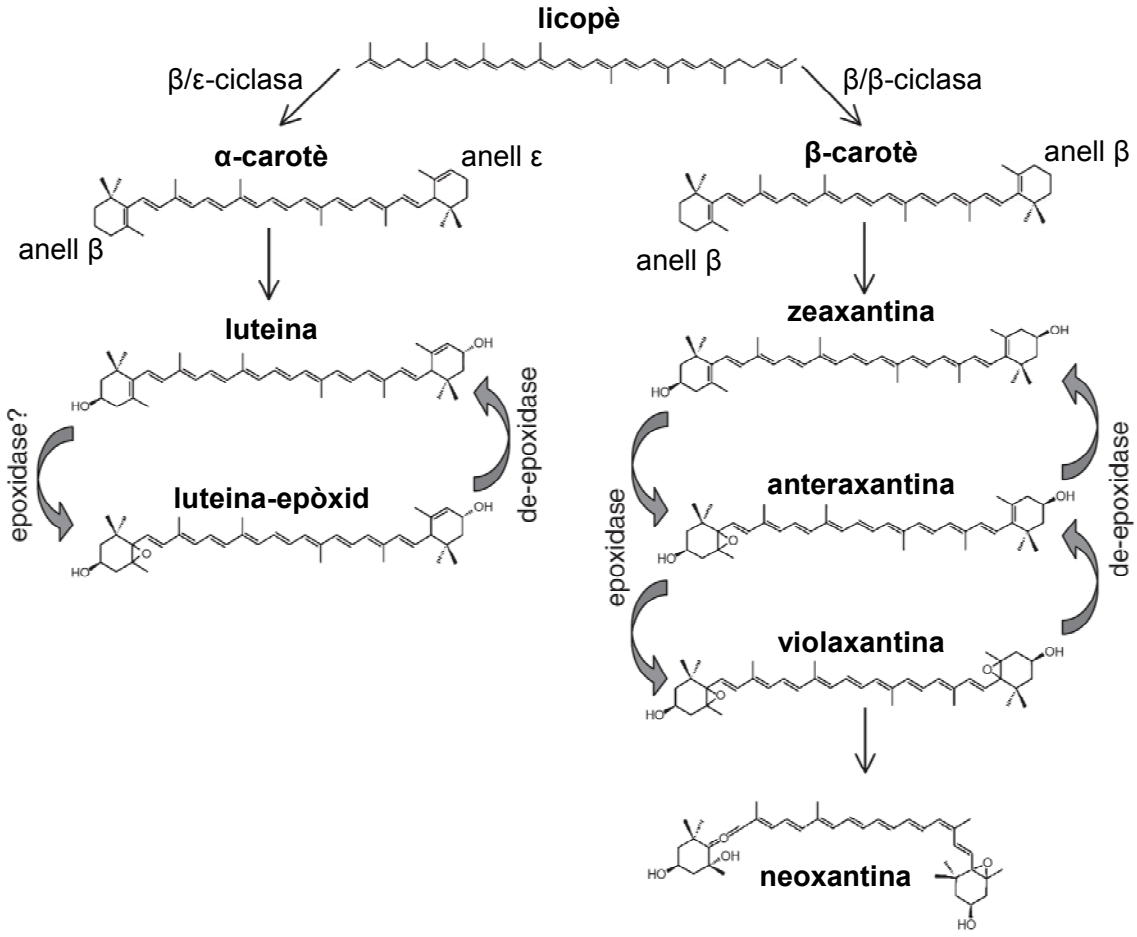

Figura 6 | Vies de síntesi de les xantofil·les. La via α condueix a la formació de la luteïna i de la luteïna epòxid, mentre que la via β genera els components del cicle de les VAZ. Adaptat de García-Plazaola *et al*. 2007.

2.4. Mecanismes antioxidants i processos de reparació

Un dels productes de múltiples vies metabòliques a diferents compartiments cel·lulars en plantes són les espècies reactives de l'oxigen (ROS). Alguns exemples de ROS (**Figura 7**) són: l'oxigen singlet ($^1O_2$), els radicals superòxid (O_2^-), els radicals hidroxil (OH^*) o el peròxid d'hidrogen (H_2O_2)) (Asada 1996, Apel i Hirt 2004). La seva presència pot malmetre, per oxidació lípids, proteïnes i àcids nuclèics provocant un estrès oxidatiu (Baier i Dietz 1999), el qual comporta una pèrdua de la integritat estructural cel·lular, podent arribar a la mort cel·lular. Al mateix temps, les ROS constitueixen a més un dels grups de senyals cel·lulars d'activació de les respostes en front a l'estrès i de les vies de defensa (Mittler 2002).

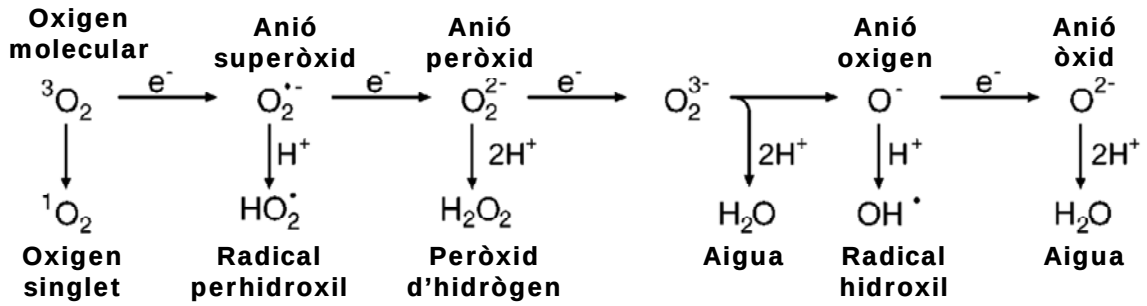

Figura 7 | Generació de les diferents espècies reactives de l'oxigen per transferència d'energia o reducció seqüencial univalent de l'oxigen molecular o en estat triplet. Adaptat d'Apel i Hirt 2004.

Les cèl·lules vegetals estan protegides contra l'oxidació de les ROS per un ampli espectre de sistemes fotoprotectors (Krinsky 1992) que eliminen els radicals lliures dins dels quals s'hi inclouen tant els enzims antioxidants, que desactiven les ROS com l'ascorbat peroxidasa, la glutatió reductasa i la superòxid dismutasa, així com altres de no-enzimàtics (Polle i Rennenberg 1994, Smirnoff 2006).

El grup dels antioxidants no-enzimàtics està compost per molècules de baix pes molecular biològicament actives que poden prevenir el dany cel·lular induït pels radicals lliures, d'entre les quals hi ha substàncies tan conegudes com les vitamines A, C (ascorbat) o E (α-tocoferol), carotenoides, flavonoids o compostos fenòlics simples.

Alguns d'aquests compostos antioxidants són hidrofílics i s'ubiquen a l'estroma del cloroplast (e.g. ascorbat, glutatió) o dins el vacúol (e.g. fenols simples i flavonoids), mentre que altres són lipofílics i es troben a les membranes del cloroplast (e.g. carotenoides, α-tocoferol). Les vitamines antioxidants controlen la peroxidació i actuen ràpidament eliminant i/o desactivant la generació de radicals lliures.

L'ascorbat és quantitativament l'antioxidant predominant en les cèl·lules vegetals i en els teixits verds; el seu "pool" pot arribar a ser superior al 10% dels carbohidrats solubles totals (Noctor i Foyer 1998) i és el principal desactivador hidrofílic de les ROS. Al cloroplast, l'ascorbat es troba a l'estroma, on actua com a cofactor de la violaxantina de-epoxidasa (Müller-Moulé *et al*. 2002) i també intervé en la regeneració de l'α-tocoferol a partir del radical tocoferoxil (Beyer 1994).

Els tocoferols són molècules liposolubles, i per tant es troben a la membrana tilacoidal; els quatre tipus (α, β, γ i δ) es diferencien per els diferents substituents de la part polar (DellaPena i Last 2006). Aquests antioxidants no només detoxifiquen les ROS (interaccionant directament amb $^1O_2$ i OH^*) (Munné-Bosch i Alegre 2002), sinó que també tenen un paper important en la estabilització de la membrana (Havaux *et al*. 2003) i actuen com a senyals intracel·lulars i en el transport cíclic d'electrons (Kruk *et al*. 2000). A més se'ls han atribuït altres funcions no-antioxidants com la inhibició de quinases de proteïnes, o la proliferació cel·lular (Azzi i Stocker 2000).

Els fenols són metabòlits secundaris molt diversos (flavonoids, tanins, èsters d'hidroxicinamat i lignines) i abundants als teixits vegetals (Grace i Logan 2000). Els polifenols/flavonols i flavonol glicòsids tenen una capacitat antioxidant superior a la de l'ascorbat i el tocoferol *in vitro*, gràcies a la seva estructura química òptima per la detoxificació de les ROS. Aquest fet es deu a tres característiques bàsiques: a) la gran reactivitat com a donadors d'hidrogen o electrons, b) la capacitat del radical derivat polifenòlic per estabilitzar l'electró desaparellat i c) la capacitat de quelar ions metàl·lics de transició (reacció de Fenton) (Rice-Evans *et al*. 1997, Rice-Evans 2001). A més, els flavonoids poden alterar la cinètica de peroxidació mitjançant la modificació de l'ordre d'apilament dels lípids i la reducció de la permeabilitat de les membranes (Arora *et al*. 2000). També s'ha comprovat que els compostos fenòlics poden estar involucrats en les cascades de senyals del peròxid d'hidrògen (Takahama i Oniki 1997)

3. Les poliamines

Les poliamines són molècules policatiòniques i fortament bàsiques que es troben de manera ubiqua a les cèl·lules de tots els organismes vius: bacteris, fongs, animals i plantes. A les plantes es troben implicades en un ampli ventall de processos i diàriament s'estan investigant noves funcions i s'està treballant en els gens que regulen el seu metabolisme, catabolisme i que intervenen en les funcions on es troben implicades.

3.1. Propietats químiques

Les principals poliamines lliures a les plantes superiors són la putrescina (Put), l'espermidina (Spd) i l'espermina (Spm). Es troben de forma natural com unió d'amines alifàtiques amb pesos moleculars baixos: 88 la putrescina (que és una diamina) 145 l'espermidina (que és una triamina) i 202 l'espermina (que és una tetraamina).

La seva estructura química és la següent:

Putrescina: $H_2N\text{-}(CH_2)_4\text{-}NH_2$

Espermidina: $H_2N\text{-}(CH_2)_3\text{-}NH\text{-}(CH_2)_4\text{-}NH_2$

Espermina: $H_2N\text{-}(CH_2)_3\text{-}NH\text{-}(CH_2)_4\text{-}NH_2\text{-}(CH_2)_3\text{-}\ NH_2$

Aquestes molècules són solubles tant en aigua com en un gran nombre de solvents orgànics. En solucions aquoses, per tant, estan totalment solvatades i de cada poliamina se'n diferencien diverses conformacions, degut a que a cada enllaç s'hi donen rotacions lliures.

A pH fisiològic, aquestes poliamines es troben totalment protonades amb dues (putrescina), tres (espermidina) i quatre (espermina) càrregues.

Les poliamines no només actuen com a molècules lliures, sinó que també poden unir-se covalentment a àcids nucleics, fosfolípids, polisacàrids i proteïnes (incloent nombrosos enzims com la transglutaminasa) als quals modulen la seva activitat o unió covalent (Votyakova *et al*. 1999, Del Duca *et al*. 2000, Kakkar i Sawhney 2002).

En cèl·lules vegetals, aquestes amines es poden trobar conjugades covalentment a àcids fenòlics petits com àcids hidroxicinàmics (Martin-Tanguy 2001), donant lloc a les poliamines conjugades solubles, o a substàncies d'elevada massa molecular com les hemicel·luloses i lignines o en petites quantitats també poden unir-se a proteïnes (poliamines conjugades insolubles).

3.2. Metabolisme i catabolisme

La biosíntesi de les poliamines s'inicia amb la síntesi de putrescina. Aquesta síntesi en cèl·lules vegetals es pot donar per dues rutes diferents a partir de dos aminoàcids bàsics: l'arginina o l'ornitina (**Figura 9**). El fet que s'opti per una o altra via de síntesi depèn de l'espècie vegetal i del punt del desenvolupament en el qual es trobi la planta (Slocum 1991).

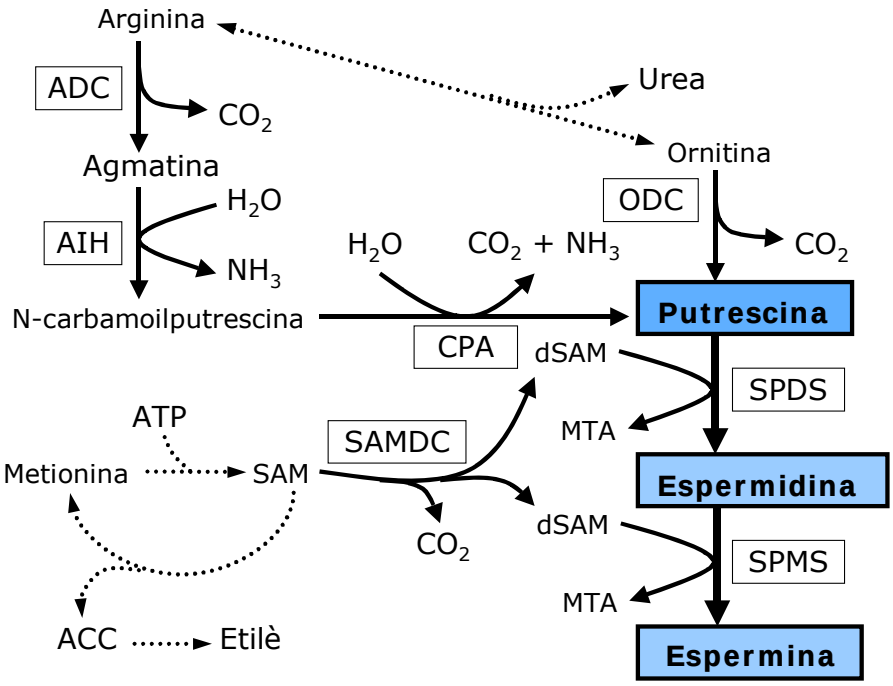

Figura 9 | Ruta de biosíntesi de les poliamines a les cèl·lules vegetals. En quadres buits s'hi representen els enzims que hi participen: arginina descarboxilasa (ADC); agmatina iminohidrolasa (AIH); N-carbamoil putrescina amidohidrolasa (CPA); ornitina descarboxilasa (ODC); S-adenosilmetionina descarboxilasa (SAMDC); espermidina sintasa (SPDS); espermina sintasa (SPMS). Altres abreviacions: S-adenosilmetionina (SAM); S-adenosilmetionina descarboxilada (dSAM); adenosil tri-fosfat (APT); àcid 1-aminociclopropà 1-carboxílic (ACC). (Modificat de Tiburcio *et al*. 1997 i Martin-Tanguy 2001).

En plantes superiors i en bacteris la descarboxilació de l'ornitina o de l'arginina es catalitzen per l'ornitina descarboxilasa (ODC) i l'arginina descarboxilasa (ADC) (Dat *et al*. 1998). Un cop obtinguda la putrescina, aquesta pot convertir-se en espermidina i en espermina mitjançant l'espermidina sintasa (SPDS) i l'espermina sintasa (SPMS), respectivament, els quals permeten

l'addició de grups amino-propil, que provenen de l'S-adenosilmetionina (SAM) en ser descarboxilada (dSAM) per l'enzim S-adenosilmetionina descarboxilasa (SAMDC) (Walters 2000).

La SAM és el precursor comú de les poliamines i de l'etilè, per tant, on es lliguen les dues rutes de síntesi (Lopez-Delgado 1998). Malgrat la SAM sigui substrat d'ambdues vies metabòliques, no s'ha trobat que la seva síntesi sigui un factor limitant per la síntesi de poliamines (Barceló-Coll *et al*. 2001).

Com en d'altres molècules vegetals, el "pool" de poliamines lliures intracel·lulars no depèn només de la seva síntesi, sinó que també depèn de varis processos de degradació (o oxidació), conjugació (ja descrita a l'apartat de propietats químiques) i transport.

La degradació o catabolització es dóna majoritàriament per la diamina oxidasa (DAO) i per la poliamina oxidasa (PAO) (Smith 1985 i Tiburcio *et al*. 1997) (**Figura 10**). Aquests enzims es troben associats a les parets vegetals dels teixits on s'està produint lignificació, suberització o augment de la rigidesa (Slocum 1991). La seva activitat depèn tant d'estímuls interns (hormones vegetals) com d'estímuls externs, de manera que les condicions ambientals com la intensitat lumínica poden afectar-ne la seva concentració cel·lular.

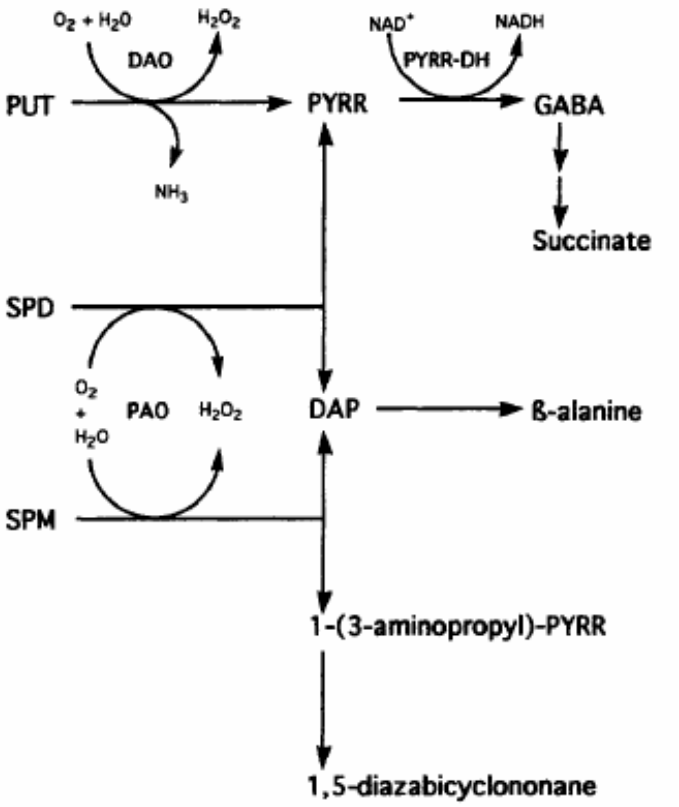

Figura 10 | Representació esquemàtica de la degradació de les poliamines per les amin-oxidases. Abreviacions: diamina oxidasa (DAO); diamino propà (DAP); àcid γ-aminobutíric (GABA); poliamina oxidasa (PAO); pirrolina (PYRR); pirrolina deshidrogenasa (PYRR-DH) (extret de Tiburcio *et al*. 1997).

3.3. Funcions

Les tres poliamines (PAs) més abundants en el regne vegetal: putrescina, espermidina i espermina s'han relacionat amb múltiples processos de regulació cel·lular, des del creixement, i la divisió cel·lular o la diferenciació vascular, embriogènesi, rizogènesi o desenvolupament floral i maduració dels fruits fins a la inhibició de la producció d'etilè i la senescència (Martin-Tanguy 1997, Kakkar i Sawhney 2002, Paschalidis i Roubelakis-Angelakis 2005).

A pH fisiològic, es troben en estat policatiònic i degut a aquesta propietat, s'ha suggerit que la majoria de les funcions fisiològiques que poden portar a terme són mitjançant la unió electrostàtica amb les càrregues negatives de molècules tals com els àcids nuclèics o els fosfolípids permetent l'estabilització dels cromosomes i de les membranes (Drolet 1986).

La seva naturalesa catiònica fa que en algunes situacions, les poliamines, puguin mimetitzar els efectes d'alguns cations com el Ca^{2+} i el Mg^{2+}. En aquests casos entren en competència pels mateixos llocs d'unió a receptors, enzims i membranes, de manera que poden modular els senyals de transducció i l'activitat enzimàtica. Aquestes interaccions bioquímiques donen a les PAs un paper important en la regulació de múltiples processos de creixement i desenvolupament (Bais i Ravishankar 2002, Martin-Tanguy 2001) així com en la resposta a diferents estressos (Bouchereau *et al*. 1999, Kumar *et al*. 1997).

Malgrat els últims anys s'han descrit les poliamines com a antioxidants eficients en diferents tipus d'estressos biòtics i abiòtics (Boucherau *et al*. 1999, Erdei *et al*. 2001, Flores i Galston 1984, Kramer i Wang 1989, Løvaas i Olsen 1998, Rácz 1996, Szalai *et al*. 1997, Tiburcio *et al*. 1997, Walters 2003), el seu paper en la protecció enfront l'estrès oxidatiu està encara sota estudi.

Així doncs, una de les funcions més interessants de les poliamines és el seu possible efecte antioxidant degut a la combinació de les seves propietats d'unió a anions i a cations. La unió a anions (fosfolípids de membrana, àcids nuclèics) contribueix a que es concentrin en zones sensibles a l'oxidació, mentre que la unió a cations evita la formació de ROS (com els radicals hidroxil o els singlets d'oxigen) en llocs específics (Løvaas 1997). La primera publicació que citava les poliamines com a possibles antioxidants data de 1979, on es va comprovar que les poliamines inhibien la peroxidació lipídica en microsomes de ronyó de rata (Kitada *et al*. 1979).

Besford *et al*. (1993) observaren que l'addició de PAs inhibia la destrucció de tilacoides i prevenia la pèrdua de pigments durant la senescència en protoplasts aïllats i Beigbeder *et al*. (1995) descrigueren la relació entre els nivells de PAs i les taxes de síntesi de clorofil·la i fotosíntesi durant el desenvolupament de pro-plàstids dins els cloroplasts.

La contribució de les PAs a la protecció de l'aparell fotosintètic en àlgues verdes s'ha ampliat recentment gràcies a l'equip dirigit pel Dr. Kiriakos Kotzabasis i la Dra. Elena Navakoudis que han treballat amb *Scenedesmus obliquus* aplicant diferents tipus d'estressos com CO_2 elevat (Logothetis *et al*. 2004), radiació UV-B (Sfichi *et al*. 2004), i contaminació per ozó (Navakoudis *et al*. 2003). Prèviament (Kotzabasis *et al*. 1993) comprovaren que en extractes de fulles d'espinacs, les pincipals PAs vegetals es trobaven associades a les membranes del tilacoid i a múltiples sub-complexes fotosintètics, mentre que al centre de reacció del PSII s'hi trobava principalment Spm.

No obstant, els treballs relacionats amb la composició en PAs de plantes superiors ubicades en el seu entorn natural són escassos, destacant els de: Erdei *et al*. (2001) amb *Phragmites australis*, Gicquiaud *et al*. (2002) amb *Bromus* o Jouve *et al*. (2004) amb *Populus tremula*. A més, la flexibilitat de la seva composició és molt alta tal i com s'ha demostrat en *Pringlea antiscorbutica* (Hennion *et al*. 2006). Per aquest motiu, en aquest treball s'ha estudiat el paper de les poliamines lliures i lligades a proteïna en alzina durant diferents condicions de llum en el seu propi hàbitat i en condicions controlades.

4. L'enzim transglutaminasa

Les transglutaminases (TGases; E. C. 2.3.2.13) (R-glutaminil-pèptid-amina-γ-glutamil transferases), són una família d'enzims intra i extracelul·lars que catalitzen modificacions post-traduccionals de proteïnes establint enllaços N-(O-glutamil) i unions covalents amb poliamines amines (Lorand i Graham 2003).

Les TGases es troben àmpliament distribuides en bacteris, animals i plantes. Tanmateix, la recerca s'ha focalitzat en els sistemes animals, en els quals es va trobar la primera activitat TGasa en mamífers (Folk i Cole 1966, Folk i Chung 1973, Folk 1980), mentre que en plantes no va ser fins al 1987 quan Icekson i Apelbum la trobaren en plàntules de pèsol (revisat a Folk 1980, Lorand i Conrad 1984). Tot i que les TGases s'han trobat a múltiples òrgans en plantes superiors i inferiors, les TGases cloroplàstiques són les que han despertat més interès i de les vegetals fou la primera a ser clonada (Torné *et al*. 2002).

4.1. Propietats químiques

Les TGases tenen una triada catalítica de cisteïna, histidina i aspartat (asparagina). Les modificacions post-traduccionals de proteïnes que efectuen les TGases (**Figura 11**) catalitzen unions amides covalents entre un grup amino primari o el grup ε-amino de la lisina (amino donadors) i un grup γ-carboxiamida d'un residu glutamil d'algunes proteïnes o pèptids (amino receptors) (Folk 1980, Lorand i Graham 2003).

El rang òptim de pH per a les TGases vegetals es troba entre 7.6 i 8.5, el qual és molt similar al de les TGases d'origen animal. Alguns resultats com el reconeixement de proteïnes de diferent pes molecular o un àmpli rang de pH òptim i diferents corbes d'activitat, són els que suggereixen la presència de múltiples formes d'aquest grup d'enzims, així com el fet de que algunes podrien ser específiques per cada òrgan.

En mesurar-se la incorporació de putrescina a N-N-dimetil caseina en la fracció precipitada d'un extracte de meristems apicals de plantes etiolades de pèsol va comprovar-se que l'enzim responsable era soluble i presentava la cinètica típica descrita per Michaelis i Menten. Aquesta activitat fou promoguda per Ca^{2+} i inhibida per coure i DTT (DL-ditio-treitol).

Les transglutaminases vegetals són capaces de reconèixer zones específiques a substrats descrits per a transglutaminases animals com la insulina, el fibrinogen, la pepsina i la trombina. Amb *Helianthus tuberosus* també va demostrar-se que les TGases vegetals són capaces de reconèixer substrats que reconeixen les TGases d'origen animal, com el dipèptid sintètic Z-L-glutaminil-L-leucina (Serafini-Fracassini *et al*. 1994). Del Duca *et al*. (1994) i Bregoli *et al*. (1994) demostraren que les TGases de plantes inferiors i superiors presenten reacció creuada amb els anticossos contra transglutaminases d'origen animal. També descriuen aquest grup d'enzims altres característiques com la dependència de calci o el reconeixement de la dimetil caseina com a substrat.

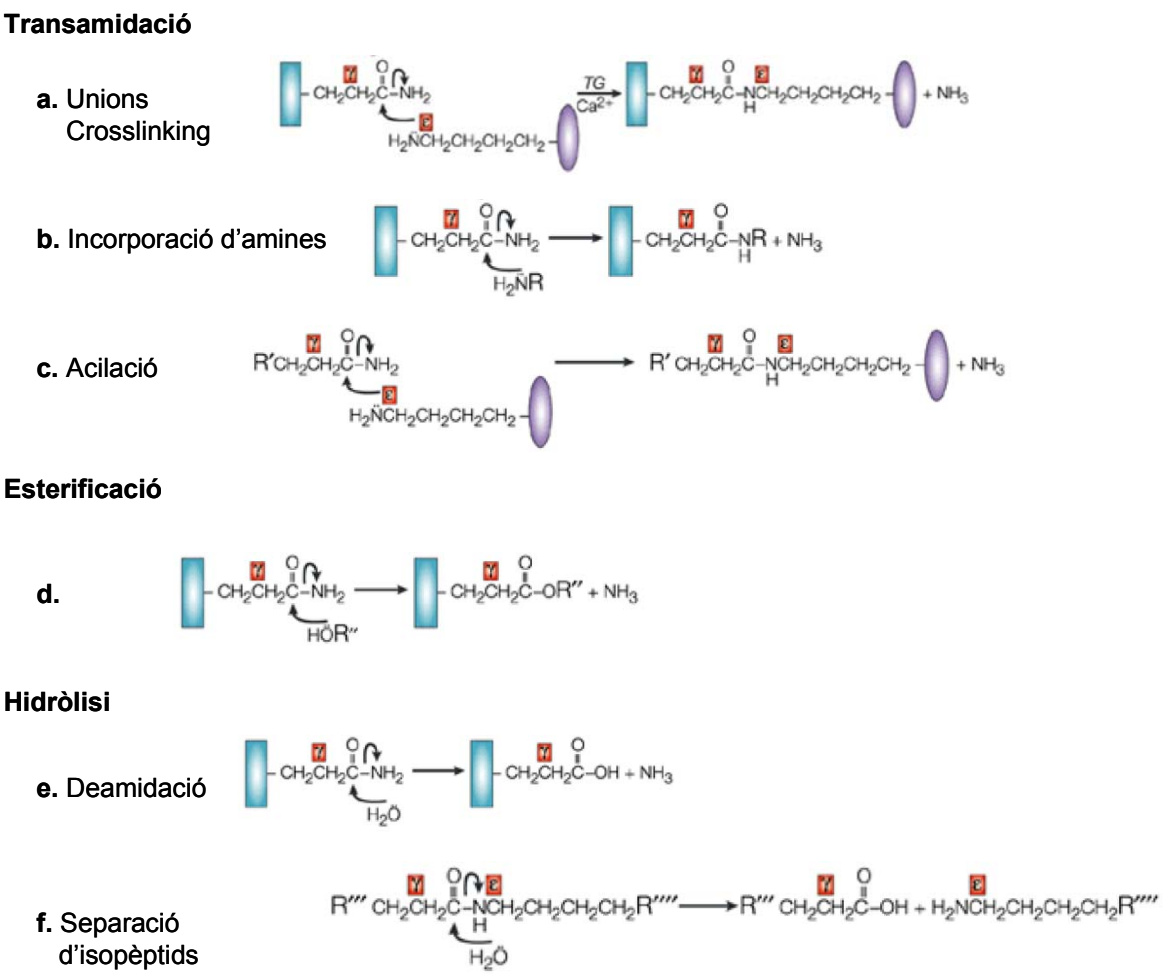

Figura 11 | Reaccions post-translacionals de les transglutaminases. La transamidació pot provocar **a |** Unió crosslinking entre proteïnes formant un pont N^{ε}(γ-glutamil) lisina isopèptid entre una lisina (Lys) desprotonada residu d'una proteïna donadora (elipse violeta) i l'acceptor glutamina (Gln) residu d'una altra (rectangle blau), **b |** la incorporació d'una amina (H_2NR) dins del residu Gln de la proteïna acceptora (les diamines i les poliamines poden actuar com a teter en un bis-glutaminil entre dues molècules acceptores) i **c |** l'acilació d'un locus Lys de la cadena de la proteïna donadora, **d |** esterificació, **e |** deamidació i **f |** separació per trencament d'isopèptids. Extret de Lorand i Graham 2003.

Les transglutaminases vegetals utilitzen les poliamines com a substrat donant del grup amino; aquest fet va demostrar-se en assajos on la incorporació de putrescina era retardada en presència d'altres diamines o poliamines. Les transglutaminases presenten diferències d'afinitat pels substrats amino, mostrant una afinitat superior per l'espermidina seguida per la espermina i finalment per la putrescina; aquests resultats es demostraren en esperma de ratolí (Paonessa *et al*. 1984), en àpexs etiolats d'*H. tuberosus* (Serafini-Fracassini *et al*. 1988) i també en el fong *Physarum polycephalum* (Klein *et al*. 1992). Les poliamines semblen tenir, tal i com s'ha descrit prèviament, un paper essencial en procesos de creixement i divisió cel·lular tant a microorganismes com a animals i plantes (Altman *et al*. 1983, Galston 1990), i per tant, la interacció entre les transglutaminases i les poliamines és d'un evident interès científic.

4.2. Funcions

Els estudis de les TGases en plantes s'han focalitzat en aspectes bioquímics relacionats amb la seva activitat, substrats sobre els que actuen i teixits en els que hi és més abundant. No obstant, la informació sobre la seva funció en els processos on intervenen és parcial, com per exemple en el creixement i desenvolupament, la morfogènesi, la fotosíntesi o la mort cel·lular programada (Margosiak *et al*. 1990, Del Duca *et al*. 1994, 2000, Lilley *et al*. 1998, Bernet *et al*. 1999, Serafini-Fracassini i Del Duca 2002).

En plantes, s'ha detectat activitat TGasa a certes cèl·lules d'alguns òrgans i orgànuls. De les dades que es disposen actualment se'n dedueix que, en cèl·lules vegetals, el paper de les transglutaminases és similar al de les cèl·lules animals, tot i que la seva presència a determinats compartiments cel·lulars específics i el tipus de substrats que utilitzen, suggereixi que a més poden tenir altres funcions. La localització de l'enzim i els seus substrats en diferents compartiments cel·lulars és un dels temes d'estudi importants, en plantes degut al baix nombre de referències (veure la revisió de Serafini-Fracassini i Del Duca 2008).

Alguns estudis inicials indicaren que als cloroplasts també s'hi detectava activitat transglutaminasa (Margosiak *et al*. 1990, Klein *et al*. 1992). Del Duca *et al*. (1994), mitjançant anticosos policlonals en front una TGasa animal, trobaren que les apoproteïnes del complexe antena (com les LHCII) eren substrats de les TGases en els cloroplasts de les fulles d'*H. tuberosus*. A més, els cloroplasts (i el

seu contingut) són altament dependents la llum, ja que sofreixen una sèrie de canvis en el desenvolupament que poden ser activats o desactivats per la presència o no de llum (Del Duca *et al*. 2000), com per exemple la síntesi de pigments de membranes. S'ha demostrat que la TGasa pot ser sensible a la llum, a canvis hormonals o als ritmes llum/foscor (Bernet 1997, Bernet *et al*. 1999, Villalobos *et al*. 2001, Villalobos 2007). Degut a aquesta sensibilitat per la llum i la possible implicació de la TGasa en els processos lumínics s'ha volgut ampliar el coneixement de les funcions de les TGases vegetals. Els nostres estudis han focalitzat el paper de les TGases en la seva funció com a lligands de les poliamines a membranes per a la protecció en front l'excés lumínic.

5. L'espectroscòpia de reflectància al vermell proper (NIRS) i la seva utilització en l'avaluació dels sistemes de fotoprotecció

El NIRS (Near Infrared Reflectance Spectroscopy) constitueix una tècnica molt pràctica i, en conseqüència, utilitzada habitualment en estudis de la composició bioquímica dels aliments o de material vegetal a través de l'anàlisi de la reflectància difosa de les mostres (Williams 1975).

5.1. Bases metodològiques

Les vibracions moleculars d'enllaços com O–H, C–N, N–H i C=O, característics de la matèria orgànica, permeten l'absorció de la llum entre 780 i 2500 nm determinant un espectre únic per cada mostra analitzada (Osborne *et al*. 1993). Per tant, l'espectre de llum que es reflecteix per una mostra conté detalls de la composició química (com per exemple el nombre i naturalesa dels enllaços que el constitueixen) d'aquell material en concret, els quals permeten estimar-ne, indirectament, el contingut (Birth i Hecht 1987). Per tal de determinar la concentració d'un constituent orgànic concret (i.e. %C, %N), es necessita construir un model basat en les relacions que existeixen entre els espectres d'absorció i la composició bioquímica concreta d'un rang ampli i nombrós de mostres (**Figura 12**).

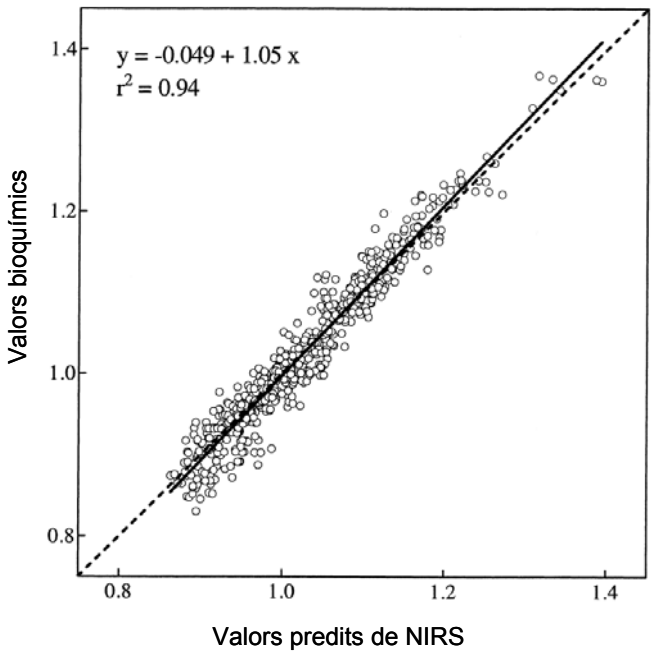

Figura 12 | Correlació entre els valors obtinguts de l'anàlisi bioquímic de cadascuna de les mostres i el seu valor respectiu en NIRS. Modificat de Joffre *et al*. 2001.

Les mesures de reflectància de llum monocromàtica entre 400 fins a 2500nm a través del NIRSystems 6500 (Foss NIR Systems, Inc, Silver Spring, MD) produeixen un espectre amb 1050 punts d'informació cada 2nm dins d'aquest rang (**Figura 13**).

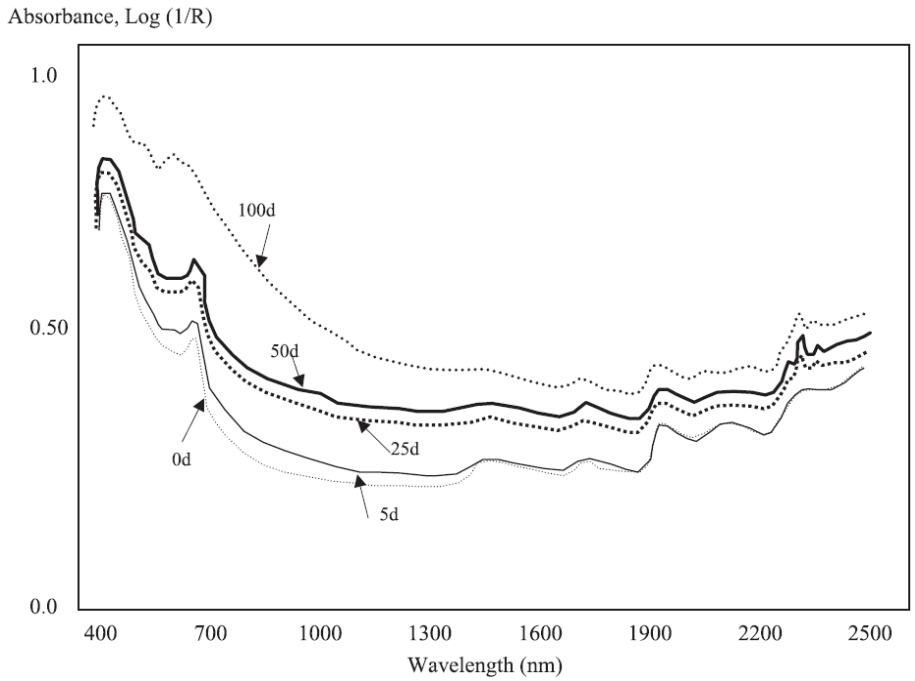

Figura 13 | Diferents espectres d'absorció NIRS de mostres d'*Atriplex portulacoides* L. Modificat de Bouchard *et al*. (2003).

5.2. Antecedents

El NIRS permet anàlisi de material vegetal ràpides, repetibles i acurades dels continguts en molècules hidrocarbonades i altres nutrients (McLellan *et al*. 1991, Joffre *et al*. 1992, Foley *et al*. 1998, Gillon *et al*. 1999, Gillon i David 2001). A *Quercus ilex*, s'ha aplicat per estudis de remobilització de nutrients (Cherbuy *et al*. 2001), de predicció de les taxes de descomposició de la matèria orgànica (Bouchard *et al*. 2003), de l'efecte de la sequera (Peña-Rojas *et al*. 2005) i d'efecte del CO_2 elevat (Staudt *et al*. 2001, Peñuelas *et al*. 2002, Aranda *et al*. 2006). En aquests estudis es determinaren les concentracions foliars de nitrogen (N), sucres solubles, midó, cel·lulosa, hemicel·lulosa, lignina i lípids en base a unes equacions de calibració construïdes amb 260 espectres i la seva respectiva composició (Meuret *et al*. 1993).

A més, en quant a la determinació d'antioxidants, en productes farmacèutics i alimentaris, l'espectroscopia del NIR s'ha utilitzat per a determinar vitamina C (àcid ascorbic) (Yang i Irudayaraj 2002) i alguns estudis han recolzat l'aplicabilitat del NIRS a l'anàlisi quantitativa del total d'antioxidants en el te verd (Luypaert *et al*. 2003, Zhang *et al*. 2004).

En aquest treball s'ha volgut ampliar la base de dades en alzina per a la quantificació de diversos components foliars relacionats amb la fotoprotecció: els pigments cloroplàstics (neoxantina, violaxantina, zeaxantina, anteraxantina, α i ß-carotè, luteïna, luteïna epòxid, clorofil·les *a* i *b*) i alguns antioxidants fotoprotectors (α-, β- i γ-tocoferol, ascorbat i fenols totals). Les noves correlacions facilitaran les anàlisi d'antioxidants i pigments cloroplàstics en fulles liofilitzades als investigadors que treballin amb alzina i desitgin l'ús d'una tècnica àgil (una sola lectura), no-destructiva i econòmica amb resultats fiables.

En quant a aplicacions pràctiques de la base de dades, s'han estudiat: 1) les variacions de les concentracions d'aquestes molècules en un bosc amb alzines adultes i rebrots després de tala a les dues estacions més estressants (estiu i hivern) i 2) les variacions a rebrots d'alzines originats i sotmesos a concentracions de CO_2 ambientals o elevades i les respostes obtingudes aplicant un estrès tèrmic a cadascun dels tractaments.

5.3. Liofilització

Donat que la majoria de reaccions en els organismes vius es donen en matriu aquosa i l'aigua és el medi on tenen lloc la majoria de reaccions de les vies de degradació (com per exemple de les proteïnes: Arakawa *et al*. 1993, Manning *et al*. 1989) per a assegurar la no-degradació de les molècules que composen les mostres vegetals, en general, es procedeix a la seva congelació en nitrogen líquid, mantenint-lo posteriorment a -80ºC. Malgrat la seguretat en la conservació de la composició de la mostra, aquest protocol a baixes temperatures en limita el transport i l'emmagatzematge. Actualment, però, la liofilització és una tècnica àmpliament estesa, que s'utilitza per la deshidratació del material vegetal permetent una extracció de l'aigua de la mostra congelada mitjançant la pressió del buit exercida directament sobre els teixits vegetals per sota del punt triple. Per tant, l'eliminació del màxim d'aigua possible mitjançant la liofilització promou la conservació de les mostres vegetals amb les mínimes alteracions bioquímiques allargant el temps de durabilitat abans de la seva anàlisi (Carpenter i Chang 1996, Carpenter *et al*. 1997, Cherian i Corona 2006).

Diversos treballs han utilitzat aquesta tècnica per la mesura d'antioxidants i pigments cloroplàstics (Tausz *et al*. 2003, Nessa *et al*. 2005, Sivakumar *et al*. 2005).

Conservants durant el procés de liofilització

El fet d'afegir antioxidants o altres conservants abans o durant la liofilització pot frenar o alentir les reaccions de formació de radicals durant el procés o al llarg del magatzematge (De Paz *et al*. 2002) i permetre així el control de la degradació bioquímica. Generalment, els antioxidants més utilitzats són agents reductors com els fenols, tiols i aldehids, ja que, en presència d'una molècula ROS, aquests agents reductors són compostos que s'oxiden més ràpidament que els altres components cel·lulars, evitant l'oxidació d'aquests últims. Alguns biocides també s'utilitzen com a conservants ja que la seva presència evita la degradació microbiana del material vegetal.

La majoria dels estudis relacionats amb aquesta temàtica han considerat com a criteri només l'estabilitat física del material (Kreilgaard *et al*. 1998) però en aquest estudi s'han volgut analitzar les modificacions en la seva composició química (pigments cloroplàstics i antioxidants lipofílics) i s'ha estandarditzat un protocol amb els passos a seguir a fi de poder assegurar la conservació del material vegetal (utilitzant espècies amb estructura física i química molt diferenciada) i evitar la degradació de diferents components cel·lulars fent especial èmfasi als diversos components cloroplàstics mitjançant l'aplicació de múltiples opcions de liofilització i comparant-les amb el material vegetal fresc. Els diferents protocols de liofilització que s'exposen en aquest treball inclouen l'addició dels següents compostos utilitzats com a conservants: els antioxidants 2,6-ditertbutil-4-metilfenol, cisteamina o 1,4-ditiotreitol; o el biocida 2-butilamino etanol.

L'objectiu general d'aquest treball és aprofundir en el coneixement dels sistemes de fotoprotecció en les fulles de *Quercus ilex*.

Per aconseguir-ho s'han desenvolupat els següents objectius específics:

1. Estandardització d'un protocol per assegurar la conservació del material vegetal durant el seu magatzematge, transport i anàlisi evitant la degradació de les molècules implicades en la fotoprotecció mitjançant:

- **a.** Comparació dels resultats obtinguts entre el material vegetal fresc i el material vegetal liofilitzat utilitzant múltiples protocols de liofilització.
- **b.** Comprovació de l'efecte de l'addició de diferents compostos utilitzats com a conservants durant la liofilització.

2. Realització d'una base de dades correlacionant els resultats de les anàlisi bioquímiques amb els obtinguts a través de l'anàlisi per espectroscòpia de reflectància al vermell proper (NIRS) del material vegetal, permetent la quantificació de:

- **a.** Pigments cloroplàstics (neoxantina, violaxantina, zeaxantina, anteraxantina, α i ß-carotè, luteïna, luteïna epòxid, clorofil·les *a* i *b*).
- **b.** Antioxidants fotoprotectors (α, β i γ-tocoferol, ascorbat i fenols totals).

3. Aplicació de la nova base de dades NIRS descrivint les respostes dels compostos antioxidants en els següents estudis:

- **a.** Caracterització en fulles d'alzines adultes i de rebrots després de tala en bosc durant les dues estacions més estressants (estiu i hivern).
- **b.** Caracterització en rebrots d'alzina sota condicions de CO_2 elevat i estrès tèrmic (per alta temperatura).

4. Estudi del paper de les poliamines (lliures i lligades a proteïna) en la fotoprotecció en alzina en el seu propi hàbitat i en condicions d'irradiància controlada.

5. Estudi del paper de les transglutaminases vegetals en la seva funció com a lligands de les poliamines a membranes per a la protecció en front a l'excés lumínic.

The main aim of the present work was to go deeper into our knowledge of photoprotection systems acting in *Quercus ilex* leaves.

To achieve this final objective, the following specific aims have been developed:

1. Standardization of a protocol to ensure the conservation of the plant material during its storage, transport and analysis avoiding the degradation of the molecules implied in photoprotection by:

   a. Comparison of the results obtained between fresh plant material and lyophilized plant material using several lyophilization procedures.

   b. Verification of the effect of the addition of different compounds used as preservatives during lyophilization.

2. Obtention of a database relating the biochemical analysis results with those obtained by the use of Near Infrared Reflectance Spectroscopy (NIRS) of the same plant material and allowing the quantification of:

   a. Chloroplastic pigments (neoxanthin, violaxanthin, zeaxanthin, anteraxanthin, α and ß-carotene, lutein, lutein epoxide, chlorophyll *a* and *b*).

   b. Antioxidant photoprotectors (α, β and γ-tocopherol, ascorbate and total phenols).

3. Application of the new NIRS database characterizing the antioxidant compounds responses in the following studies:

   a. Characterization in leaves of adult holm oak and resprouts after clear cut in their natural environment during the two stressing seasons (summer and winter).

   b. Characterization in holm oak resprouts under elevated CO_2 conditions and high temperature stress.

4. The study of the role of polyamines (free and protein-bound) in photoprotection in holm oak in its natural habitat or under controlled-irradiance conditions.

5. The study of the role of the plant's transglutaminases and its function binding polyamines to membrane proteins in the protection against high light stress.

INFORME DE LA DIRECTORA DEL FACTOR D'IMPACTE DELS ARTÍCLES PUBLICATS I PARTICIPACIÓ DE LA DOCTORANDA

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La Dra. Isabel Fleck Bou, com a directora de la tesi que porta per títol: "Sistemes de fotoprotecció a *Quercus ilex* L. i aplicació de la NIRS (espectroscòpia de reflectància a l'infraroig proper) com a tècnica ecofisiològica per la detecció d'estrès oxidatiu"que ha dut a terme la doctoranda Marta Pintó Marijuan,

Informa sobre la participació de la doctoranda en cadascun dels articles inclosos en la memòria de l'esmentada Tesi.

Capítol 1. Article: "Freeze-drying conditions for pigment and antioxidant evaluation in plant material", no publicat, la versió que es presenta en la memòria de tesi es la versió revisada i consensuada pels diversos coautors i enviada a una revista especialitzada internacional. Tracta de la optimització del procés de liofilització a fi de disposar d'un material inalterat cara a la seva utilització en la realització d'una base de dades mitjançant NiRS (veure capítol 2). El disseny experimental s'ha dut a terme conjuntament entre la Dra. I. Fleck, la doctorand i el Dr. I. Casals, director del servei d'Anàlisi dels Serveis Científico-Tècnics de la Universitat de Barcelona. La doctorand ha realitzat tots els processos de liofilització amb addició de diversos conservants. La comprovació de la idoneïtat de la metodologia es realitzà avaluant el contingut en pigments cloroplàstics mitjançant HPLC al Departamento de Biología Vegetal y Ecología, Universidad del País Vasco, Bilbao, sota la supervisió dels doctors JI García-Plazaola i JM Becerril i la col·laboració de R. Esteban. La doctoranda ha redactat l'article assessorada pels diversos coautors.

Capítol 2. Article: "Antioxidative and photoprotective defence systems in *Quercus ilex* L. evaluated by Near Infrared Reflectance Spectroscopy", no publicat, la versió que es presenta en la memòria de tesi es la versió revisada i consensuada pels diversos coautors i enviada a una revista especialitzada internacional. En aquest estudi s'ha realitzat una base de dades mitjançant l'aplicació de la espectroscòpia per reflectància en el vermell proper (NIRS) a fi d'avaluar en una sola mesura el contingut en compostos de caràcter antioxidant en fulles d'alzina. El disseny experimental s'ha fet conjuntament entre la directora i la doctoranda, l'assessorament bioquímic ha estat realitzat per Dr. I. Casals, director del servei d'Anàlisi dels Serveis Científico-Tècnics de la Universitat de Barcelona. La doctoranda ha intervingut en el mostreig i realització de les anàlisis bioquímiques corresponents a la quantificació de pigments cloroplàstics i tocoferols realitzades al Departamento de Biología Vegetal y Ecología, Universidad del País Vasco, Bilbao, sota la supervisió dels doctors JI García-Plazaola. Les anàlisi de la quantificació d'ascorbat i fenols totals foren realitzats al IBAF- CNR de Roma per l'equip dels Drs. Zacchini i de Agazio. Les lectures NIRS foren realitzades per la doctoranda en el CEFE-CNRS de Montpellier gràcies a una subvenció per part del GDRE (Grup de Recerca

Europeu) ja que aquest treball forma part del programa Ecosistemes en un món canviant del que forma part l'equip de la doctoranda. Les equacions de cal·libració foren desenvolupades pel Dr. Richard Joffre del CEFE-CNR, en el marc de la citada col·laboració. La doctoranda també ha realitzat les mesures fisiològiques de característiques hídriques, estructurals i fotoquímiques i ha redactat l'article assessorada pels diversos coautors.

Capítol 3. Article: "Antioxidant protection during heat stress in holm-oak resprouts originated from plants grown at elevated CO_2", no publicat , la versió que es presenta en la memòria de tesi es la versió revisada i consensuada pels diversos coautors per ser enviada a una revista especialitzada internacional. El disseny experimental s'ha realitzat entre la directora i la doctoranda. En aquest treball s'ha aplicat la base de dades realitzada mitjançant l'aplicació de la NIRS a un estudi sobre l'efecte del CO_2 elevat i altes temperatures sobre les respostes antioxidants de rebrots d'alzina. Els resultats de la utilització de la base de dades NIRS han estat calculats pel Dr. Richard Joffre del CEFE-CNR. M. Guàrdia ha estat la responsable del comptatge estomàtic. La doctoranda ha redactat l'article assessorada pels diversos coautors.

Capítol 4. Article: "Seasonal and diurnal monitoring of leaf polyamine content in *Quercus ilex* L. resprouts after fire in relation to changes in irradiation and photosynthetic parameters", publicat a la revista Trees: Structure and function, índex d'impacte (2008) 1.4676. Aquest article descriu les variacions estacionals i diàries en les tres principals poliamines, els seus canvis en alzines de bosc i els seu paper en la fotoprotecció de l'aparell fotosintètic. El disseny experimental s'ha fet conjuntament entre la directora i la doctoranda. La determinació del contingut en poliamines lliures va ser realitzat per la doctoranda al IBAF-CNR de Roma durant diverses estades) sota la supervisió dels Drs. De Agazio i Zacchini. Les mesures de bescanvi de gasos, fluorescència de les clorofil·les i corbes de resposta de la fotosíntesi a les variacions de llum i CO_2 foren realitzades al bosc per la doctoranda. La doctoranda ha redactat l'article assessorat pels diversos coautors.

Capítol 5. Article: "Response of transgluminase activity and bound putrescine to changes in light intensity under natural or controlled conditions in *Quercus ilex* leaves" publicat a la revista Physyiologia Plantarum, índex d'impacte (2008) 2,192. En aquest treball s'aprofundí en les relacions entre poliamines i fotoprotecció avaluant el paper de les poliamines lligades a proteïnes en diverses situacions d'iluminació natural en plantes en test. El disseny experimental s'ha fet conjuntament entre la directora, la doctoranda i els Drs. JM. Torné i M. Santos del Departament de Genètica Molecular, Laboratori de Genètica Molecular Vegetal, CSIC. L'avaluació dels contingut en poliamines lliures i lligades a proteïnes es realitzà al IBAF-CNRS sota la supervisió dels Drs. De Agazio i Zacchini. La determinació de l'activitat TGasa es realitzà al CSIC sota la supervisió dels Drs. Torné i Santos. La doctoranda realitzà les mesures fotosintètiques i de fluorescència de les clorofil·les i ha redactat l'article assessorat pels diversos coautors.

Annex 1. Article: "Alternative methods for sampling and preservation of photosynthetic pigments and tocopherols in plant material from remote locations", no publicat , la versió que es presenta en la memòria de tesi es la versió revisada i consensuada pels diversos coautors i enviada a una revista especialitzada internacional. Realitzat en col·laboració amb diversos organismes: Departamento de Biología Vegetal y Ecología Universidad del País Vasco, Departamento de Biología Vegetal; Facultad de Biología Universidad Complutense de Madrid; Departamento de Fisiología y Ecología

Vegetal CCMA-CSIC, Madrid; Serveis Científico-Tècnics Universitat de Barcelona i Departamento de Biología Vegetal. Universidad de La Laguna, Tenerife. La doctoranda ha intervingut en el disseny experimental així com en el mostreig i preparació dels diferents protocols de conservació de diversos materials.

Annex 2. Article: " Leaf flavonoid content in *Quercus ilex* L. resprouts and its seasonal variation". Acceptat per a la seva publicació a Trees: Structure and function, índex d'impacte (2008) 1.4676. La doctoranda participà en la presa de mostres i en l'anàlisi de les característiques fotoquímiques del material vegetal .

Annex 3. Article: "Transplastomic tobacco plants over-expressing maize chloroplast transglutaminase: Modifications of the thylakoid appression pattern and photochemistry parameters". No publicat, la versió que es presenta ha estat revisada i consensuada pels diversos coautors i enviada a una revista especialitzada internacional. La doctoranda s'ha responsabilitzat de la realització de les mesures de la activitat TGasa i dels paràmetres de fluorescència de les clorofil·les així com de la determinació dels pigments cloroplàstics.

I per que consti als efectes oportuns,

Dra. Isabel Fleck Bou

Barcelona, 30 de setembre del 2008

CAPÍTOL 1.

FREEZE-DRYING CONDITIONS FOR PIGMENT AND ANTIOXIDANT EVALUATION IN PLANT MATERIAL

Marta Pintó-Marijuan [a], José-Ignacio García-Plazaola [b], José Maria Becerril [b], Raquel Esteban [b], Isidre Casals [c], Isabel Fleck [a]

Phytochemical Analysis (submitted)

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Condicions de liofilització per l'avaluació de pigments i antioxidants en material vegetal

La majoria d'anàlisi de pigments vegetals impliquen la congelació de les mostres en nitrogen líquid seguides per un posterior magatzematge a -80ºC fins al moment de l'extracció. Sense aquestes mesures és difícil poder garantir la integritat dels pigments cloroplàstics i dels antioxidants. En aquest treball s'estandarditzen les condicions a utilitzar durant la liofilització per obtenir material vegetal sec amb la composició bioquímica sense alterar. Van utilitzar-se fulles de sol i d'ombra de *Taraxacum officinale* Weber (herbàcia) i de *Quercus ilex* L. (escleròfil·la) per a comprovar protocols de liofilització amb l'addició de quatre conservants diferents: els antioxidants 2,6-ditertbutil-4-metilfenol, cisteamina, 1,4-ditiotreitol i el biocida 2-butilamino etanol. A cadascún dels tractaments van mesurar-s'hi un ampli rang de pigments cloroplàstics (neoxantina, violaxantina, zeaxantina, anteraxantina, α i β-carotè, luteina, epoxiluteina, clorofil·la *a* i *b*) així com α- i γ-tocoferol. Els resultats van comparar-se amb els obtinguts de les fulles liofilitzades sense l'addició de cap conservant o amb els de les fulles únicament congelades i sense liofilitzar. Es va avaluar per separat el millor mètode de conservació per cada grup de molècules. Tots els conservants induiren canvis en la composició de pigments (formació de feofitina, reducció de violaxantina, isomerització de la luteina) excepte 2-butilamino etanol, el qual protegí efectivament la majoria dels compostos en ambdues espècies i mantingué l'estat de de-epoxidació del cicle de les xantofil·les.

Paraules clau

Antioxidants; conservants; liofilització; pigments fotosintètics.

Freeze-drying conditions for pigment and antioxidant evaluation in plant material

Most methods of plant pigment analysis involve the freezing of samples in liquid nitrogen and their subsequent storage at –80°C until extraction. Without these measures it is difficult to guarantee the integrity of chloroplast pigments and antioxidants. Here we standardize the conditions used during freeze-drying to obtain dry plant material with an unaltered biochemical composition. Sun and shade leaves of a herbaceous (*Taraxacum officinale* Weber) and a sclerophyllous (*Quercus ilex* L.) species were used to test several lyophilization protocols involving the addition of four preservatives: the anti-oxidants 2,6-ditertbutyl-4-methylphenol, cysteamine, or 1,4-dithiothreitol; or the biocide 2-butylamino ethanol. A wide range of chloroplast pigments (neoxanthin, violaxanthin, zeaxanthin, antheraxanthin, α and β-carotene, lutein, epoxilutein, chlorophyll *a* and *b*) as well as α- and γ-tocopherol were measured in the all treatments. Results were compared with those obtained from freeze-dried leaves without the addition of preservative or with frozen non-lyophilized leaves. The best conservation method for each kind of molecule was evaluated. All preservatives induced pigment changes (phaeophytin formation, violaxanthin reduction, lutein isomerization) except 2-butylamino ethanol, which effectively protected most compounds in both species and maintained the de-epoxidation state of the xanthophyll cycle.

Keywords

Antioxidants; lyophilization; preservatives; photosynthetic pigments.

1.1. Introduction

The conservation of plant samples usually requires their freezing in liquid nitrogen and storage at –80°C until analysis. These conditions ensure there are no chemical transformations or pigment degradation, but they greatly restrict the storage and transport of samples when dry ice or liquid nitrogen are not available or, for instance, when they cannot be taken on planes as frozen hand luggage. Moreover, several days or even months may pass from the sampling date to the time of analysis. It is therefore necessary to establish a protocol to preserve plant samples against changes caused by the degradation of unstable molecules, air oxidation, enzyme reactions, etc. Protection from light and heat should be the first measure to be taken since these factors can stimulate the chemical changes responsible for modifications in target-molecules.

Given that most reactions in living organisms occur in an aqueous matrix and water facilitates or mediates a variety of physical and/or chemical degradation pathways, e.g. in proteins (Arakawa *et al*., 1993; Manning *et al*., 1989), the elimination of water by lyophilization or freeze-drying facilitates conservation. Lyophilization implies rapid freezing of the sample and subsequent drying of the frozen liquids by vacuum below the triple point. Lyophilization achieves better stability, decreased temperature sensitivity and extended shelf-life of the resultant product (Carpenter and Chang, 1996; Carpenter *et al*., 1997; Cherian and Corona, 2006). Moreover, freeze-dried material is also easier to grind, which implies that a homogeneous powder can be obtained (Tausz *et al*., 2003). There are several reports concerning the application of lyophilization for pigment and antioxidants measurements in the literature (Tausz *et al*.; 2003; Nessa *et al*., 2005; Sivakumar *et al*., 2005).

The addition of antioxidants or other preservatives before or during lyophilization can stop or slow down radical-forming reactions during the process as well as during storage (De Paz *et al*., 2002); most studies on this topic have considered only the physical stability of the material (Kreilgaard *et al*., 1998). Here we examine the effect on chemical degradation of adding a number of preservatives during the lyophilization of plant material. Typically, reducing agents such as phenols, thiols and aldehydes are used as antioxidants because they are oxidized earlier than other compounds. When a redox reaction occurs in the presence of one of these substances, if radicals appear, they are trapped by these agents and the oxidation chain is stopped. Several biocides are also used

as preservatives since microbiological growth spoils harvested vegetables. Moreover, most antioxidants are also biocides because they block biochemical reactions.

The aim of this study was first to standardize the conditions and requirements for the lyophilization of leaf material and second to test the effect of several preservatives on a number of chloroplast pigments and antioxidants. To this end, we used leaves from two species with contrasting foliar characteristics: a sclerophyllous (*Quercus ilex)* and a herbaceous (*Taraxacum officinale*) species. We tested the following preservatives: 2,6-ditertbutyl-4-methylphenol, cysteamine, 1,4-dithiothreitol and 2-butylamino ethanol. These molecules were chosen because they do not interfere with later HPLC analysis, they facilitate the preservation of chemical and physical structure, and they have the capacity to stop degradation. Moreover, as controls, we used lyophilized samples that were not treated with preservatives as well as frozen non-lyophilized leaves. To estimate the state of degradation/preservation of each sample and the effectiveness of the preservatives, we analysed several chloroplast pigments and lipophilic antioxidants.

1.2. Material and methods

Experimental sites and plant material

We used leaves from two structurally distinct plants: a sclerophyllous tree, the evergreen holm oak (*Q. ilex*), characteristic of the Mediterranean forests, and a perennial herbaceous plant, *T. officinale*, which is common throughout temperate regions.

Sampling to determine the optimal freeze-drying conditions was done in the winter on individually potted *Q. ilex* plants in the Experimental Fields of the University of Barcelona as well as in *Q. ilex* trees in Sant Medir forest (Serra de Collserola, Barcelona), where two plots were established: one undisturbed with control plants (C) and another which had been burned the previous summer and which showed post-fire resprouts (R).

We also performed pigment analysis after leaf treatment with the distinct preservative treatments used during lyophilization. Sampling was done in winter on seven *Q. ilex* potted plants in the Experimental Fields of the University of Barcelona and on six spontaneously-growing *T. officinale* plants. Leaves (fully-expanded and mature) of both species were collected at midday to avoid daily

variations of some molecules. They were then immediately frozen in liquid nitrogen and stored at -80ºC until analysis. In *Q. ilex,* sun and shade leaves were distinguished.

Lyophilization and sample preparation

To determine the optimal freeze-drying conditions of sclerophyllous material *Q. ilex* leaves stored at –80ºC were cut in liquid N_2 before lyophilization. Freeze-drying was carried out in a Virtis Lyophiliser (Freezemobile 6EL, Gardiner, N.Y., USA) in vials of 1.5 cm and 3.0 cm diameter respectively, for a range of times at the *Laboratori de Tècniques Separatives*, *Serveis Cientifico-Tècnics*, University of Barcelona. Immediately after freeze-drying, samples were weighed and lyophilized weight (LW) was obtained. All samples were stored under low humidity and dark conditions until analysis, during which samples were protected from light and atmospheric oxygen.

Water content determination of lyophilized samples: Karl Fischer titration and % dry weight

Two tests were performed to ensure the reliability of the freeze-drying process. Lyophilized samples were ground in an agate mortar (Mortar Grinder RM 100, Retsch, Haan, Germany) and immediately evaluated for water content. Karl Fischer (KF) titrations were carried out with a KF Titrino 701 from Metrohm, Herisau, Switzerland, at the *Servei d'Anàlisi Geoquímica, Serveis Cientifico-Tècnics UB*, by applying the two-component technique, with Hydranal-Titrant 5 and Hydranal-Solvent from Riedel-de Haën, Seelze, Germany. The KF titration is an extensively used technique for the determination of water content in a wide range of samples (Isengard and Schultheiβ, 2003; Grünke, 2001; De Caro *et al*., 2001; Schöffsky, 2001). This technique consists of the electrochemical measurement of sample humidity by means of a specific reagent (RN), a base.

$$I_2 + SO_2 + 3\ RN + CH_3OH + H_2O = 2\ RN{\cdot}HI + RNB{\cdot}HSO_4CH_3$$

Methanol, CH_3OH, is most commonly used as solvent in KF titration. The % mass of water/mass of the lyophilized sample was calculated.

% Dry weight determination

Freeze-dried leaves were weighed and either ground in an agate mortar (Mortar Grinder RM 100, Retsch, Haan, Germany) or kept intact. Both kinds of

material were placed in a forced-air oven at 60ºC; lyophilized dry weight (LDW) was calculated on various occasions in the course of one week of drying, as %LDW = (LDW/LW) x 100.

Frozen forest leaves were weighed (FW), allowed to defrost and thereafter maintained in an oven at 60ºC until constant weight. We also calculated % leaf dry weight (% DW= DW/FW x 100) in order to compare these values with those obtained from lyophilized material.

Preservatives

To evaluate the effect of several preservatives during lyophilization pools of 3-8 frozen leaves of *Q. ilex* or *T. officinale* leaves from 6-7 individuals were separated in five homogeneous parts; each received a different treatment before being stored at -80ºC. Four preservation treatments were tested: the antioxidant agents 1,4-dithiothreitol (DTT), cysteamine (CA), and 2,6-ditertbutyl-4-methylphenol (BHT); and the biocide (bactericide and algaecide) 2-butylamino ethanol (BAE) (1 ml of aqueous 0.25 M preservative per g of fresh weight (g FW)). The fifth part was left untreated and used as a control. Thereafter all samples were lyophilized.

DTT is a widespread antioxidant used for *in vitro* reactions and for incubating oxidized disulphides to yield free thiols. CA is an antioxidant amino acid whose chemical behaviour is quite similar to that of DTT. CA is a precursor of cysteine and other sulphur-containing molecules in metabolic pathways. BHT is an antioxidant used as a food preservative. It captures free radicals, thereby stopping typical chain reactions. BAE inhibits bacterial, mycobacterial and algal growth and can therefore interact with chemical reactions that are necessary to sustain life, such as redox reactions and others that could lead to the degradation of pigments and antioxidants.

Analysis of chloroplast pigment and lipophilic antioxidants were repeated by comparing the results obtained with the preservative treatment used during lyophilization with those from frozen non-freeze-dried samples.

Chloroplast pigment and tocopherol determination

For the determination of lipophilic antioxidants (α-, β-, δ- and γ-tocopherol) and chloroplast pigments (VAZ cycle components (violaxanthin (V), antheraxanthin (A), zeaxanthin (Z)), neoxanthin, α and β-carotene, lutein, epoxilutein, chlorophylls *a* and *b*), acetone extracts from lyophilized and frozen

leaves were analysed by reverse-phase high performance liquid chromatography (HPLC) following the method by García-Plazaola and Becerril (1999), with the modifications described in García-Plazaola and Becerril (2001). The de-epoxidation state is defined as the (A+Z)/VAZ ratio. Detection was carried out with a fluorescence detector (Waters model 474) set to λ_{exc} = 295 nm and λ_{em} = 340 nm calibrated with α-, β, δ- and γ-tocopherol standards (Calbiochem, San Diego, CA). Under these chromatographic conditions, we were unable to distinguish β- from γ-tocopherol neither to detect δ-tocopherol. The values of Chl *a* and Chl *b* correspond to the addition of their epimers to their respective values (van Leeuwe *et al*., 2006).

Statistical analysis

All statistical procedures were done using SPSS for Windows (SPSS for Windows v. 12.0, SPSS Inc., Chicago, IL, USA). Analyses of variance (ANOVA) were used to test the main effects and interactions, against appropriate error terms, on % of dry weight (LW *vs*. DW; Control *vs*. Resprouts) and on chloroplast pigment and tocopherol concentrations (between the lyophilization treatments; frozen samples *vs*. BAE-Lyophilization). Post-hoc Duncan test was applied when appropriate. Statistical significance was set at $p \leq 0.05$. The number of replicates is indicated in the table and figure legends. Correlation coefficients between frozen and BAE-Lyophilization values were calculated.

1.3. Results and discussion

Freeze-drying of sclerophyllous material

To establish a protocol to extract the maximum volume of water during freeze-drying for sclerophyllous leaves, several lengths of lyophilization were tested. Our results indicate that after five days of freeze-drying, samples were at their minimum weight (i.e. minimum water content). To ascertain the reliability of the method, after the material was lyophilized we took several measurements of its water content. First, KF titration was applied on two replicates per plant (4 individuals) and our results showed that the water content after lyophilization was very small (5.35 ± 0.22 %). Secondly, ground and intact lyophilized leaves were kept in a forced air-oven and the %LDW was calculated on various occasions in the course of one week of drying. After 120 h, LDW was constant around 5% without significant differences between both kinds of material (Fig.

1). These results indicate that a 5-day lyophilization produces the greatest reduction of water content in sclerophyllous material.

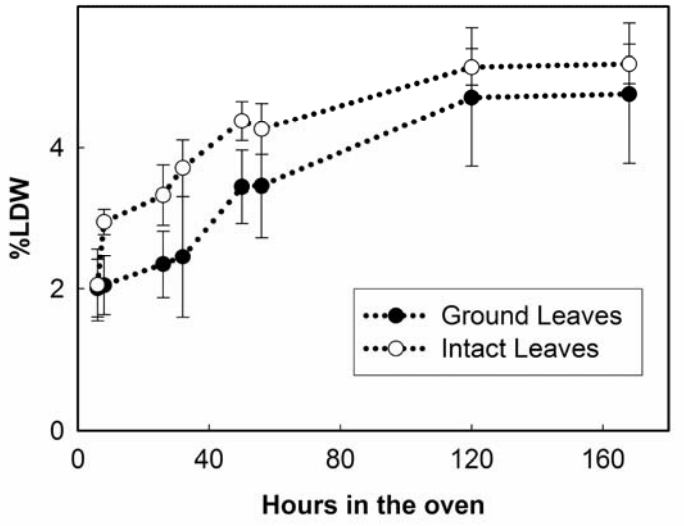

Figure 1. % Dry weight variation of lyophilized *Quercus ilex* leaf samples (%LDW) kept for one week in a forced air-oven at 60ºC. Leaves ground in agate mortar and intact leaves.

Finally, we compared the leaves of *Q. ilex* forest trees with distinct ecophysiological characteristics: undisturbed controls and resprouts originated after a summer fire. Significant differences in structural parameters between controls and resprouts were detected (Fig. 2), with greater water availability for small resprouting shoots (reflected in their lower %DW and %LW), which is consistent with the findings of a previous study (Peña-Rojas *et al*., 2004). In addition, we examined how these differences were maintained in lyophilized and in oven-dried material. No differences between %LW and %DW were detected, indicating that the freeze-drying protocol was appropriate.

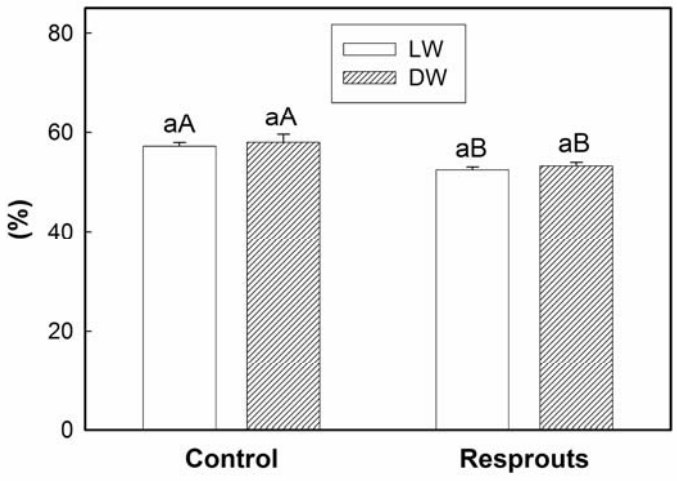

Figure 2. Percentage of lyophilized weight (%LW) and percentage of dry weight (%DW) in control trees and resprouts after fire. Values are mean ± SE of three pools, each with 3-5 leaves from 7 *Q. ilex* plants. Different letters indicate significant differences ($p<0.05$) between %LW and %DW (a, b) or between kinds of leaf (control, resprouts) (A, B).

Preservatives

Chloroplast pigments and lipophilic antioxidants were analysed in *Q. ilex* and *T. officinale* leaves to compare the effectiveness of DTT-, CA-, BHT- or BAE-

Lyo (lyophilization) treatments with that of simple lyophilization (S-Lyo) (Tables 1, 2, 3, 4). The parameters used to characterize the reliability of each treatment were phaeophytin formation (from chlorophyll), xanthophyll cycle (VAZ) pigments alteration, changes in carotenoid/Chl ratios and increases in the rate of carotenoid isomerization.

No differences in Chl *a* in either *Q. ilex* or *T. officinale* were detected between the treatments used during lyophilization (Table 1). The least suitable method to determine total Chl content, and especially Chl *b,* was DTT-Lyo. In *Q. ilex*, two treatments induced the formation of phaeophytin: DTT- and BHT-Lyo (around 1.45% phaeophytin·Chl^{-1}) while the formation of this pigment in BAE-Lyo and S-Lyo was very low (0.6%). In *T. officinale*, the CA-Lyo did not prevent phaeophytin formation (4% phaeophytin·Chl^{-1}) while addition of BAE stopped this process (0.39% phaeophytin·Chl^{-1}). Therefore, the treatment that prevented chlorophyll degradation in both species was BAE-Lyo.

		Q. ilex		***T. officinale***	
Chl a	DTT	1620.8 ± 43.3	a	5962.9 ± 169.4	a
(nmol · g DW^{-1})	CA	1656.3 ± 9.4	a	6329.2 ± 298.8	a
	BHT	1697.6 ± 42.6	a	5174.7 ± 671.9	a
	BAE	1613.2 ± 42.7	a	5721.0 ± 777.5	a
	S-Lyo	1629.1 ± 15.7	a	6796.6 ± 481.8	a
Chl b	DTT	387.5 ± 6.1	a	1727.2 ± 33.3	ab
(nmol · g DW-1)	CA	588.9 ± 4.5	b	2516.7 ± 118.9	a
	BHT	595.1 ± 13.3	b	2049.8 ± 270.5	ab
	BAE	497.0 ± 11.9	c	1304.8 ± 379.1	b
	S-Lyo	548.3 ± 4.6	d	2663.2 ± 168.5	a
Total Chl	DTT	2008.2 ± 49.0	a	7690.1 ± 201.5	a
(nmol · g DW-1)	CA	2245.2 ± 13.5	bc	8846.0 ± 416.7	a
	BHT	2292.6 ± 55.8	c	7224.4 ± 941.8	a
	BAE	2110.3 ± 54.2	ab	7025.8 ± 1070.8	a
	S-Lyo	2177.3 ± 20.3	abc	9459.8 ± 650.3	a
Phaeophytin	DTT	28.58 ± 0.87	ab	54.32 ± 1.71	ab
(nmol · g DW^{-1})	CA	20.01 ± 0.45	ab	358.48 ± 24.57	c
	BHT	35.08 ± 7.44	a	80.80 ± 16.32	a
	BAE	13.11 ± 0.53	b	31.31 ± 7.60	b
	S-Lyo	13.76 ± 0.30	b	54.23 ± 2.71	ab
%Phaeophytin · Chl (a+b)$^{-1}$	DTT	1.423 ± 0.028	a	0.703 ± 0.005	ab
(% mol · mol^{-1})	CA	0.893 ± 0.019	ab	4.045 ± 0.157	c
	BHT	1.498 ± 0.301	a	1.090 ± 0.191	a
	BAE	0.619 ± 0.019	b	0.395 ± 0.061	b
	S-Lyo	0.633 ± 0.009	b	0.575 ± 0.012	b

Table 1. Chlorophylls (Chlorophyll a: Chl *a*; Chlorophyll b: Chl *b*) and phaeophytin concentrations and rates in *Q. ilex* and *T. officinale* leaves treated with distinct preservatives during lyophilization (1,4-dithiothreitol (DTT), cysteamine (CA), 2,6-ditertbutyl-4-methylphenol (BHT) or 2-butylamino ethanol (BAE)), or without any preservative (simple lyophilization: S-Lyo)). Values are mean of 4-8 replicates ± SE. Different letters indicate significant differences ($p<0.05$) between lyophilization treatments.

DTT-Lyo reduced V to A in both species (Table 2). DTT is a strong reducing agent, which, at the concentration used in this study, modifies the proportions of VAZ cycle components. In *Q. ilex*, DTT-Lyo reduced V concentration by more than 50% with respect to the other treatments and more than doubled the concentration of A. In *T. officinale*, a V reduction trend, although not significant, was also observed for DTT-Lyo, whereas the increase in A (on FW or Chl basis) was remarkable. The best preservative for Z conservation expressed on a Chl basis was BAE. The de-epoxidation index (A+Z/VAZ) and the epoxidation index (EI = 0.5(A+V)/VAZ) showed a significant influence of DTT: highest A+Z/VAZ (indicating an accumulation of the reduced forms) and lowest EI (indicating a reduction of the oxidized forms). In short, the BAE-Lyo method is the most appropriate for the study of VAZ cycle components, whereas DTT-Lyo (at 0.25 M or higher) is not appropriate because of its strong reducing properties.

As shown in Table 3, in *Q. ilex,* the best results for neoxanthin preservation were BAE-Lyo and BHT-Lyo whereas in *T. officinale*, the former was the best option. In *Q. ilex*, DTT-Lyo caused a reduction in Lutein-5,6-epoxide (Lx) without a significant increase in lutein (L) concentration. Lx and L constitute a de-epoxidation cycle in addition to the VAZ cycle and are also involved in *Q. ilex* photoprotection (Llorens *et al*., 2002; García-Plazaola *et al*., 2007). The amount of L is usually one or two orders of magnitude higher than that of Lx and it is difficult to detect the reduction of Lx to L. In *T. officinale* the results for Lx and L were not clear. In both species DTT-Lyo increased the isomerization of neoxanthin and L. In contrast, the lowest percentage of neoxanthin isomerization was in the S-Lyo treatment. Finally, BAE- and BHT-Lyo were the best treatments for the prevention of degradation of the total carotenoids (Total Car) in *Q. ilex*, while BAE-Lyo was the best treatment for this purpose in *T. officinale*. In conclusion, BAE-Lyo and BHT-Lyo showed the best results for the preservation of neoxanthin, L, Lx and Total Car.

		Q. ilex		*T. officinale*	
Violaxanthin	DTT	16.99 ± 0.63	a	246.54 ± 4.96	a
(nmol · g DW^{-1})	CA	45.55 ± 0.85	b	373.29 ± 63.33	ab
	BHT	50.89 ± 4.52	b	364.70 ± 58.25	ab
	BAE	77.75 ± 2.88	c	408.59 ± 55.65	ab
	S-Lyo	70.37 ± 0.96	c	521.69 ± 85.78	b
Antheraxanthin	DTT	62.58 ± 1.36	a	267.16 ± 6.89	a
(nmol · g DW^{-1})	CA	21.47 ± 0.26	b	32.50 ± 13.34	c
	BHT	27.34 ± 0.83	c	58.81 ± 7.54	bc
	BAE	31.72 ± 1.35	d	78.16 ± 12.24	b
	S-Lyo	29.05 ± 0.71	cd	65.26 ± 16.39	bc
Zeaxanthin	DTT	85.51 ± 3.40	ab	108.86 ± 2.17	ab
(nmol · g DW^{-1})	CA	86.51 ± 2.01	ab	101.07 ± 11.86	ab
	BHT	103.65 ± 6.76	a	68.93 ± 9.75	a
	BAE	96.44 ± 4.73	ab	139.79 ± 25.46	b
	S-Lyo	83.52 ± 2.37	b	122.22 ± 16.91	ab
Violaxanthin · Chl (a+b)$^{-1}$	DTT	8.50 ± 0.29	a	32.25 ± 0.25	a
(mmol · mol^{-1})	CA	20.25 ± 0.48	b	42.00 ± 6.36	ab
	BHT	22.50 ± 2.38	b	51.13 ± 3.37	bc
	BAE	36.86 ± 1.26	c	63.75 ± 6.82	c
	S-Lyo	32.25 ± 0.25	c	54.25 ± 6.42	bc
Antheraxanthin · Chl (a+b)$^{-1}$	DTT	31.25 ± 0.48	a	34.50 ± 0.65	a
(mmol · mol^{-1})	CA	9.50 ± 0.29	b	3.50 ± 1.50	b
	BHT	11.88 ± 0.13	c	8.00 ± 0.00	c
	BAE	15.00 ± 0.44	d	11.13 ± 0.13	d
	S-Lyo	13.25 ± 0.25	e	6.50 ± 1.50	c
Zeaxanthin · Chl (a+b)$^{-1}$	DTT	42.50 ± 0.87	ab	14.25 ± 0.63	a
(mmol · mol^{-1})	CA	38.50 ± 0.65	a	11.50 ± 1.19	ab
	BHT	44.88 ± 2.14	b	9.63 ± 0.38	b
	BAE	45.57 ± 1.76	b	18.88 ± 1.17	c
	S-Lyo	38.50 ± 1.32	a	12.75 ± 1.03	ab
VAZ	DTT	165.1 ± 5.0	ab	622.6 ± 10.3	a
(nmol · g DW^{-1})	CA	153.5 ± 1.6	a	506.9 ± 87.8	a
	BHT	181.9 ± 4.5	b	492.4 ± 74.5	a
	BAE	205.9 ± 7.8	c	626.5 ± 82.7	a
	S-Lyo	182.9 ± 1.9	d	709.2 ± 118.2	a
VAZ · Chl (a+b)$^{-1}$	DTT	82.25 ± 0.95	a	81.00 ± 1.47	ab
(mmol · mol^{-1})	CA	68.25 ± 0.48	b	56.75 ± 9.10	c
	BHT	79.38 ± 0.68	a	69.00 ± 3.70	bc
	BAE	97.43 ± 2.61	c	93.75 ± 5.61	a
	S-Lyo	84.00 ± 1.08	a	73.50 ± 8.84	bc
A+Z/VAZ	DTT	0.90 ± 0.00	a	0.60 ± 0.00	a
	CA	0.70 ± 0.01	b	0.26 ± 0.01	b
	BHT	0.72 ± 0.03	b	0.26 ± 0.01	b
	BAE	0.62 ± 0.01	c	0.33 ± 0.03	b
	S-Lyo	0.62 ± 0.01	c	0.26 ± 0.01	b
O.5·(A+V)/VAZ	DTT	0.29 ± 0.00	a	0.61 ± 0.00	a
	CA	0.37 ± 0.01	b	0.77 ± 0.01	bc
	BHT	0.36 ± 0.03	b	0.80 ± 0.01	c
	BAE	0.46 ± 0.01	c	0.73 ± 0.03	b
	S-Lyo	0.46 ± 0.01	c	0.78 ± 0.01	bc

Table 2. VAZ cycle chloroplast pigment concentrations and ratios (expressed as mmol·mol chlorophyll^{-1}) and indexes (De-epoxidation index: A+Z/VAZ; Epoxidation index: 0.5·(A+V)/VAZ) in *Q. ilex* and *T. officinale* leaves treated with distinct preservatives during lyophilization (1,4-dithiothreitol (DTT), cysteamine (CA), 2,6-ditertbutyl-4-methylphenol (BHT) or 2-butylamino ethanol (BAE)), or without any preservative (simple lyophilization: S-Lyo)). Values are mean of 4-8 replicates ± SE. Different letters indicate significant differences ($p<0.05$) between lyophilization treatments.

		Q. ilex		*T. officinale*	
Neoxanthin	DTT	85.09 ± 2.33	a	331.45 ± 8.48	a
(nmol · g DW^{-1})	CA	97.75 ± 0.77	b	322.73 ± 25.12	a
	BHT	109.26 ± 3.44	c	289.70 ± 40.19	a
	BAE	101.14 ± 3.41	bc	334.60 ± 44.44	a
	S-Lyo	96.12 ± 0.63	b	379.78 ± 40.43	a
Neoxanthin · Chl (a+b)$^{-1}$	DTT	42.50 ± 0.29	a	42.75 ± 0.25	a
(mmol · mol^{-1})	CA	43.50 ± 0.29	a	36.50 ± 1.85	a
	BHT	47.63 ± 0.56	b	40.13 ± 1.01	a
	BAE	48.14 ± 0.80	b	49.63 ± 2.52	b
	S-Lyo	44.00 ± 0.00	a	39.75 ± 1.93	a
% Trans Neox · Nx total^{-1}	DTT	19.72 ± 0.40	a	18.18 ± 0.98	a
	CA	12.11 ± 1.12	b	11.52 ± 0.43	bc
	BHT	15.04 ± 1.72	b	11.03 ± 0.23	bc
	BAE	12.07 ± 0.71	b	14.46 ± 2.73	ab
	S-Lyo	7.66 ± 0.50	c	7.24 ± 1.57	c
Lutein Epoxide	DTT	5.76 ± 0.26	a	18.77 ± 2.79	a
(nmol · g DW^{-1})	CA	9.07 ± 0.10	b	14.27 ± 0.60	ab
	BHT	10.87 ± 0.42	c	11.69 ± 1.48	b
	BAE	12.64 ± 0.65	d	13.73 ± 1.82	ab
	S-Lyo	10.88 ± 0.38	c	14.62 ± 0.85	ab
Lutein	DTT	322.7 ± 7.3	ab	1024.1 ± 27.2	a
(nmol · g DW^{-1})	CA	303.8 ± 2.6	a	982.3 ± 57.8	a
	BHT	358.6 ± 17.4	b	955.9 ± 122.7	a
	BAE	320.7 ± 10.6	ab	1035.9 ± 142.0	a
	S-Lyo	303.0 ± 4.0	a	1111.5 ± 98.3	a
Lutein Epoxide · Chl (a+b)$^{-1}$	DTT	3.00 ± 0.00	a	2.50 ± 0.29	a
(mmol · mol^{-1})	CA	4.00 ± 0.00	b	2.00 ± 0.00	b
	BHT	4.75 ± 0.16	c	1.88 ± 0.13	b
	BAE	6.00 ± 0.22	d	2.00 ± 0.00	b
	S-Lyo	5.00 ± 0.00	c	1.50 ± 0.29	b
Lutein · Chl (a+b)$^{-1}$	DTT	156.8 ± 0.9	a	129.5 ± 0.3	a
(mmol · mol^{-1})	CA	133.8 ± 0.8	b	109.5 ± 3.0	c
	BHT	154.0 ± 4.7	a	126.5 ± 2.1	ab
	BAE	149.7 ± 3.1	a	149.4 ± 5.4	d
	S-Lyo	137.5 ± 0.6	b	115.5 ± 3.2	bc
% Cis Lut · Lut total^{-1}	DTT	2.49 ± 0.13	a	2.92 ± 0.20	ab
	CA	1.11 ± 0.02	b	1.13 ± 0.08	b
	BHT	1.27 ± 0.07	bc	3.95 ± 0.97	a
	BAE	1.44 ± 0.10	c	1.86 ± 0.17	ab
	S-Lyo	1.21 ± 0.07	bc	1.08 ± 0.15	b
Total Car	DTT	830.3 ± 21.5	a	2839.2 ± 62.9	a
(nmol · g DW^{-1})	CA	823.2 ± 5.7	a	2840.3 ± 201.6	a
	BHT	968.8 ± 41.6	b	2566.8 ± 337.5	a
	BAE	915.0 ± 33.6	ab	2943.3 ± 394.1	a
	S-Lyo	848.4 ± 8.5	a	3277.6 ± 322.6	a
Total Car · Chl (a+b)$^{-1}$	DTT	413.5 ± 2.1	ab	369.3 ± 2.5	a
(mmol · mol^{-1})	CA	366.5 ± 1.0	c	320.3 ± 13.6	a
	BHT	421.6 ± 10.0	a	355.6 ± 1.8	a
	BAE	433.4 ± 10.5	a	436.5 ± 21.5	b
	S-Lyo	389.8 ± 1.3	bc	344.0 ± 13.0	a

Table 3. Neoxanthin and Lutein epoxide cycle pigment concentrations, ratios (expressed as mmol·mol chlorophyll^{-1}), percentages of carotenoid isomerization and total carotenoid (Total Car) concentration and rate in *Q. ilex* and *T. officinale* leaves treated with distinct preservatives during lyophilization (1,4-dithiothreitol (DTT), cysteamine (CA), 2,6-ditertbutyl-4-methylphenol (BHT) or 2-butylamino ethanol (BAE)), or without any preservative (simple lyophilization: S-Lyo)). Values are mean of 4-8 replicates ± SE. Different letters indicate significant differences ($p<0.05$) among lyophilization treatments.

For both species, BAE-, DTT- and S-Lyo were the best preservatives for tocopherol content, while CA in *T. officinale* and BHT in *Q. ilex* were associated with a decrease in tocopherol (Table 4).

		Q. ilex		*T. officinale*	
α-Tocopherol (nmol · g DW^{-1})	DTT	4623.8 ± 117.6	a	472.3 ± 7.2	ab
	CA	4592.9 ± 30.6	a	330.3 ± 18.4	b
	BHT	3811.1 ± 528.0	a	331.9 ± 44.8	b
	BAE	3801.5 ± 192.3	a	360.4 ± 47.3	b
	S-Lyo	4470.9 ± 108.7	a	508.6 ± 21.4	a
γ-Tocopherol (nmol · g DW^{-1})	DTT	187.52 ± 5.86	a	23.71 ± 0.85	a
	CA	184.75 ± 2.34	a	19.38 ± 1.00	a
	BHT	141.39 ± 23.25	a	37.17 ± 6.61	a
	BAE	138.86 ± 17.39	a	32.73 ± 7.36	a
	S-Lyo	175.43 ± 3.66	a	34.21 ± 2.42	a
Total Tocopherol (nmol · g DW^{-1})	DTT	4811.3 ± 123.3	a	496.0 ± 7.2	ab
	CA	4777.7 ± 32.7	a	349.7 ± 19.2	a
	BHT	3952.5 ± 548.2	a	369.0 ± 48.2	a
	BAE	3940.4 ± 209.3	a	393.2 ± 51.6	ab
	S-Lyo	4646.3 ± 112.2	a	542.8 ± 23.0	b
α-Tocopherol · Chl (a+b)$^{-1}$ (mmol · mol^{-1})	DTT	2303.3 ± 42.1	a	61.5 ± 0.9	a
	CA	2046.0 ± 19.1	ab	37.3 ± 1.3	c
	BHT	1677.5 ± 235.0	b	45.8 ± 0.9	bc
	BAE	1802.1 ± 79.9	ab	54.9 ± 4.6	ab
	S-Lyo	2053.5 ± 49.3	ab	54.3 ± 1.7	ab
γ-Tocopherol · Chl (a+b)$^{-1}$ (mmol · mol^{-1})	DTT	93.25 ± 1.80	a	3.00 ± 0.00	ab
	CA	82.25 ± 1.11	ab	2.00 ± 0.00	b
	BHT	62.75 ± 10.83	b	4.88 ± 0.69	a
	BAE	65.71 ± 7.74	ab	4.25 ± 0.49	a
	S-Lyo	80.25 ± 1.75	ab	3.75 ± 0.25	ab
Total Tocopherol · Chl (a+b)$^{-1}$ (mmol · mol^{-1})	DTT	2396.8 ± 43.6	a	64.5 ± 0.9	a
	CA	2128.0 ± 19.8	ab	39.5 ± 1.0	c
	BHT	1740.3 ± 244.6	b	51.3 ± 0.7	b
	BAE	1867.7 ± 87.3	ab	59.3 ± 4.1	ab
	S-Lyo	2134.5 ± 51.0	ab	57.8 ± 1.7	ab

Table 4. Tocopherol concentrations and ratios (expressed as mmol·mol chlorophyll^{-1}) in *Q. ilex* and *T. officinale* leaves treated with distinct preservatives during lyophilization (1,4-dithiothreitol (DTT), cysteamine (CA), 2,6-ditertbutyl-4-methylphenol (BHT) or 2-butylamino ethanol (BAE)), or without any preservative (simple lyophilization: S-Lyo)). Values are mean of 4-8 replicates ± SE. Different letters indicate significant differences ($p<0.05$) between lyophilization treatments.

We also compared our results with the least damaging lyophilization technique (i.e. BAE-Lyo) with results obtained from frozen non-lyophilized leaves. We determined chloroplast pigments and lipophilic antioxidants in *Q. ilex* sun and shade leaves, correlated the two methods and expressed measurements per concentration and per Chl rate (Figs. 3 and 4 respectively). Results from frozen non-lyophilized samples showed a very high standard error, which implies non-significant differences between sun and shade leaves (e.g. Chl *a* values,

letter A in Fig. 3). In contrast, the results for samples treated with BAE-Lyo showed statistically significant differences and confirmed the distinct pigment composition of sun *vs.* shade leaves (i.e. higher Chl *b* values in the shade; letter B in Fig. 3). This finding can be attributed to the fact that lyophilization facilitates the homogenization of plant material and variability of results can be reduced (Tausz *et al*., 2003).

VAZ cycle pigments in sun or shade leaves showed similar values in frozen non-lyophilized and BAE-Lyo samples, although in some cases such as V content, a decrease was detected in the latter (letter C in Fig. 3). Although standard errors in the results of frozen samples were quite high, differences between the VAZ cycle components of sun and shade leaves were statistically significant, as reflected in the results from the BAE-Lyo treatment. A and Z (letter A and B respectively in Fig. 4) were higher in *Q. ilex* sun leaves because of a greater need to dissipate energy as heat through the VAZ cycle (Demmig-Adams and Adams, 1996; Fleck *et al*., 1998). Accordingly, the de-epoxidation index and the VAZ concentration were also significantly higher in sun leaves, while the epoxidation index was lower in both preservation methods (data not shown).

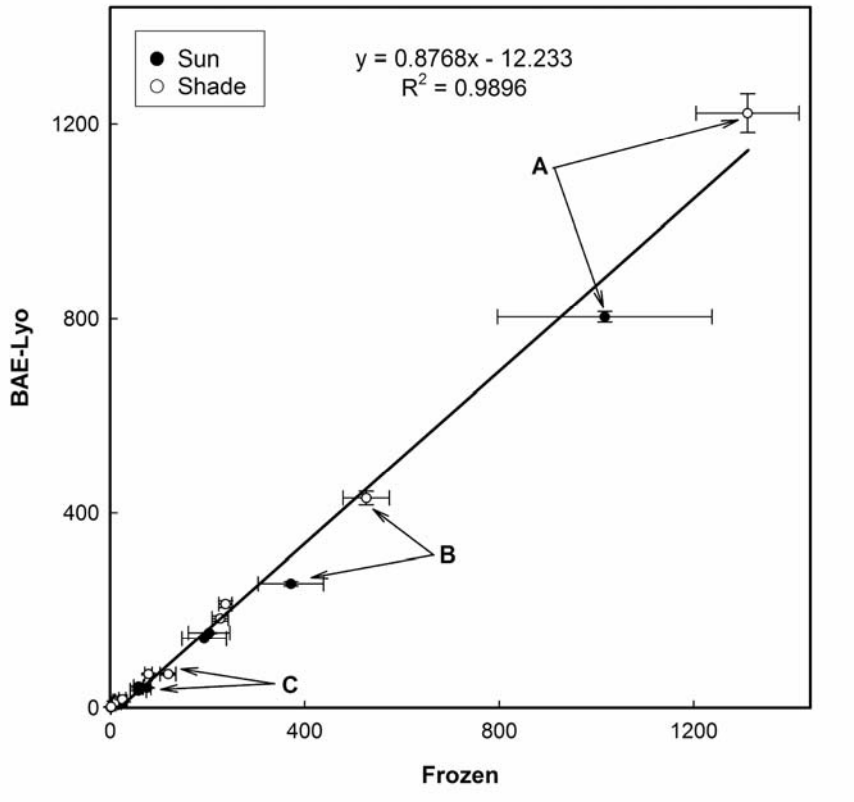

Figure 3. Correlation between sun and shade leaves of *Q. ilex* that have been frozen (Frozen) and those that have been lyophilized with butylamino ethanol (BAE-Lyo). Values are mean of 3 replicates ± SE per pool of 10-12 leaves each of the following compounds in nmol pigment·g^{-1} FW: **A**, chlorophyll *a*; **B**, chlorophyll *b*; phaeophytin; **C**, violaxanthin; antheraxanthin; zeaxanthin; neoxanthin; lutein; lutein epoxide; α- carotene; β-carotene.

No significant differences in Chl *a* or Chl *b* concentration were detected between frozen non-lyophilized samples and BAE-Lyo material. However, phaeophytin on Chl basis increased in the BAE-Lyo treatment. Nevertheless, the mean increase in both sun and shade leaves was about 0.47% higher in Lyo-samples than in frozen material, thereby indicating very low chlorophyll degradation during lyophilization. No differences between frozen or BAE-Lyo samples were observed in neoxanthin, Lx, L, Total Car or α-Toc. Increased concentration of the most abundant L and β carotene in sun leaves (letter C and D respectively in Fig. 4) confirms the increased photoprotection requirements under high light.

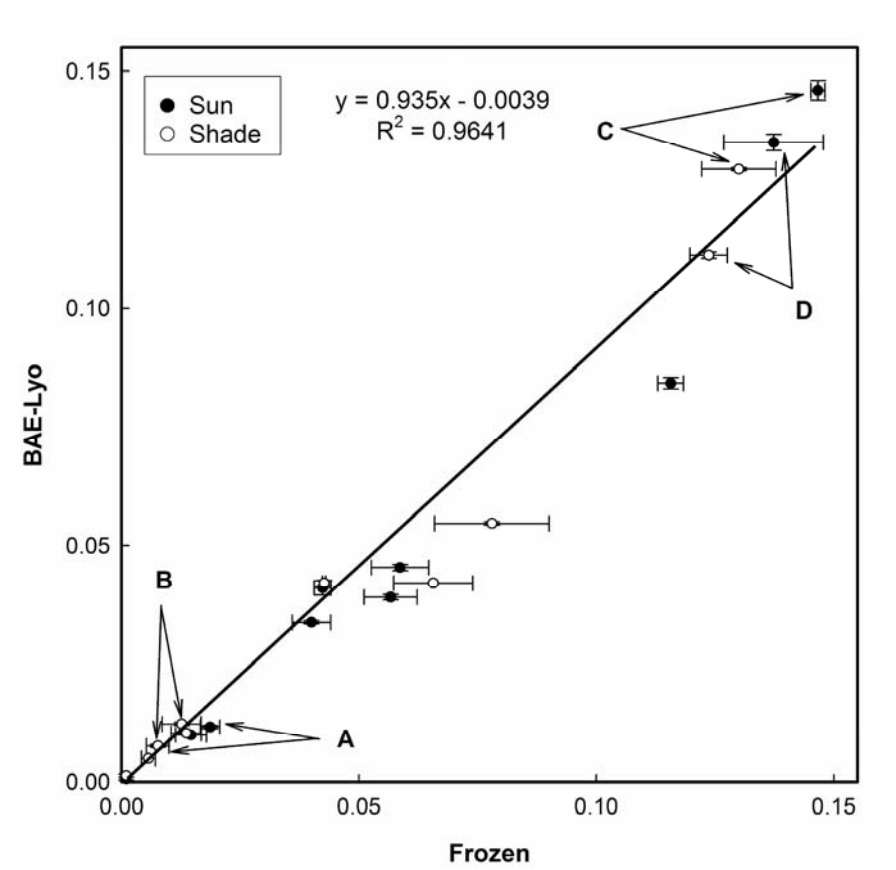

Figure 4. Correlation between sun and shade leaves of *Q. ilex* that have been frozen (Frozen) and those that have been lyophilized with butylamino ethanol (BAE-Lyo). Values are mean of 3 replicates ± SE per pool of 10-12 leaves each of the following compounds in mol pigment · mol^{-1} chlorophyll: violaxanthin; **A**, antheraxanthin; **B**, zeaxanthin; neoxanthin; **C**, lutein; lutein epoxide; α- carotene; **D**, β-carotene.

On the basis of our results, as listed in Table 5, we conclude that the use of BAE during a 5-day lyophilization treatment in the dark provides the most effective preservation of pigments and antioxidants in *Q. ilex* and *T. officinale*.

	Quercus ilex		*Taraxacum officinale*	
	Best	**Worst**	**Best**	**Worst**
Phaeophytin	BAE-, S-Lyo	DTT-, BHT-Lyo	BAE-Lyo	CA-Lyo
Neoxanthin isomerization	S-Lyo	DTT-Lyo	S-Lyo	DTT-Lyo
Lutein isomerization	-	DTT-Lyo	S-Lyo	BHT-Lyo
VAZ	BAE-Lyo	DTT-Lyo	BAE-Lyo	DTT-Lyo
Total Carotenoids	BAE-, BHT-Lyo	-	BAE-Lyo	-

Table 5. Evaluation of lyophilization in the presence of various preservatives (1,4-dithiothreitol (DTT-Lyo), cysteamine (CA-Lyo), 2,6-ditertbutyl-4-methylphenol (BHT-Lyo) or 2-butylamino ethanol (BAE-Lyo)), or lyophilization without any preservative (simple lyophilization: S-Lyo)) in *Q. ilex* and *T. officinale* leaves. The best and the worst methods to avoid phaeophytin formation and neoxanthin and lutein isomerisation and maintain VAZ pigment concentration and total carotenoid content are described and briefly summarised.

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CAPÍTOL 2.

ANTIOXIDATIVE AND PHOTOPROTECTIVE DEFENCE SYSTEMS IN *QUERCUS ILEX* L. EVALUATED BY NEAR INFRARED REFLECTANCE SPECTROSCOPY

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Avaluació per espectroscòpia de reflectància al vermell proper dels sistemes de defensa antioxidant i fotoprotectors a *Quercus ilex* L.

- La quantificació de compostos fotoprotectors i antioxidants és costosa en temps i diners. Per a aconseguir-ne una avaluació ràpida i àgil es construí una base de dades correlacionant l'espectroscòpia de reflectància al vermell proper (NIRS) amb les anàlisi químiques de fulles de *Quercus ilex* L.
- S'establiren les equacions de cal·libració predictives per la quantificació dels pigments cloroplàstics (clorofil·les *a* i *b*, neoxantina, violaxantina, zeaxantina, anteraxantina, α- i ß-carotè, luteïna i luteïna epòxid) i compostos antioxidants (α-, (β+γ)-tocoferol, ascorbat i fenols totals) mitjançant l'algoritme per regressió de mínims quadrats parcials (PLC).
- Les equacions de cal·libració de clorofil·les *a* i *b*, feofitina, β-carotè, neoxantina, luteina, fenols totals i α-tocoferol foren cal·librades acuradament mentre que les de la luteina epòxid i l'α-carotè mostraren resultats de cal·libració no tant bons degut a les baixes concentracions. Els pigments involucrats en el cicle VAZ (violaxantina, anteraxantina, zeaxantina) mostraren millors correlacions amb els valors de la violaxantina. L'ascorbat presentà una repartició desigual dels resultats. Les prediccions extretes de la base de dades NIRS s'utilitzaren per comparar les variacions estacions dels compostos fotoprotectors durant la regeneració de *Quercus ilex*. Els resultats demostraren la validesa del mètode.
- El NIRS constitueix una eina ecofisiològica vàlida per caracteritzar els sistemes fotoprotectors permentent estalviar temps i diners. Només amb una sola mesura, es pot obtenir la quantificació de múltiples variables fisiològiques. Aquesta és la primera vegada que la NIRS s'utiltza per caracteritzar la composició de pigments en plantes.

Paraules clau

Antioxidants; espectroscòpia de reflectància al vermell proper (NIRS); fotoprotecció; *Quercus ilex* (alzina); pigments cloroplastics

Antioxidative and photoprotective defence systems in *Quercus ilex* L. evaluated by Near Infrared Reflectance Spectroscopy

- Quantifying photoprotective and antioxidant compounds is laborious and expensive. For their rapid evaluation, a database using Near Infrared Reflectance Spectroscopy (NIRS) and chemical analyses of *Quercus ilex* L. leaves was built.
- Predictive calibration equations for concentration of chloroplast pigments (chlorophylls *a* and *b*, phaeophytin, neoxanthin, violaxanthin, zeaxanthin, antheraxanthin, α- and β-carotene, lutein and lutein epoxide) and antioxidants (α-, (β+γ)-tocopherol, ascorbate and total phenolics) were established using partial least squares regression algorithm.
- Calibration equations of chlorophylls *a* and *b*, phaeophytin, β-carotene, neoxanthin, lutein, total phenolics and α-tocopherol were accurately calibrated whilst lutein epoxide and α-carotene calibration showed poorer results due to their lower concentration. The pigments involved in the VAZ cycle (violaxanthin, antheraxanthin, zeaxanthin) showed contrasted results with more satisfactory calibrations for violaxanthin. Ascorbate presented an unequal repartition of values. NIRS predictions were used in a forest study comparing seasonal trends of photoprotective compounds during *Quercus ilex* regeneration. Results demonstrated the validity of the method.
- NIRS constitutes a valid tool to characterize photoprotection compounds allowing time and money saving. With only one single measurement, results can be obtained on multiple physiological attributes. This is the first time that NIRS is used to characterize pigment composition in plants.

Keywords

Antioxidants, chloroplast pigments, *Quercus ilex* (holm oak), Near Infrared Reflectance Spectroscopy (NIRS), photoprotection

2.1. Introduction

The imbalance between light energy absorption and its use in photosynthesis leads to the generation in chloroplasts of activated oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radicals (O_2^-), hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2) which can be highly reactive (Asada, 1996, Apel & Hirt, 2004). The enhanced production of ROS can produce injury to cell structures (Baier & Dietz, 1999), but they also act as signals for the activation of stress-response and defence pathways (Mittler, 2002).

Several defence systems protect the photosynthetic apparatus in front of excess energy excitation by decreasing the rate of ROS formation; these include photorespiration (Osmond *et al.*, 1997) and dissipation as heat by the VAZ cycle (Demmig Adams & Adams, 1992) and in some species by the lutein epoxide cycle (Llorens *et al*., 2002; García-Plazaola *et al.*, 2007). Moreover, the VAZ cycle may have a direct antioxidant action by enhancing the tolerance of thylakoid membranes to lipid peroxidation (Niyogi, 1999). ROS detoxification by antioxidant systems is another main photoprotective mechanism against photodamage (Krinsky, 1992) that includes enzymatic and non-enzymatic mechanisms (Asada, 1996; Smirnoff, 2006). Among the latter, naturally occurring plant substances such as vitamins A, C (ascorbic acid) and E (α-tocopherol), carotenoids, flavonoids, and simple phenolic compounds are antioxidants and scavenge ROS directly in the pigment bed (Polle & Rennenberg, 1994). The antioxidant vitamins control peroxidation and rapidly quenching, eliminating and/or deactivating free radical generation. Ascorbate is quantitatively the predominant antioxidant in plant cells and in green tissues its pool can be over 10% of the soluble carbohydrate (Noctor & Foyer, 1998). It is also required for the formation of zeaxanthin (Müller-Moulé *et al.*, 2002) and for the regeneration of α-tocopherol (Beyer, 1994). α-tocopherol not only detoxifies ROS and lipid peroxyl radicals, but has a significant membrane stabilizing function (Havaux *et al.*, 2003) and has been suggested to participate in intracellular signalling and in cyclic electron transport around photosystem II (Munné-Bosch & Alegre, 2002). Phenolics are diverse secondary metabolites (flavonoids, tannins, hydroxycinnamate esters and lignin) abundant in plant tissues (see review by Grace & Logan, 2000), that together with ascorbate have the major role in the antioxidant defence in plant cells. Due to its importance, photoprotective metabolites are currently analysed and quantified in ecophysiological studies using different spectrophotometric and chromatographic methods. Most of them are expensive and time-consuming, and a standard

characterization of the photoprotective profile of a single leaf involves 3 or 4 separate chromatographic separations.

NIRS (Near Infrared Reflectance Spectroscopy) constitutes a powerful analytical tool for studies of the biochemical composition of aliments or plant material by means of the analyses of the diffuse reflectance of the samples (Williams, 1975). The absorbance of light between 780 and 2500 nm due to molecular vibrations (overtones and combinations of fundamental vibrations) of bonds like O–H, C–N, N–H and C=O characteristic to organic matter determines a unique spectral signature for each analysed sample (Osborne *et al.*, 1993). Therefore, the spectrum of light that is reflected by a sample contains details on the chemical composition (i.e., number and nature of bonds present) of that material (Birth & Hecht, 1987). Nevertheless, NIRS is not a direct analytical method but an indirect one. In order to determine the concentration of a particular organic constituent of interest, a calibration model has to be built based on the relationship between measurements with a reference technique and spectral features. NIRS allows rapid, repeatable, and accurate measurement of hydrocarbonated molecules and nutrients fraction in plant material (McLellan *et al.*, 1991; Joffre *et al.*, 1992; Foley *et al.*, 1998; Gillon *et al.*, 1999). In the evergreen holm-oak (*Quercus ilex* L.), dominant species of the Mediterranean forests, NIRS has been applied in studies of nutrient remobilization (Cherbuy *et al.*, 2001), prediction of litter decomposition rates (Bouchard *et al.*, 2003), effects of drought (Peña-Rojas *et al.*, 2005) and of elevated CO_2 (Staudt *et al.*, 2001, Peñuelas *et al.*, 2002, Aranda *et al.*, 2006). In these studies leaf concentrations of nitrogen (N), soluble sugars, starch, cellulose, hemicellulose, lignin, and lipids were determined based on calibration equations built on the spectral and wet chemical database of 260 samples including *Q. ilex* leaves collected throughout Mediterranean area (Meuret *et al.*, 1993). In food and pharmaceutical products, NIRS has been used to determine vitamin C (ascorbic acid) (Yang & Irudayaraj, 2002) and recent studies have assessed the applicability of NIRS to quantitative analysis of total antioxidant capacity in green tea (Luypaert *et al.*, 2003, Zhang *et al.*, 2004).

Q. ilex, like other species of the Mediterranean forest experience stress periods with high irradiance combined with drought or low temperatures (Larcher, 2003) that induce several photoprotective responses. This species shows a great resprouting capacity after perturbations (fire, clear-cut, grazing) and rapid growth attributed to the stimulated resprouts photosynthesis because of greater water and nutrient availability with respect to the original plants (the

pre-existing root system is associated with a much smaller aerial biomass) (Fleck *et al.*, 1998, El Omari *et al.*, 2003a).

The main objective of this study was to test the feasibility of NIRS to accurately quantify photoprotective and antioxidant compounds acting in *Q. ilex* leaves. This species was chosen because it represents one of the key species in the fragile Mediterranean ecosystem and their photoprotection mechanisms have been deeply studied, allowing the comparison with previous studies. Therefore, a chemical and spectral database of *Q. ilex* leaves was created with biochemical quantification of chloroplast pigments (chlorophylls *a* and *b*, neoxanthin, violaxanthin, zeaxanthin, antheraxanthin, α- and β-carotene, lutein, lutein epoxide,) and antioxidant compounds (α- tocopherol, ascorbate and total phenolics) and near-infrared spectra on the same samples. The use of this database will allow in just a single measurement the characterization of all the above described metabolites avoiding the time-consuming and expensive biochemical analyses for each pigment or antioxidant separately. Moreover, we performed the NIRS database validation in a field study comparing photoprotective and antioxidant responses during holm-oak regeneration, by resprouting, under stressful conditions in winter and summer.

The characterization of photoprotective and antioxidant compounds that act in *Q. ilex* under stress conditions will enable to quantify damages at an early stage and establish its adaptation capacity in the context of global change and biodiversity conservation of the Mediterranean ecosystem.

2.2. Material and methods

a. NIRS data base creation

Experimental sites and plant material

In order to build accurate calibration models between chemical constituents and spectral absorbance, the widest range of values in the different studied compounds should be obtained. Therefore, samplings of *Q. ilex* leaves were carried out under different conditions: 1) In two different locations: Serra de les Forques forest, Castellbisbal, Barcelona, Spain (41º48'45'' N, 01º96'35'' E, 144 m asl and oriented N–NW) and Experimental Fields of the Faculty of Biology, University of Barcelona, Spain (41º22'59'' N, 02º06'44'' E, 60 m asl ; 2) In different populations: a) 25-year-old undisturbed individuals, b) resprouts originated after a previous summer fire, c) 6-year-old *Q. ilex* plants grown in 9–l

pots filled with a mixture of soil from the area (40%) (by volume), peat (20%), vermiculite (20%) and perlite (20%) watered with 500 ml of Hoagland solution per day; 3) Different leaf characteristic: wealthy, infected by pathogens, chlorotic; 4) Different leaf age (old, young) on the same tree; 5) Throughout the year on sunny and cloudy days during the four seasons; 6) At different times of the day: in the morning (9:00–11:30 h local time), at midday (13:30–16:30 h) and in the evening (18:00–20:00 h). For each sample, groups of 30 leaves were frozen in liquid nitrogen and stored at -80ºC until lyophilisation.

Lyophilisation conditions

Lyophilisation was thought to be the most comfortable way to handle and transport leaf material to be analysed by NIRS. For that purpose, we standardised the conditions and requirements during the freeze-drying process. Frozen leaves were smashed into pieces in a mortar with liquid nitrogen before lyophilisation. 1 ml of 0.25 M of the preservative 2-butylamino ethanol (BAE) was added per g FW. This biocide has been shown to be the best preservative in front of material oxidation (Pintó-Marijuan *pers.comm)*. Freeze-drying was carried out in a Virtis Lyophiliser (Freezemobile 6EL, Gardiner, N.Y., USA) in vials of 3.0 cm diameter, at the Laboratori de Tècniques Separatives dels Serveis Cientifico-Tècnics, UB. All samples were stored under low humidity and dark conditions until biochemical and NIRS analysis. Lyophilised plant leaves were milled in a Cyclotec 1093 Sample Mill (Tecator, Höganäs, Sweden).

Chloroplast pigments and tocopherol determination

For the determination of lipophilic antioxidants (α-, δ- and (β+γ)-tocopherol; α-Toc, δ-Toc and (β+γ)-Toc, respectively) and chloroplast pigments (VAZ cycle components (violaxanthin (V), antheraxanthin (A), zeaxanthin (Z)), neoxanthin (Neo), α- and β-carotene (α-Car and β-Car, respectively), lutein (Lut), lutein epoxide (Lx), chlorophylls *a* and *b* (Chl*a* and Chl*b*, respectively)), acetone extracts from lyophilized and frozen leaves were analysed by reverse-phase high performance liquid chromatography (HPLC) following the method by García-Plazaola & Becerril (1999), with the modifications described in García-Plazaola & Becerril (2001). The de-epoxidation state is defined as the AZ/VAZ ratio (calculated as (A/2+Z)/(V+A+Z)). Detection was carried out with a fluorescence detector (Waters model 474) set to λ_{exc} = 295 nm and λ_{em} = 340 nm calibrated with α-, β-, δ- and γ-tocopherol standards (Calbiochem, San Diego, CA). Under these chromatographic conditions, we were unable to distinguish β-Toc from γ-Toc neither to detect δ-Toc in *Q. ilex* leaves.

Ascorbic acid analyses

Lyophilised leaves were reduced to powder with liquid N_2 in a pre-chilled mortar and pestle and homogenised with 7 vols of a 5% (w/v) cold metaphosphoric acid solution by Polytron (Kinematica AG, Switzerland), by interpolation into Grantz *et al.* (1995). The homogenates were centrifuged at 14000xg for 15 min. The supernatants were filtered by using 0.22 µm filters and injected on a C18 reverse phase column (250x4.6 mm, 5 µm pore size, Grace Davison, Deerfield, IL, USA). Elution was performed with a multi step linear gradient of acetonitrile in 0.05 M sodium acetate solution (pH 5.2): 5% acetonitrile for 4 min, 5 to 50% acetonitrile in 1 min, 50% for 5 min, 50 to 5% acetonitrile in 1 min, at a flow rate of 1 mL min^{-1}. Ascorbic acid (Asc) was detected and quantified by a UV/VIS Diode Array Detector (System Gold mod. 168, Beckman-Coulter, Fullerton, CA, USA), according to standard curves. Chromatograms were acquired at 254 nm and integrated by the 32 Karat™ Software v 5.0, using Peak purity test (Beckman-Coulter, Fullerton, CA, USA).

Total phenolics analyses

Lyophilised leaves were reduced to powder with liquid N_2 in a pre-chilled mortar and pestle that was homogenised with 20 vols of an 80% methanol solution and extracted twice. After vortexing, the homogenates were sonicated for 5 min, centrifuged at 14000xg for 10 min and phenol content determined according to Singleton & Rossi (1965). The supernatants were filtered by using 0.45 µm filters and 10 µl were added to 750 µl of 0.2N Folin-Ciocalteau reagent. After five minutes in darkness at room temperature, 600 µl of 7.5% sodium carbonate were added and the samples incubated for two hours in darkness at room temperature. Sample absorbance (nm 735) was read and total phenol content (TPhe) was expressed as equivalents of gallic acid (GAE), calculated by interpolation in its corresponding standard curve.

NIRS analysis and calibration

All samples were scanned with a near-infrared spectrometer working in reflectance mode (NIR Systems 6500, Foss NIR Systems, Inc, Silver Spring, MD) following the procedure described by Joffre *et al.* (1992). Each sample was packed into a sample cell having a quartz-glass cover. For each sample, one reflectance measurement was made from 400 to 2500 nm obtaining a spectrum with 1050 data points at 2-nm intervals over this range. Data analysis was conducted using ISI software system (Shenk & Westerhaus, 1991). Independent calibration equations between spectral and chemical data were built for each analysed chemical constituent using partial least squares (PLS) algorithm

(Martens & Naes, 1989; Shenk & Westerhaus, 1991). PLS calibrations were developed and compared using six math treatments corresponding to first and second derivative and gap of 5, 10, and 15 data points or 10, 20, and 30 nm. For all these previous math treatments, results obtained with and without detrending method (Barnes *et al.*, 1989) were compared. Cross validations were used to estimate the optimal number of terms in the calibration and to avoid overfitting.

b. Forest study

Experimental site and plant material

The forest study was conducted in Can Coll, Serra de Collserola forest, Barcelona, Spain; 41º28'28'' N, 2º7'32'' E at an elevation of 140 m. The climate is Mediterranean, with cold winters, cool, wet springs and autumns, and hot, dry summers. The area has a mean annual temperature of 13–14ºC and an annual rainfall of 500–700 mm. The 35-year-old forest is dominated by *Quercus ilex* L. and *Pinus halepensis* Mill. Here we set out two plots: one was left intact; the other was clear-cut. Six *Quercus ilex* L. plants on the intact site were randomly selected and designated as CF (forest controls). On the clear-cut plot, the shoots from six randomly selected plants were completely excised, and resprouts originating thereafter were designated as R. Six plants were left undisturbed, and designated as controls of the clear-cut site (C). Ecophysiological characteristics of C, CF and R plants were studied in winter and summer. Climatic data during one month before sampling in the nearest to the study site meteorological station (Observatori Fabra), have been recorded (Fig. 1). Temperatures on days previous to sampling were never below freezing point in winter or above 35°C in summer, indicating the absence of severe thermal stress. However summer precipitation was almost non-existent as corresponds to the typical Mediterranean summer drought.

Lyophilization conditions

Lyophilisation and milling of *Q. ilex* leaves were performed as described above, after the addition of 0.25 M 2-butylamino ethanol (BAE). Freeze-dried samples were stored under low humidity and dark conditions until NIRs analyses.

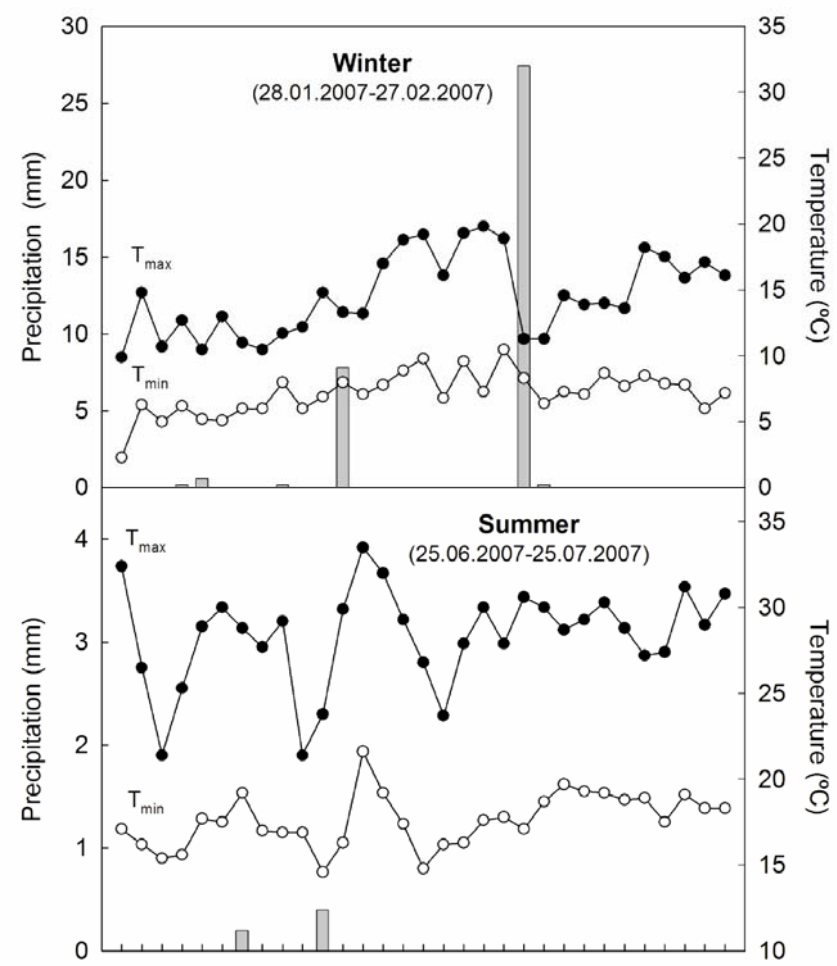

Figure 1. Climatological data recorded during one month before sampling in the nearest to the study site meteorological station (Observatori Fabra). On lines (right axis scale): T_{min}, minimal daily temperature; T_{max}, maximal daily temperature. On bars (left axis): Precipitation.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured with a portable modulated fluorimeter (Mini-Pam Photosynthesis Yield Analyser, Walz, Effeltrich, Germany). The light-adapted components of chlorophyll fluorescence (steady-state yield (F), maximum fluorescence yield (F'_m) and quantum yield of photosystem II (PSII) photochemistry (Φ_{PSII}; equivalent to $(F'_m-F)/F'_m$)) (Genty *et al.*, 1989) were measured. Parameters F'_o, minimum fluorescence yield in light-adapted state; qP, photochemical quenching (equivalent to $(F'_m-F)/(F'_m-F'_o)$), and F'_v/F'_m, intrinsic efficiency of open PSII centres during illumination (equivalent to $(F'_m-F'_o)/F'_m$) were estimated following Oxborough & Baker (1997). To obtain minimum fluorescence yield (F_o), maximum fluorescence yield (F_m) and maximum quantum yield of PSII photochemistry (F_v/F_m) (equivalent to $(F_m-F_o)/F_m$), leaves were dark-adapted for at least 20 min, after which F_v/F_m values reach about 95% of the pre-dawn values in *Q. ilex* (Fleck *et al.*, 1998).

Relative water content and leaf biomass parameters

Five current-year leaves of the same five plants per treatment used for chlorophyll fluorescence measurements in winter and summer were used to calculate the following parameters: a) relative water content (RWC) calculated as [(FW-DW)/(FSW-DW)·100] (where FW, fresh weight; FSW, fresh saturated weight (after rehydrating samples for 24h in the dark); DW, dry weight (after oven-drying samples at 65ºC until constant weight)); b) leaf mass per area, LMA calculated as DW/LA (where LA, leaf area); c) Leaf Dry Weight over Fresh Weight calculated as DW/FW·100. To assess leaf area, images were obtained with a flat-bed scanner Epson GT5000 and processed using image analyser software supplied by Serveis Científico-Tècnics (UB).

Leaf chemistry

All freeze-dried samples were scanned with a near-infrared reflectance spectrophotometer (NIR Systems 6500, Foss NIR Systems, Inc, Silver Spring, MD) following the procedure described by Joffre *et al*., (1992). Leaf concentrations of nitrogen (N), soluble sugars, starch, cellulose, hemicellulose, lignin, and lipids were determined based on calibration equations built on the spectral and wet chemical database of 260 samples including *Q. ilex* leaves collected throughout Mediterranean area (Meuret *et al.*, 1993).

Chloroplast pigments and antioxidant compounds

The determination of chloroplast pigments (Neo, V, Z, A, α-Car, β-Car, Lut, Lx, Chl*a*, Chl*b*), α-Toc, (β+γ)-Toc, Asc and TPhe were performed on the same samples used for leaf chemistry determination applying the new created NIRS data base.

Statistical analyses

All statistical procedures were done using SPSS for Windows (SPSS for Windows v. 14.0, SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to test the main effects and interactions, against appropriate error terms between seasons (winter and summer) and among populations (C, CF and R) of chlorophyll fluorescence parameters, RWC and leaf biomass parameters, leaf chemistry, chloroplast pigments and antioxidant compounds. The post-hoc Duncan test was applied when appropriate. Due to the complexity of the experimental design and the statistical analyses, the differences between these parameters were not always evident and were discussed in the text. Statistical significance was set at $p \leq 0.05$. The number of replicates is indicated in the table and figure legends.

2.3. Results

Sample preparation and NIRS calibration

Freeze-drying of samples for 5 days enabled the optimal maintenance of the analyzed compounds since obtained concentrations on fresh samples did not differ significantly from those lyophilized (data not shown).

As shown in Table 1 and Fig. 2 calibrations were enough accurate for all major photosynthetic pigments. Thus, calibration equations for chlorophylls, phaeophytin and carotene pigments were generally satisfactory except for α-carotene with a low R^2 (0.72) and a low percentage of variance (1-VR) explained by the calibration model. Neoxanthin and lutein were accurately calibrated whilst lutein epoxide calibration showed poorer results that could be partly explained by the unequal repartition of values as shown in Fig. 2.

	n	Mean	SD	SEC	R^2	SECV	1-VR	RPD	Math	Nt
Chloroplast pigments										
Chl*a*	62	1648.7	670.2	117.1	0.969	228.2	0.882	2.94	2 5 5	6
Chl*b*	62	567.6	274.5	57.7	0.955	96.3	0.876	2.85	2 5 5	6
Phaeo	57	94.9	30.5	8.5	0.922	14.8	0.762	2.05	2 5 5	6
α-Car	52	3.21	1.81	0.796	0.717	0.866	0.676	2.09	2 5 5	4
ß-Car	60	278.6	102.1	24.7	0.941	36.2	0.871	2.82	2 5 5	4
Neo	62	107.8	48	10.1	0.955	13.6	0.918	3.53	2 5 5	4
Lx	59	21.1	15.7	7.14	0.793	8.01	0.693	1.96	2 5 5	7
Lut	62	348	107.5	23.4	0.952	48.2	0.796	2.23	2 5 5	7
VAZ cycle components										
V	62	97.6	57	14.4	0.935	28.2	0.756	2.02	2 5 5	7
A	46	20.6	11.6	4.4	0.792	6.1	0.692	1.9	2 10 10	5
Z	59	71.2	35	16.8	0.769	18.4	0.579	1.9	2 10 10	6
Antioxidant compounds										
TPhe	54	95.1	11.2	3.2	0.917	3.9	0.88	2.87	2 5 5	5
Asc	46	5	2.4	0.9	0.841	1.2	0.755	2	2 10 10	3
α-Toc	63	1374	1394	196	0.98	505	0.876	2.76	2 5 5	7
(β+γ)-Toc	54	71.2	35.39	20.09	0.778	18.23	0.603	1.94	1 5 5	5

Table 1. Calibration statistics for the considered constituents. The values given in the columns refer to number of samples (n), the mean of measured values (mean), standard deviation of measured values (SD), standard error of calibration (SEC), R squared (RSQ), standard error of cross validation (SECV), residual prediction deviation (RPD = SD/SECV). Math: mathematical treatment of the spectral data: the first number is the order of the derivative function, the second is the segment length data points over which the derivative was taken and the third is the segment length over which the function was smoothed; number of terms (Nt): number of terms of the PLS models. Data are presented as $nmol \cdot g^{-1}DW$ except for total phenolics expressed as mg gallic acid equivalents$\cdot g^{-1}DW$ and Asc expressed as $\mu mol \cdot g^{-1}DW$.

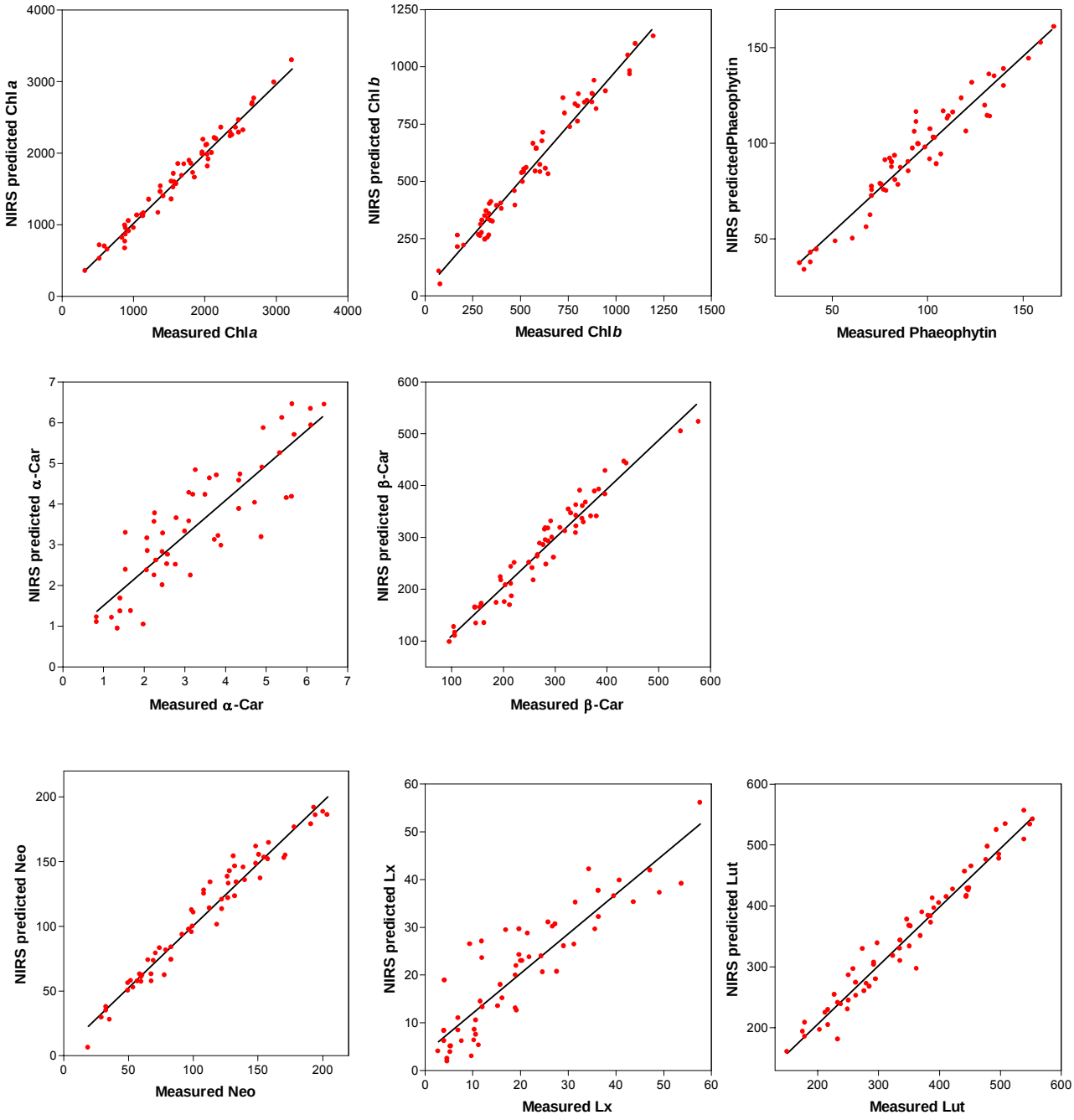

Figure 2. Correlation graphs between biochemicaly measured pigments (Chl*a*, Chl*b*, Phaeophytin, α-carotene (α-Car), β-carotene (β-Car), neoxanthin (Neo), lutein epoxide (Lx) and lutein (Lut)) and their respective NIRs predicted values.

The pigments involved in the VAZ cycle (Fig. 3) showed contrasted results with higher calibration results for violaxanthin (Table 1). A great dispersion of values for A led to numerous outliers computed on the T distance between predicted and measured values during the process of calibration; as result, the final model was built on 46 samples. TPhe results were satisfactory with a high accuracy (Table 1). Regarding on antioxidant compounds, the quantitative dominant tocopherol form, α-Toc, was satisfactory correlated, while (β+γ)-Toc showed a high dispersion and therefore the correlation was not so

accurate. Asc results presented an unequal repartition of values from the selected calibration samples (Fig. 3), preventing good calibration results.

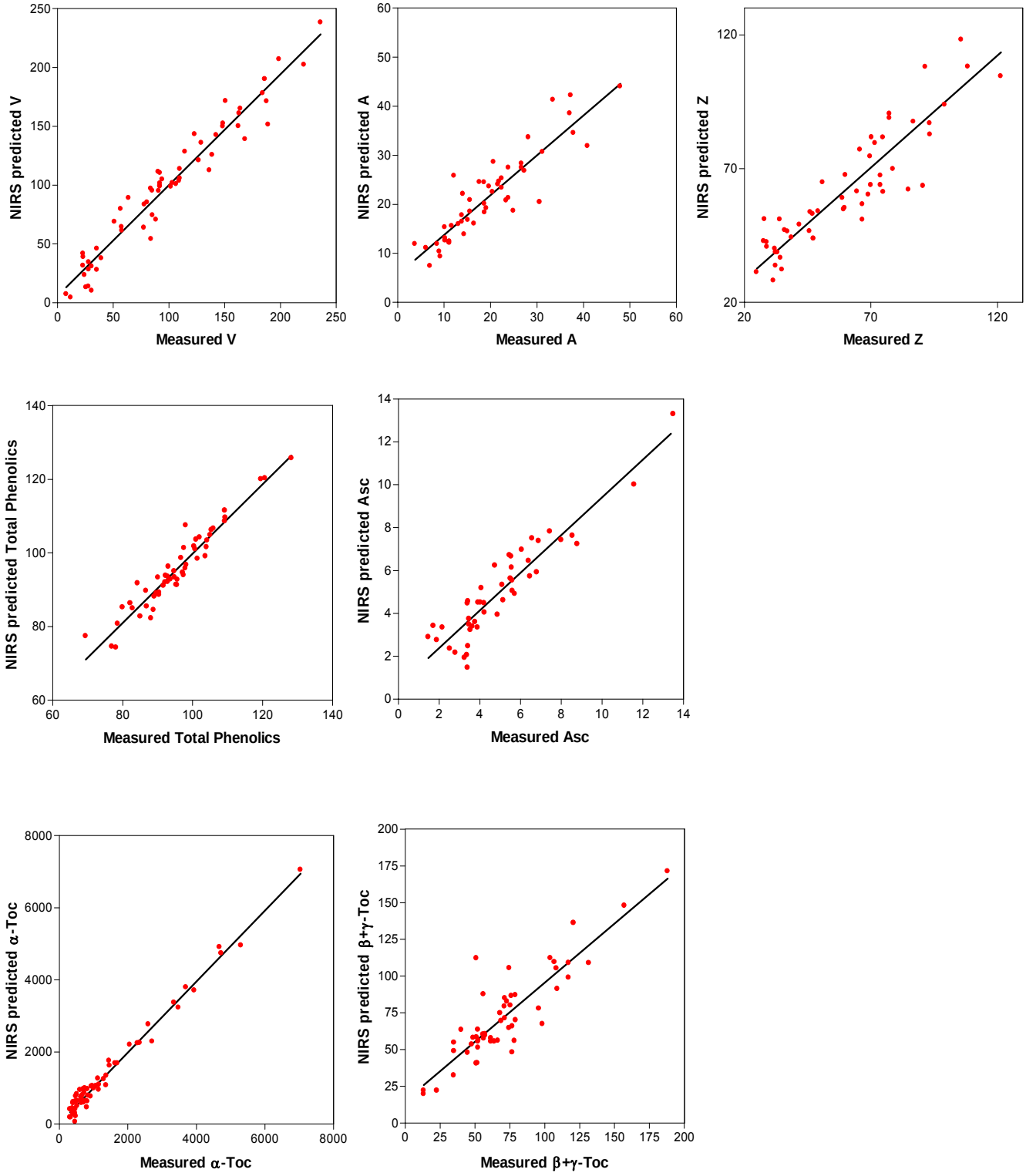

Figure 3. Correlation graphs between biochemicaly measured VAZ xantophylls (violaxanthin (V), antheraxanthin (A), zeaxanthin (Z)) or antioxidants (total phenolic (TPhe), ascorbate (Asc), α-Tocopherol (α-Toc), (β+γ)-Tocopherol ((β+γ)-Toc)) and their respective NIRs predicted values.

Forest study

Samples collected in winter and summer showed different responses to seasonality. The results on RWC and leaf biomass parameters (Table 2a) showed that midday RWC had its lowest value in C plants. RWC values fell from winter to summer in CF and R. % leaf DW was very similar in C and CF and was maintained from winter to summer; in R values were lowest in winter with respect to CF and C and increased from winter to summer. LMA was similar in CF and R in the winter, increasing from winter to summer in R; C showed highest values in the winter with respect to other treatments, showing no seasonal differences.

a)		Control Forest	Control	Resprouts
RWC (%)	Winter	77.62 ± 1.76 $^{a\ A}$	63.86 ± 3.10 $^{b\ A}$	82.11 ± 1.14 $^{a\ A}$
	Summer	71.36 ± 2.24 $^{a\ B}$	66.99 ± 2.11 $^{a\ A}$	70.28 ± 1.58 $^{a\ B}$
DW (%)	Winter	53.96 ± 2.22 $^{ab\ A}$	59.89 ± 4.44 $^{a\ A}$	51.22 ± 0.41 $^{b\ A}$
	Summer	54.70 ± 0.88 $^{a\ A}$	54.84 ± 0.51 $^{a\ A}$	56.61 ± 0.48 $^{a\ B}$
LMA (g m^{-2})	Winter	85.34 ± 7.42 $^{a\ A}$	124.41 ± 19.23 $^{b\ A}$	89.73 ± 2.91 $^{a\ A}$
	Summer	85.45 ± 2.63 $^{a\ A}$	109.08 ± 6.77 $^{b\ A}$	112.57 ± 3.77 $^{b\ B}$
b)				
Nitrogen	Winter	1.96 ± 0.07 $^{a\ A}$	2.12 ± 0.03 $^{b\ A}$	2.14 ± 0.02 $^{b\ A}$
	Summer	1.94 ± 0.07 $^{a\ B}$	2.00 ± 0.04 $^{a\ A}$	1.74 ± 0.03 $^{b\ B}$
Cellulose	Winter	17.33 ± 0.92 $^{a\ A}$	19.66 ± 0.28 $^{b\ A}$	18.23 ± 0.29 $^{a\ A}$
	Summer	19.24 ± 0.54 $^{a\ B}$	20.99 ± 0.30 $^{b\ A}$	18.66 ± 0.41 $^{a\ A}$
Hemicellulose	Winter	19.42 ± 0.67 $^{a\ A}$	21.79 ± 0.37 $^{b\ A}$	20.40 ± 0.29 $^{a\ A}$
	Summer	21.39 ± 1.14 $^{a\ B}$	23.79 ± 0.36 $^{b\ A}$	20.23 ± 0.29 $^{a\ A}$
Lignin	Winter	7.82 ± 0.59 $^{a\ A}$	7.12 ± 0.30 $^{a\ A}$	7.10 ± 0.37 $^{a\ A}$
	Summer	5.69 ± 0.95 $^{a\ B}$	5.90 ± 0.48 $^{a\ A}$	7.25 ± 0.17 $^{a\ A}$
Soluble Sugars	Winter	13.62 ± 0.37 $^{a\ A}$	13.98 ± 0.15 $^{ab\ A}$	14.39 ± 0.12 $^{b\ A}$
	Summer	13.07 ± 0.28 $^{a\ B}$	12.87 ± 0.28 $^{a\ A}$	14.02 ± 0.26 $^{b\ A}$
Starch	Winter	0.57 ± 0.25 $^{a\ A}$	1.30 ± 0.26 $^{a\ A}$	2.45 ± 0.23 $^{b\ A}$
	Summer	2.12 ± 0.58 $^{a\ A}$	1.34 ± 0.36 $^{a\ B}$	2.00 ± 0.38 $^{a\ A}$
TNC	Winter	14.19 ± 0.50 $^{a\ A}$	15.28 ± 0.38 $^{b\ A}$	16.85 ± 0.24 $^{c\ A}$
	Summer	15.19 ± 0.42 $^{ab\ A}$	14.21 ± 0.43 $^{a\ A}$	16.02 ± 0.45 $^{b\ A}$
Lipids	Winter	19.29 ± 0.64 $^{a\ A}$	18.40 ± 0.32 $^{a\ A}$	16.59 ± 0.17 $^{b\ A}$
	Summer	18.01 ± 0.42 $^{a\ A}$	17.36 ± 0.33 $^{ab\ A}$	17.00 ± 0.11 $^{b\ A}$

Table 2. a) Relative water content (RWC), % leaf dry weight (% DW) and leaf mass per area (LMA); b) leaf chemical composition as %DW. CF, forest control; C, control; R, resprouts. Each value represents the mean ± S.E. of 5 measurements (a) and of 6-18 measurements (b). Significant differences at $p<0.05$ are indicated by superscript letters: treatment differences (a,b) and seasonal differences (A,B).

Leaf chemical composition results (Table 2b) indicate that leaf N content on DW basis was lowest in CF in the winter and showed a decreasing trend from winter to summer being especially marked in R. Cellulose and hemicellulose were higher in C, and lignin content was similar for all treatments. Only CF showed declining content from winter to summer. TNC content showed higher values in R with respect to CF and C in both seasons (both soluble sugars and starch were higher); and values were maintained in all treatments from winter to summer whereas on area basis TNC increased in R due to both soluble sugars accumulation. Lipid content was lower in R and did not change between seasons in all treatments.

The analysis of the chlorophyll fluorescence parameters (Fig. 4) indicate that Φ_{PSII} and qP values were always higher in CF leaves and decreased from winter to summer especially in C. Values of F'_v/F'_m were lower in the summer than in the winter, especially in C and R. Midday F_v/F_m fell in the summer only in R plants and, values followed the trend CF>C>R in both seasons.

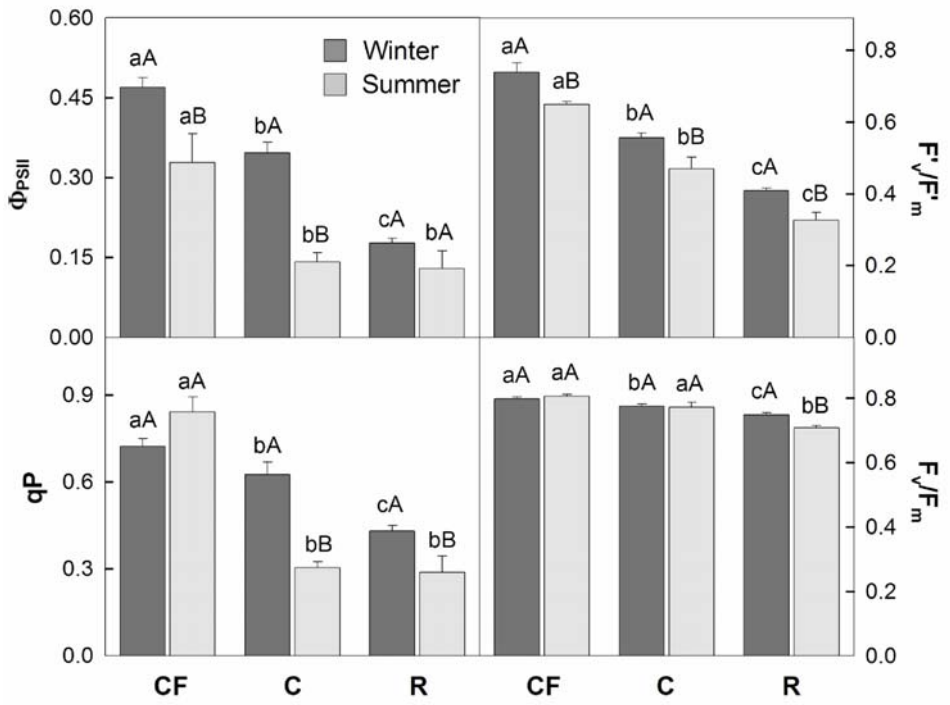

Figure 4. Chlorophyll fluorescence parameters. Φ_{PSII}, quantum yield of PSII; qP, photochemical quenching; F'_v/F'_m, intrinsic efficiency of open PSII centres; F_v/F_m, maximum quantum yield of PSII photochemistry. CF, forest control; C, control; R, resprouts. Each value represents the mean ± S.E. of 6-12 measurements. Significant differences at $p<0.05$ are indicated by superscript letters: treatment differences (a,b) and seasonal differences (A,B).

When observing the results analyzed by the NIRS database (Table 3) and comparing among populations, Chl*a* and Chl*b* showed higher concentrations in the most shaded sites being lower in resprouts. Phaeo followed the same

trend as chlorophylls. The Chl*a*/Chl*b* ratio (Chl*a*/*b*) was highest in R and similar for CF and C in both seasons. α-Car and β-Car presented similar concentrations in CF and C with higher values than in R. Neo, Lx and Lut content showed the same trend: CF>C>R. The highest amount of A was in R while V concentration was the lowest. The sum of the total concentration of the VAZ cycle components did not change between populations. The de-epoxidation state (AZ/VAZ) was higher in the more light exposed sites (R and C) than into the forest (CF). In R, TPhe and Asc content were highest, whereas α-Toc and (β+γ)-Toc, were lowest.

		Control Forest		Control		Resprouts	
Chl*a*	Winter	3667.2 ± 96.4	a A	3381.8 ± 88.0	b A	3015.0 ± 72.8	c A
	Summer	3213.8 ± 159.7	a B	3226.6 ± 171.6	a A	2374.7 ± 124.8	b B
Chl*b*	Winter	1200.6 ± 61.1	a A	1001.6 ± 35.4	b A	783.4 ± 33.0	c A
	Summer	1175.9 ± 54.7	a A	1137.8 ± 67.6	a A	807.9 ± 59.1	b A
Chl*a*/Chl*b*	Winter	3.07 ± 0.11	a A	3.39 ± 0.06	a A	3.92 ± 0.11	b A
	Summer	2.72 ± 0.06	a B	2.85 ± 0.04	ab B	2.99 ± 0.09	b B
Phaeo	Winter	175.7 ± 5.4	a A	172.4 ± 5.4	a A	144.7 ± 1.6	b A
	Summer	149.9 ± 3.7	a B	154.5 ± 7.5	a A	109.2 ± 6.0	b B
α-Car	Winter	6.57 ± 0.47	a A	6.05 ± 0.22	ab A	5.43 ± 0.22	b A
	Summer	6.02 ± 0.40	a A	6.01 ± 0.36	a A	4.15 ± 0.18	b B
β-Car	Winter	552.2 ± 18.4	a A	536.6 ± 11.8	a A	464.7 ± 7.9	b A
	Summer	485.9 ± 15.8	a B	472.7 ± 12.3	a B	354.6 ± 13.5	b B
Neo	Winter	221.7 ± 10.7	a A	186.0 ± 5.3	b A	146.8 ± 5.2	c A
	Summer	192.5 ± 10.5	a A	172.9 ± 9.6	a A	118.8 ± 7.2	b B
Lx	Winter	47.33 ± 2.99	a A	43.03 ± 1.68	a A	23.96 ± 1.55	b A
	Summer	40.88 ± 2.91	a A	36.61 ± 2.88	a A	14.25 ± 2.66	b B
Lut	Winter	602.9 ± 28.8	a A	540.6 ± 20.7	a A	454.0 ± 18.7	b A
	Summer	474.3 ± 42.9	a B	484.7 ± 37.6	a A	344.3 ± 27.5	b B
V	Winter	161.7 ± 8.4	a A	132.4 ± 8.0	b A	105.8 ± 7.9	b A
	Summer	126.0 ± 11.7	a B	114.4 ± 8.4	a A	62.7 ± 12.0	b B
A	Winter	10.00 ± 1.98	a A	11.62 ± 1.47	a A	23.46 ± 0.55	b A
	Summer	15.87 ± 2.03	a A	13.98 ± 2.10	a A	26.96 ± 1.54	b B
Z	Winter	67.20 ± 2.82	a A	94.83 ± 4.11	b A	89.32 ± 2.82	b A
	Summer	87.33 ± 4.86	a B	100.32 ± 3.01	a A	70.99 ± 4.88	b B
VAZ	Winter	238.9 ± 5.4	a A	238.8 ± 6.3	a A	218.6 ± 6.1	a A
	Summer	229.3 ± 11.1	a A	228.7 ± 4.7	a A	160.6 ± 8.9	b B
AZ/VAZ	Winter	0.368 ± 0.030	a A	0.501 ± 0.028	b A	0.636 ± 0.030	c A
	Summer	0.525 ± 0.042	a B	0.564 ± 0.038	a A	0.800 ± 0.073	b B
TPhe	Winter	92.07 ± 3.38	a A	84.64 ± 2.67	b A	98.12 ± 1.05	a A
	Summer	94.22 ± 3.49	a A	81.92 ± 2.74	b A	108.06 ± 2.82	c B
Asc	Winter	218.1 ± 89.4	a A	343.4 ± 36.1	ab A	455.7 ± 38.3	b A
	Summer	327.7 ± 81.2	a A	529.1 ± 66.8	ab B	715.0 ± 62.3	b B
α-Toc	Winter	1782.3 ± 121.3	a A	2404.6 ± 223.0	b A	761.1 ± 85.4	c A
	Summer	700.1 ± 79.3	a B	929.5 ± 255.9	a B	884.9 ± 142.2	a A
(β+γ)-Toc	Winter	121.48 ± 6.45	a A	95.35 ± 4.89	b A	68.59 ± 2.15	c A
	Summer	75.37 ± 7.35	a B	73.57 ± 2.47	a B	66.87 ± 4.82	a A

Table 3. NIRS-predicted values of leaf content of chloroplastic pigments and antioxidant systems. Chlorophyll (Chl); phaeophytn (Phaeo), carotene (Car); neoxanthin (Neo); lutein epoxide (Lx); lutein (Lut); violaxanthin (V); antheraxanthin (A); zeaxanthin (Z); V+A+Z (VAZ); de-epoxidation index (AZ/VAZ); total phenolics (TPhe); ascorbate (Asc); tocopherols (Toc). CF, forest control; C, control; R, resprouts. Data are presented as nmol·g^{-1}DW except for total phenolics expressed as mg gallic acid equivalents·g^{-1}DW and Asc expressed as µmol·g^{-1}DW. Each value represents the mean ± S.E. of 6-18 measurements. Significant differences at $p<0.05$ are indicated by superscript letters: treatment differences (a,b) and seasonal differences (A,B).

From winter to summer, we found a decline in Chl*a* and Phaeo, especially in CF and R, while no variations were observed in Chl*b* concentrations, and therefore the ratio Chl*a*/*b* was higher in winter for each kind of vegetation. Regarding seasonal differences in the carotenoid content in the two control populations, most of them (α-Car, β-Car, Neo, Lx, Lut and V) had higher values in winter, but A and Z, as well as VAZ, were higher in summer; significant differences in both C and CF were only found in β-Car; in resprouts all analyzed carotenoid concentrations, even VAZ content, were significantly higher in the winter except A, that was higher in the summer. AZ/VAZ increased from winter to summer in R and CF, while C remained stable. TPhe presented no differences between seasons in CF and C, but in R was higher in summer. Asc content was higher in the summer, but only significantly in the most sun exposed populations (C and R). α-Toc and (β+γ)-Toc declined in CF and C from winter to summer, while R showed no variation on its concentration.

2.4. Discussion

NIRS Database

An effective preservation of pigments and antioxidants in *Q. ilex* was obtained in lyophilized samples. Lyophilisation achieves better stability, decreased temperature sensitivity and extended shelf-life of the resultant product (Carpenter *et al*., 1997). Moreover, freeze-dried material was also easier to grind in the mill, which implies that a homogeneous powder could be obtained for their scanning with the near-infrared reflectance spectrophotometer. There are several reports concerning the application of lyophilization for pigment and antioxidants measurements in the literature (Tausz *et al*.; 2003; Nessa *et al*., 2005; Sivakumar *et al*., 2005).

With respect to the data base results, when comparing the dispersion in the calibration equations (Table 1, Figs 2 and 3), a clear correspondence between the smaller R^2 values (with a mean ± SE of 0.77 ± 0.01) and the photoprotectors with a lower concentration per g DW (α-Car, Lx, A, Z and (β+γ)-Toc; ranging from 3.2 to 71.2 nmol·g^{-1}DW) was observed. This fact evidenced that the lesser concentration of component to analyze the higher likelihood to do any error in the quantification. Thus, all major components of the photoprotective system can be adequately calculated.

Briefly, the results show that NIRS allows the quantification of the antioxidative and photoprotective defence compounds in *Q. ilex* lyophilized

leaves. However, the accuracy of the calibration equations differed depending on the compound's concentration. This result could be partly attributed to a higher accuracy and precision of the reference methods when the concentration of the compound is higher than 100 $nmol \cdot g^{-1}DW$, when punctual measures are less precise (Horwitz *et al*., 1980) and, in consequence, the calibration equations tend to lose trustworthiness. If *Horwitz trumpet* model had been used for NIRS predictions, standard errors for little concentrations would have been higher (Rittler & Meyer, 2005).

As already mentioned by a number of authors (e.g., Foley *et al.*, 1998), this method can then be preferred over conventional analysis for large sets of samples as it ensures rapid, non-destructive, and economical analysis of material. NIRS offers several advantages over wet chemistry analysis for ecological studies (Foley *et al.*, 1998). After the establishment of predictive equation, NIRS rapidly predicts the chemical composition of other samples without further analysis (Joffre *et al.*, 1992; Gillon & David, 2001). Finally, the method requires only a small amount of material (ca. 2 g DW) and short time preparation (lyophilization and grinding).

The database application to a forest study

To assess the suitability of NIRS determinations for ecophysiological purposes, we performed a field study comparing *Q. ilex* plants growing under different conditions. In fact, the two studied sites differed in several environmental parameters that can affect the physiology of plants: 1) water availability: due to differences in evapotranspiration (canopy and substrate properties) or in water retention (substrate properties); 2) light availability: CF trees were placed in a dense forest with low incident photosynthetic photon flux density (PPFD) on most individuals whereas in the clear-cut area, C and especially R, received increased radiation, being not so manifest in C due to the self shading effect in developed trees; 3) root/shoot ratio: in the clear-cut area, the high ratio in R account for their improved water relations with respect to undisturbed individuals of the same area (C) as previously reported by Peña-Rojas *et al.* (2005).

The different environmental constraints in the two studied sites were reflected in the functional, structural and biochemical differences in the three populations (CF, C, R) observed by analysing NIRS results in summer and winter. In the former, Mediterranean forests are submitted to high temperature, DPV, and irradiance together with little precipitation (Fig. 1) inducing a marked

drought. Water limitation accounts for the observed RWC lowest values in C (65%); RWC below 70% was reported to affect the photosynthetic apparatus (Cornic & Massacci, 1996). Another indicator of the water limitation in accordance with Gratani (1996) is the higher sclerophylly i.e. high LMA and structural carbohydrates (cellulose and hemicellulose; Table 2) as also observed in C. Mediterranean ecosystems are also exposed to a variable degree of winter stress that may result in severe winter photoinhibition and upregulation of photoprotective systems (García-Plazaola *et al.*, 1999). In our study all parameters indicative of photoprotection shift to lower values in winter (except tocopherols in controls), indicating that temperatures were not enough low as to cause severe stress.

Differences in light availability were reflected in the decreasing trend of Φ_{PSII} from CF to R and from winter to summer (Fig. 4) and in parameters obtained by the utilization of the new database. In fact, Chl*a* and Chl*b* were lower in R as expected for sun-exposed expanding leaves (García-Plazaola *et al*., 1997). From winter to summer, the chlorophyll decline, especially Chl*a* in R, was correlated with N decline (Tausz *et al*., 1996). The chlorophyll degradation under stressing conditions, such as drought, has been reported (Tausz *et al*., 2004; Loggini *et al*., 1999). Chl*a*/*b* values were higher in the clear-cut site with higher light availability, whereas the lower values in CF reflected an acclimation to a more shaded environment (García-Plazaola *et al*., 1997, Lichtenthaler *et al*., 2007).

The seasonal onset of photoprotective mechanisms were observed in all populations. In the winter, the content of several carotenoids (α-Car, β-car, Neo, Lx and Lut) were observed to be highest as also reported for β-car in *Pistacia lentiscus* (Munné-Bosch & Peñuelas, 2003a). Resprouts showed the lowest carotenoid content (El Omari *et al*., 2003b) and a decline from winter to summer of about 34%. In general, pigment and antioxidant contents were in the range of previous works with this species. As an example, averaging VAZ contents in four independent studies (Faria *et al*., 1998; Fleck *et al*., 2000; García-Plazaola *et al*., 1999; Martínez-Ferri *et al*., 2000), unstressed leaves contained 74 mmol VAZ·mol^{-1}Chl and it increased to 89 mmol VAZ·mol^{-1}Chl when the same plants were exposed to environmental stress. In the present work this parameter ranged from 74 to 88 mmol VAZ mol^{-1}Chl in the different treatments.

The activation of xanthophyll cycles (VAZ and Lx cycles), differed between populations and seasons. In R, (submitted to highest PPFD), the de-epoxidation state (AZ/VAZ) was always higher than in controls and increased markedly from winter to summer. Also the Lx content (the epoxidated product of

Lut) decreased seasonally with increasing irradiance and was lowest in R. These results are in accordance with the observed increased thermal dissipation expressed by the low values of the chlorophyll fluorescence parameter F'_v/F'_m (Fig. 4).

The antioxidant function was shared by two groups: the lipophyllic (carotenoids and tocopherols) and the hydrophyllic (Asc). In fact, in the summer, carotenoids were lower in R and higher in controls, while Asc (the main hydrophilic antioxidant in *Q. ilex*, (García-Plazaola *et al*., 1999)) showed the opposite trend showing a higher content in R. Asc rose with increasing irradiance being highest in the summer and in R in accordance with García-Plazaola & Becerril (2001), Verdaguer *et al.* (2003) and Tausz *et al.* (2004). Also controls showed different extent of photoprotective strategies: α-Toc was high in the winter whereas their Asc content was lowest. However it should be noted that age differences between summer and winter may account for α-Toc increase since this molecule accumulates continuously during leaf development (Hormaetxe *et al*., 2005). The (β+γ)-Toc appeared in very low concentration compared with α-Toc (10-fold higher) as in other forest species (García-Plazaola & Becerril 2001, García-Plazaola *et al*., 2004). A clear negative relationship between α-Toc content and RWC was observed as already reported (Munné-Bosch *et al*., 1999; Munné-Bosch & Peñuelas, 2003b; Blokhina *et al*., 2003) indicating a possible prevention of chlorophyll photooxidation (Wise & Naylor, 1987; Simontacchi *et al*., 1993). In all treatments the contribution of hydrophilic and lipophilic antioxidant compounds was complementary as earlier observed (El Omari *et al*., 2003b).

In summary, the coherency and reliability of the obtained results in a forest study, as supported by the bibliography, confirms the usefulness of the described methodology for evaluation of photoprotectors in ecophysiological studies and makes the NIRS susceptible to be preferred over conventional analysis for large sets of samples as it ensures rapid and economical analysis of plant material.

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CAPÍTOL 3.

ANTIOXIDANT PROTECTION DURING HEAT STRESS IN HOLM-OAK RESPROUTS ORIGINATED FROM PLANTS GROWN AT ELEVATED CO_2

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Avaluació de la protecció antioxidant i fotoprotectora durant l'estrès per altes temperatures en rebrots originats d'alzines crescudes a CO_2

Es compararen les respostes fotosintètiques i antioxidants de rebrots d'alzina (*Quercus ilex*) originats de plantes crescudes sota diferents concentracions de CO_2: ambiental (350 $\mu L \cdot L^{-1}$) (RA) i elevada (750 $\mu L \cdot L^{-1}$) (RE). A les seves concentracions de CO_2 respectives, els RE mostraren una major transpiració (E) i conductància total (g_T), malgrat no s'observaren diferències de la densitat estomàtica i paràmetres fluorescència de les clorofil·les. Els RE tingueren una acumulació de carbohidrats no estructurals totals (TNC), fet que explica la regulació a la baixa de la taxa de fotosíntesi neta (A) i els alts nivells d'ascorbat. Degut a la baixada fotosintètica s'observà una major participació del cicle de les xantofil·les (amb un Z/VAZ superior) i del cicle de la luteina epòxid, indicant un augment de la dissipació tèrmica de l'excés d'energia. A l'aplicar estrès per altes temperatures, els RE mostraren una baixada de la fotosíntesi superior respecte als RA, mentre que les respostes estomàtiques, en els RE, foren els més estables. Els RE van experimentar pocs canvis al llarg del tractament d'estrès en les concentracions dels components antioxidants, demostrant una resistència superior a l'estrès tèrmic. En canvi, els RA mostraren un augment dels seus components lipofílics (clorofil·les, carotenoids i tocoferols) en ser sotmesos a l'estrès tèrmic. Els resultats mostren que la millor protecció antioxidant i la tolerància a les altes temperatures dels rebrots desenvolupats plenament sota condicions de CO_2 elevat, podrien mitigar l'efecte de l'aclimatació de la fotosíntesi durant la regeneració de plantes de *Q. ilex*.

Antioxidant protection during heat stress
in holm-oak resprouts originated from plants grown at elevated CO_2

The antioxidant responses during high temperature stress in holm-oak (*Quercus ilex*) resprouts originated from plants grown under current CO_2 concentration (350 $\mu L \cdot L^{-1}$) (RA) were compared with those of resprouts originated from plants grown under elevated CO_2 (750 $\mu L \cdot L^{-1}$) (RE). At their respective CO_2 growth concentration, RE evidenced lower transpiration (E) and total conductance (g_T), but similar stomatal density and chlorophyll fluorescence parameters. Total non structural carbohydrate (TNC) accumulation in RE accounted for the observed down-regulation of net photosynthesis (A) and high ascorbate content observed. The reduction of the photosynthetic sink account for the observed increases in the participation of the xanthophyll (with inereased Z/VAZ) and lutein epoxide (with lower Lx) cycles and enhanced thermal energy dissipation. When high temperature stress was applied, RE showed a more marked photosynthesis decrease with respect to RA whereas stomata responses were less affected. RE showed higher resistance to the heat treatment showing a little response in their antioxidant composition. At the contrary, a different response to heat stress was observed in RA which showed a significant increase in lipophilic compounds (chlorophylls, carotenes, tocopherols). The results show that the higher antioxidant protection and higher temperature tolerance of resprouts entirely developed under elevated CO_2 would mitigate the effect of photosynthesis acclimation during the regeneration of *Q. ilex* plants.

3.1. Introduction

Quercus ilex (L.) is a deep-rooted evergreen dominant species in the Mediterranean forest that shows a great resprouting capacity after perturbations (fire, tree-fell, grazing) and rapid growth attributed to the stimulated resprouts photosynthesis because of greater water and nutrient availability with respect to the original plants (the pre-existing root system is associated with a much smaller aerial biomass) (Fleck *et al*. 1998, El Omari *et al*. 2003). The evergreen holm-oak is exposed to multiple environmental stress factors such as drought, heat shock, chilling, nutrient deprivation and high light stress among others (Di Castri 1981). Although *Q. ilex* trees grow on sites where temperatures reach easily 40–50°C, leaves may suffer thermal stress with temperatures above 35°C (Larcher 2000).

Increased probability of drought, heat and rising atmospheric CO_2 concentration during the next decades can be particularly important in the Mediterranean basin (Christensen *et al*. 2007) affecting vegetation structure and plant productivity (Schär *et al*. 2004, Ciais *et al*. 2003). Associated with the predicted climate change, increased risk of uncontrolled fire episodes are expected (Mouillot *et al*. 2002). The progressive increase in fire frequency and severity (Davis and Michaelsen 1995) can lead to the exhaustion of several species generating a decline in their resprouting capacity and recovery. Furthermore, increased CO_2 concentration can be also, in part, responsible for the predicted temperature increase (1.4 - 5ºC by 2100) (Houghton *et al*. 2001) and drought induces higher leaf temperature due to the loss of transpirational cooling as reported in *Quercus rubra* (Singsaas and Sharkey 1998).

The information on how increases in atmospheric CO_2 concentration and temperature will influence photosynthesis-related processes is critical for understanding how the climate change will affect the structure, functioning and productivity of forest ecosystems. During stressing conditions, impaired photosynthetic electron flow results in an imbalance between the generation and the utilization of electrons, likely resulting in highly reactive ROS (oxygen radical species) formation (Asada 1996, Apel and Hirt 2004). The enhanced production of ROS can produce injury to photosynthesizing cells (Asada 1996, Baier and Dietz 1999) but they also act as signals for the activation of stress-response and defence pathways (Mittler 2002). Photosynthetic tissues are protected by several mechanisms that limit the absorption of light energy by chlorophyll, increase the rate of thermal energy dissipation by the participation of the xanthophyll

(Demmig-Adams and Adams 1996) and the lutein-epoxide cycle (García-Plazaola *et al*. 2007) or directly detoxify ROS (Krinsky 1992, Asada 1999, Smirnoff 2006). Ascorbate, α-tocopherol, carotenoids, flavonoids and simple phenolic compounds are antioxidants and scavenge ROS directly in the pigment bed (Polle and Rennenberg 1994). Ascorbate is quantitatively the most abundant in plant tissues (Noctor and Foyer 1998); it is required for the regeneration of α-tocopherol (Beyer 1994) and for the formation of zeaxanthin (Müller-Moulé *et al*. 2002).

With increasing ambient CO_2 concentration, the basal rate of oxygen activation and ROS formation could be reduced leading to a depression in the antioxidant defence system (Halliwell and Gutteridge1988). However, Badiani *et al*. (1998) and Sanità di Toppi et al. (2002) challenged this hypothesis since the antioxidant status under elevated CO_2 in herbaceous plants was found to be extremely variable (Rao *et al*. 1995; Marabottini *et al*. 2001, Schwanz and Polle 2001). We measured leaf gas-exchange, chlorophyll fluorescence, structural molecules and metabolites involved in photoprotection of *Q. ilex* resprouts grown under elevated CO_2 during their whole development and analyzed their responses with increasing temperatures up to 45°C; finaly, we compared them with those observed in resprouts grown and exposed to current CO_2. The leaf concentration of several leaf chemicals (nitrogen, soluble sugars, starch, cellulose, hemicellulose, lignin and lipids), chloroplast pigments (chlorophylls *a* and *b*, neoxanthin, violaxanthin, zeaxanthin, antheraxanthin, α- and β-carotene, lutein and lutein epoxide) and antioxidants (α-, β- and γ-tocopherol, ascorbate and total phenolics) were determined in a single measurement using a NIRS (near infrared spectroscopy) database previously developed by the authors.

The aim of this study was to determine, in resprouting holm-oak, if increases in ambient CO_2 would result in changes in the antioxidant defence system and their response when applying a high temperature stress.

The characterization of photoprotective and antioxidant systems under stress acting in *Q. ilex* during resprouting will contribute to the knowledge of their future adaptation after perturbations. We worked on plants developed entirely under higher CO_2 since we consider it a good model to test future effects as earlier reported (Aranda *et al*. 2006).

3.2. Material and methods

Plant material, growth conditions and experimental design

2-year-old holm oaks were obtained from a nursery (Bioriza, Cornellà de Terri, Spain); their gas-exchange, chlorophyll fluorescence and biomass characteristics before the application of the different treatments (data not shown) demonstrated the homogeneity between the different individuals. Plants were then transferred into 6-l pots filled with peat and perlite (2:1, v/v) and a slow-release fertilizer (2 $kg \cdot m^{-3}$, Osmocote OSMP 1214, MYC 5, Spain), and irrigated daily. For three months, 20 plants were grown in two controlled-environment greenhouses at two different CO_2 concentrations: ambient (A-plants, 350 $\mu L \cdot L^{-1}$) and elevated (E-plants, 700 $\mu L \cdot L^{-1}$). Measurements were conducted after those three months. Thereafter, all plants were excised and aerial biomass was removed above the point of attachment of cotyledons (cotyledonary node). Excised plants rested for four more months while resprouting in chambers (Conviron, Winnipeg, Canada) at their respective original CO_2 concentrations: ambient (RA, 350 $\mu L \cdot L^{-1}$) and elevated (RE, 700 $\mu L \cdot L^{-1}$). Environmental conditions were programmed: temperature: 25ºC; relative humidity: 70%; mean photosynthetic photon flux density (PPFD): 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$; day/night photoperiod: 14/10 h. Each chamber contained 10 plants that were irrigated daily with water (200-500 mL depending on the water demand during different periods of growth). To avoid position effects, pots were rotated inside each chamber and moved from one chamber to the other but keeping the corresponding CO_2 treatment throughout the experiment. Measurements after 4 months in chambers were performed on RE and RA. Finally, plants of the same height (c. 0.45 m) were exposed to temperature increases from 25°C to 45°C in 10ºC steps, followed by a final recovery at 25°C (25R). Plants were kept at specified temperatures for 20 h before measurements. All measurements were performed using green, fully-developed leaves photosynthetically active even at 45ºC.

Gas-exchange measurements

A Walz GFS-3000 portable photosynthesis system (Heinz Walz GmbH, Effeltrich, Germany) was used to estimate net photosynthesis (A), transpiration (E) and total conductance (g_T). Boundary layer conductance (g_{bl}), estimated from the transpiration of a wet filter paper enclosed in the leaf chamber, was

assumed to be constant between measurements (500 μmols·m^{-2}·s^{-1}). Water use efficiency (WUE) was calculated as A/E. To test the homogeneity of the initial population, nine randomly individuals were measured. During the A- and E-plants sampling, nine to eleven individuals per treatment were measured. To evaluate the CO_2 effect on resprouting and the high temperature stress, one fully expanded leaf per individual (five to six individuals per treatment and temperature) was measured. In the leaf chamber of the GFS-3000, light and humidity conditions were kept similar to those in the greenhouses or in the growing chambers (500, 1200 or 300 μmol·m^{-2}·s^{-1} for PPFD and absolute humidity). Temperature was kept constant at the value the plants had been exposed to during the previous 20 h (25ºC, 35ºC, 45ºC and 25R). Air flow was kept at 750 μmol·s^{-1}.

Chlorophyll fluorescence measurements

Steady-state modulated chlorophyll fluorescence was determined with a portable fluorimeter (Mini-PAM; Walz, Effeltrich, Germany) as described in Peña-Rojas et al. 2004. Light-adapted components of chlorophyll fluorescence were measured: Steady-state fluorescence (F), maximal fluorescence (F'_m), variable fluorescence F'_v equivalent to (F'_m- F) and quantum yield of PS II photochemistry (Φ_{PSII}) equivalent to (F'_m-F)/F'_m. Leaves were then dark-adapted to obtain F_o (minimum fluorescence), F_m (maximum fluorescence), F_v variable fluorescence (equivalent to F_m-F_o) and F_v/F_m (maximum quantum yield of PSII photochemistry, equivalent to (F_m-F_o)/F_m). Adaptation took at least 20 min, after which F_v/F_m values reach about 95% of the pre-dawn values in *Q. ilex* (Fleck et al. 1998). F'_o was estimated after Oxborough and Baker (1997) and used to calculate qP, photochemical quenching of fluorescence (equivalent to (F'_m-F)/(F'_m-F'_o)), and F'_v/F'_m, the intrinsic efficiency of open PSII centres during illumination (equivalent to (F'_m-F'_o)/F'_m). Three different leaves of each individual were measured. All data were corrected for changes in the fluorescence detector sensitivity induced by temperature variation of the Mini-Pam.

Relative water content, biomass and leaf area determination

The relative water content (RWC) of leaves was obtained as ((FW-DW)/(FSW-DW))x100 where FW is the fresh weight, FSW is the fresh saturated weight after rehydrating samples for 24 h in the dark at 4ºC, and DW is the dry weight after oven-drying samples at 65ºC until constant weight). Mean leaf area

(LA) was obtained with a flat-bed scanner (Epson GT5000) and processed using image analyzer software supplied by Serveis Científic-Tècnics (Universitat de Barcelona). Leaf dry weight over fresh weight (DW/FW), leaf mass per area (LMA, equivalent to DM/LA) and shoot/ root (aerial (leaf + stem) to subterranean) biomass ratio were calculated.

Stomatal density

To asses the stomatal density (number of stomata·mm^{-2}), four adult leaves per tree, both in elevated and ambient CO_2 were collected. The total amount of plants was seven per treatment. Each leaf was air dried, the tricomes were scraped and a circular portion of 1 cm of diameter was taken. Each sample was sputtered with carbon and gold (Paoletti and Gellini 1993) and observed trough a scanning electron microscope (JEOL 840), at 15 Kv. Finally, five random zones per portion were counted. The stomatal density was counted both in A-plants and E-plants and in RA and RE collected at 25ºC.

Leaf chemistry

Twenty to twenty-five leaves per plant were lyophilised following Pintó-Marijuan et al. (submitted.) protocol and then milled in a Cyclotec 1093 Sample Mill (Tecator, Höganäs, Sweden). All samples were scanned with a near-infrared reflectance spectrophotometer (NIR Systems 6500, Foss NIR Systems, Inc, Silver Spring, MD) following the procedure described by Joffre et al. (1992). Leaf concentrations of nitrogen (N), soluble sugars, starch, cellulose, hemicellulose, lignin, and lipids were determined based on calibration equations built on the spectral and wet chemical database of 260 samples including *Quercus ilex* leaves collected throughout Mediterranean area (Meuret et al. 1993). Nitrogen content was determined with a Perkin Elmer elemental analyser (PE 2400 CHN). Fiber fractions (cellulose, hemicellulose and lignin) were determined using the Fibertec procedure (van Soest and Robertson 1985). Starch and soluble sugars analysis were carried out following the method of Farrar (Farrar 1993). Independent calibration equations were built for each chemical constituent using partial least squares algorithm (Martens and Naes 1989) allowing the percentage concentration of biochemical constituents to be determined from the spectra, using partial least squares regression, with a standard error of prediction of 0.13% for nitrogen, 0.97% for cellulose, 0.87% for hemicellulose, 0.94% for lignin, 0.55% for soluble sugars, 1.10% for starch and 0.66% for ash. Total non

structural carbohydrates (TNC) correspond to the sum of soluble sugars and starch.

Chloroplast pigments and antioxidants

Leaf concentrations of chloroplast pigments (neoxanthin, violaxanthin, zeaxanthin, antheraxanthin, α- and β-carotene, lutein, lutein epoxide, chlorophylls *a* and *b*), lipophilic antioxidants (α-, β- and γ-tocopherol), hydrophilic antioxidants (ascorbate) and total phenolics were determined, with one single measurement on the same samples used for leaf chemistry determination, applying the new created NIRS data base based on calibration equations obtained (Pintó-Marijuan *et al*. (submitted). Chloroplast pigments and tocopherols were analysed by reverse-phase high performance liquid chromatography (HPLC) following the method by García-Plazaola and Becerril (1999a), with the modifications described in García-Plazaola and Becerril (2001). Ascorbic acid analyses according to Grantz *et al*. (1995) and phenol content determined according to Singleton and Rossi (1965).

Statistical analyses

All statistical procedures were done using SPSS for Windows (SPSS for Windows v. 15.0, SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to test the main effects against appropriate error terms between treatments (A-Plants with E-Plants and RA with RE) and among temperatures (25º, 35º, 45º and 25ºR) of gas exchange and chlorophyll fluorescence parameters, relative water content and leaf biomass parameters, leaf chemistry, chloroplast pigments and antioxidant compounds. The post-hoc Duncan test was applied when appropriate. Stomatal density data was also analysed by ANOVA test, comparing A and E-plants plants with their respective resprouts (RA, RE). Statistical significance was set at $p \leq 0.05$. The number of replicates is indicated in each table and figure legends.

3.3. Results

Gas-exchange and chlorophyll fluorescence

Plants grown for three months in greenhouses with either ambient or elevated CO_2 concentration (A- or E-plants respectively) and measured at their respective growth CO_2 concentration (Figure 1) showed no differences in assimilation rate and in the chlorophyll fluorescence measured parameters (Φ_{PSII}, F_v/F_m, qP, and F'_v/F'_m), whereas E-plants showed lower transpiration (E) and total conductance (g_T) and higher WUE. Resprouts grown for four months in chambers with ambient or elevated CO_2 concentration (RA or RE respectively) and measured at their respective growth CO_2 concentration (Figure 2) showed also lower E and g_T. Stomatal density showed significant differences between A-plants and RA, but no differences were observed between E-plants and RE (Figure 3). When a heat stress was applied, RA and RE maintained photosynthesis rates (A) when temperature rose from 25ºC to 35 ºC decreasing thereafter (Figure 4). E and g_T were higher in RA especially with increasing temperature, while RE were slightly affected. Regarding chlorophyll fluorescence parameters, both kinds of resprout showed maintained values of Φ_{PSII}, F_v/F_m, qP, and F'_v/F'_m when temperature rose from 25ºC to 35ºC, but these values decreased at 45ºC (Figure 5), especially in RE. A final recovery of gas-exchange and chlorophyll fluorescence values when submitting plants back to 25°C for 24h was not possible for either treatment: gas-exchange values decreased with respect to values at 45ºC whereas chlorophyll fluorescence values were similar to those obtained at 45ºC.

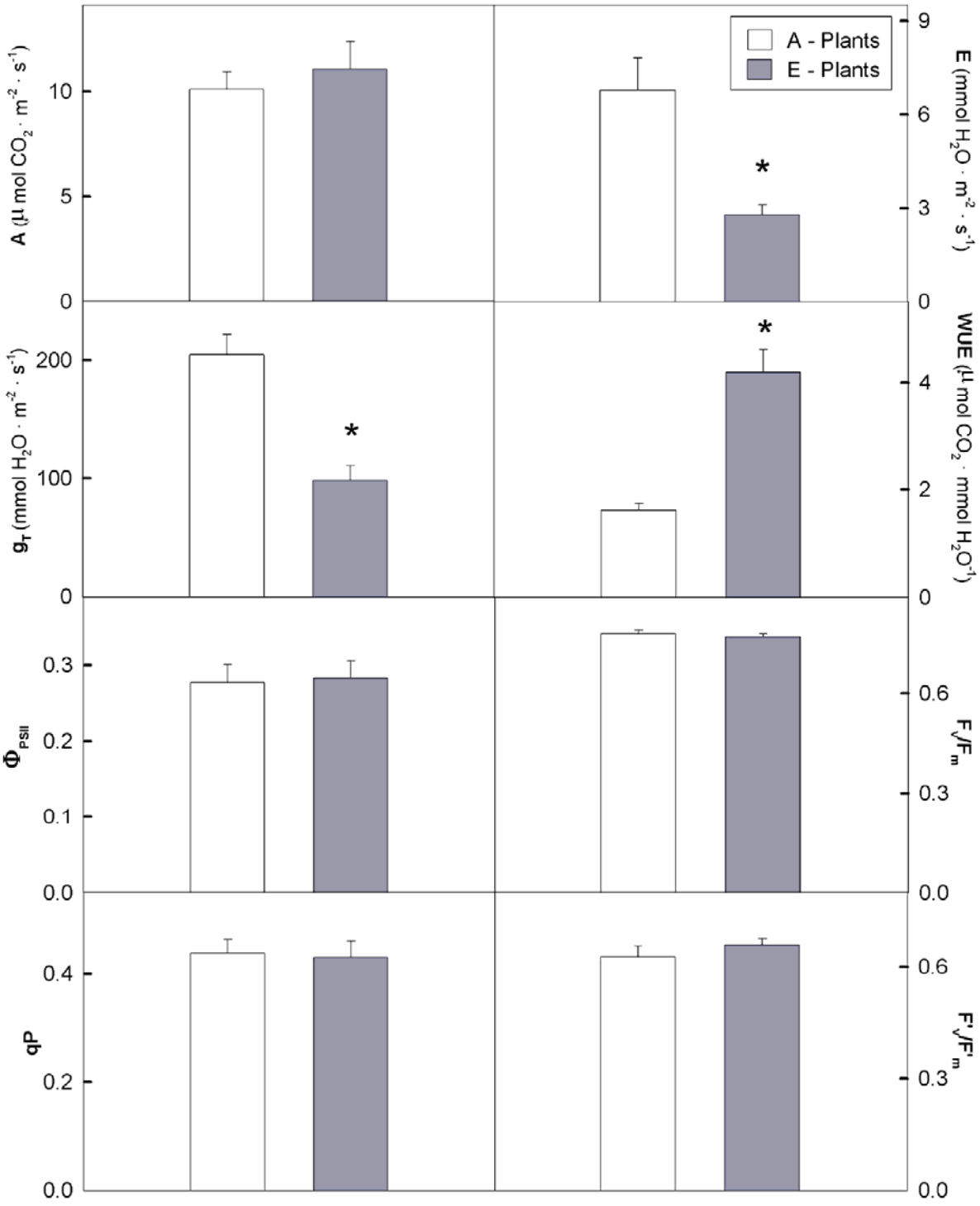

Figure 1. Gas-exchange and chlorophyll fluorescence parameters in *Q. ilex* plants grown at ambient (A-Plants) or elevated CO_2 concentration (E-Plants) for three months: net CO_2 assimilation rate (A), transpiration (E), total conductance (g_T), water use eficiency (WUE), photochemical PSII efficiency (Φ_{PSII}), potential PSII quantum yield (F_v/F_m), photochemical quenching of fluorescence (qP) and, intrinsic efficiency of open PSII centers (F'_v/F'_m). Each value represents the mean ± S.E. of nine plants per treatment (fluorescence values are calculated from the mean of 3 measurements per plant). Asterisks indicate significant differences at $p<0.05$.

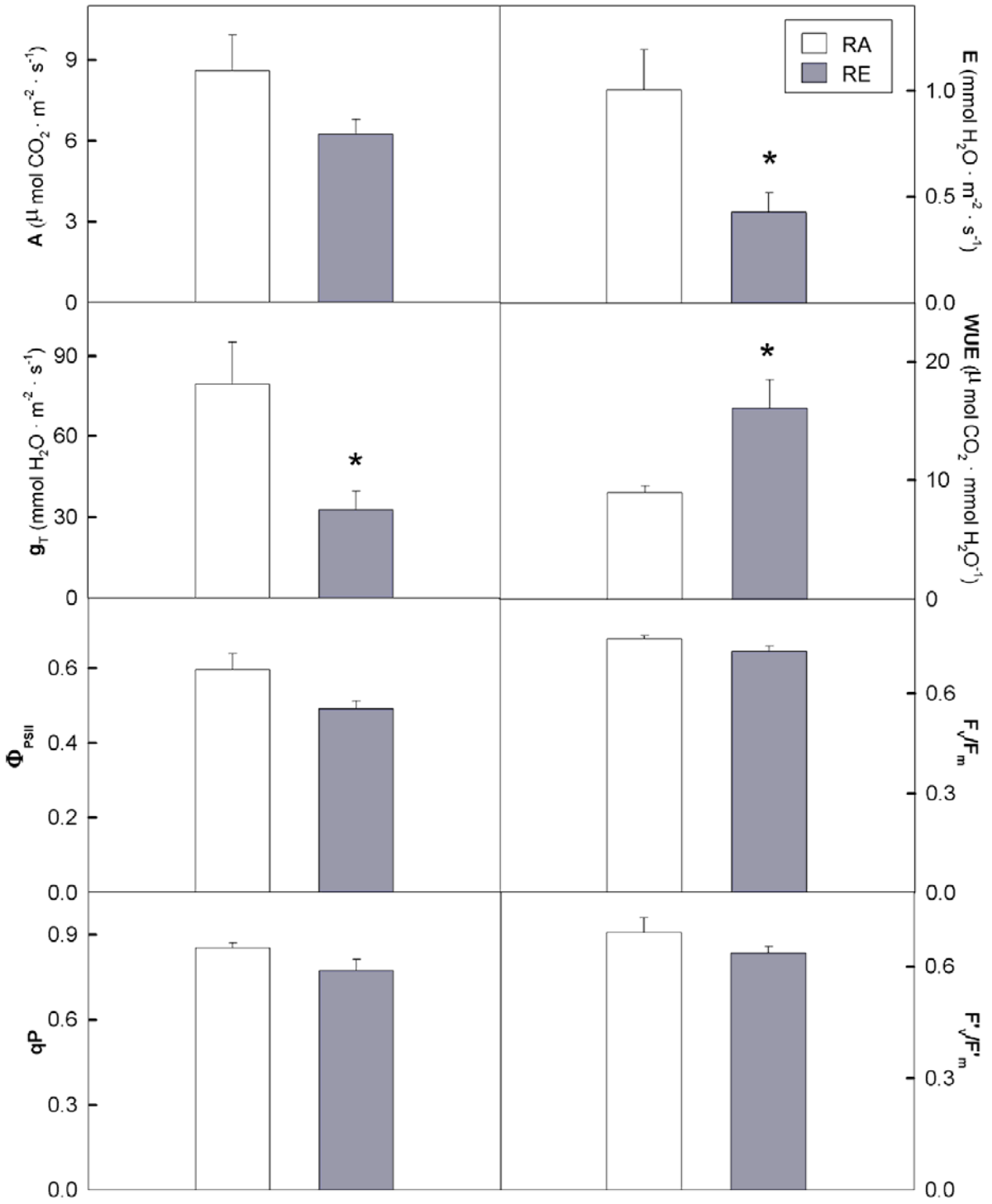

Figure 2. Gas-exchange and chlorophyll fluorescence parameters in *Q. ilex* resprouts grown at ambient (RA) or elevated CO_2 concentration (RE) for four months: net CO_2 assimilation rate (A), transpiration (E), total conductance (g_T), water use eficiency (WUE), photochemical PSII efficiency (Φ_{PSII}), potential PSII quantum yield (F_v/F_m), photochemical quenching of fluorescence (qP) and, intrinsic efficiency of open PSII centers (F'_v/F'_m). Each value represents the mean ± S.E. of five plants per treatment (fluorescence values are calculated from the mean of 3 measurements per plant). Asterisks indicate significant differences at $p<0.05$.

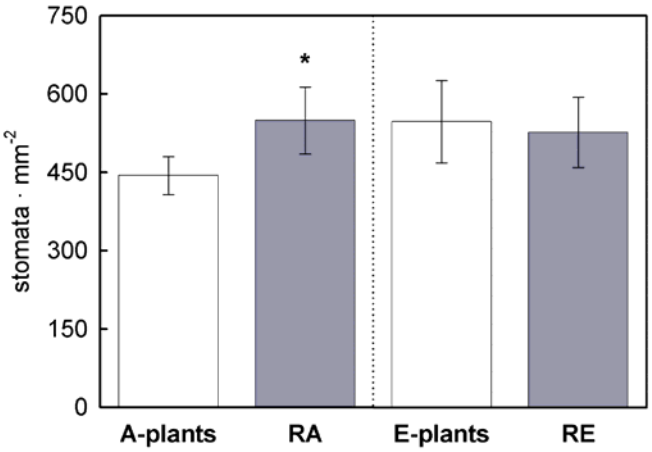

Figure 3. Stomatal density (stomata·mm^{-2}) in *Q. ilex* A-plants and their resprouts (RA) grown at ambient CO_2 concentration and in E-plants and their resprouts (RE) grown at elevated CO_2 concentration. Each value represents the mean ± S.E. of seven plants per treatment. In each plant, five measurements per leaf on four leaves were carried out. Asterisks indicate significant differences at $p<0.05$.

Relative water content and growth parameters

RE showed higher shoot (leaf and stem) and root biomass whereas shoot/root ratio, % leaf and stem dry weight, RWC and LMA were similar between kinds of resprout (Table 1) and height was not significantly different (46.00±2.11 and 42.40±2.57cm respectively). No differences between A- and E-plants were obtained in any parameter (data not shown).

	RA	RE
RWC (%)	87.64 ± 1.66 ^{a}	88.34 ± 2.19 ^{a}
LMA (g·m^{-2})	142.71 ± 4.68 ^{a}	159.20 ± 7.10 ^{a}
% leaf DM	51.35 ± 0.43 ^{a}	53.00 ± 0.89 ^{a}
% stem DM	46.17 ± 0.58 ^{a}	46.94 ± 0.97 ^{a}
g DM leaf · plant^{-1}	17.04 ± 1.86 ^{a}	22.84 ± 1.23 ^{b}
g DM stem · plant^{-1}	9.14 ± 0.97 ^{a}	11.88 ± 0.85 ^{b}
g DM root · plant^{-1}	35.63 ± 2.37 ^{a}	60.14 ± 6.60 ^{b}
Shoot DM/Root DM	0.60 ± 0.04 ^{a}	0.67 ± 0.08 ^{a}

Table 1. Relative water content (RWC), leaf mass per area (LMA), % leaf and stems dry weight over fresh weight, aerial and underground biomass and shoot/root dry weight ratio in *Q. ilex* resprouts grown at ambient (RA) or elevated CO_2 concentration (RE). Values are mean ± S.E. of one leaf measurement on 5-9 *Quercus ilex* individuals. Different letters indicate significant differences between kinds of plant ($p<0.05$).

Leaf chemistry

RA and RE resprouts showed no differences in the content of nitrogen, cellulose, hemicellulose, lignin and soluble sugar, whereas starch, TNC and lipid content were higher in RE (Table 2). No differences in these parameters were obtained between A- and E-plants (data not shown).

	RA	RE
Nitrogen	2.29 ± 0.06 ^{A}	2.26 ± 0.06 ^{A}
Cellulose	19.94 ± 0.69 ^{A}	17.68 ± 1.37 ^{A}
Hemicellulose	17.74 ± 0.39 ^{A}	19.00 ± 0.73 ^{A}
Lignin	7.46 ± 0.40 ^{A}	4.76 ± 1.43 ^{A}
Soluble-Sugar	13.82 ± 0.40 ^{A}	14.54 ± 0.58 ^{A}
Starch	5.51 ± 0.63 ^{A}	9.74 ± 0.97 ^{B}
TNC	19.33 ± 1.02 ^{A}	24.29 ± 1.53 ^{B}
Sclerophyll Index	40.95 ± 1.08 ^{A}	38.82 ± 1.71 ^{A}
Starch · Nitrogen^{-1}	2.44 ± 0.35 ^{A}	4.33 ± 0.48 ^{B}
Lipids	14.37 ± 0.61 ^{A}	17.26 ± 0.97 ^{B}

Table 2. Leaf chemical composition (% dry weight) in *Q. ilex* resprouts grown at ambient (RA) or elevated CO_2 concentration (RE). Values are mean ± S.E. of one leaf measurement on 5-9 *Quercus ilex* individuals. Different letters indicate significant differences between kinds of plant ($p<0.05$).

Chloroplast pigments and antioxidants

Although not always significant α- and β-carotene (α-Car and β-Car, respectively), neoxanthin (Neo), lutein epoxide (Lx), lutein (Lut), the total amount of chlorophylls (TChl: Chl*a*+Chl*b*) and α- and γ-tocopherol (α-Toc and γ-Toc, respectively) showed higher concentration in RA, while ascorbate (Asc) and total phenolics (TPhe) concentration as well as the de-epoxidation state (defined as Z/VAZ) were higher in RE (Figs. 6 and 7).

When a temperature stress was applied, the response of some studied compound tended to increase. For instance, in RA individuals throughout the stress period α-Car, Lut, TChl and α-Toc suffered a gradual significant increase from 25ºC to 45ºC. The recovery treatment was not effective. Differences between RA and RE in all chloroplast pigments became significant at 35ºC, whereas regarding cellular oxidative-state indicators such as Asc, TPhe or Z/VAZ, differences were significant during almost the whole heat stress treatment.

3.4. Discussion

Results obtained in two homogeneous populations of *Q. ilex* growing for three months under current (350 $\mu L \cdot L^{-1}$) (A-plants) or under elevated (750 $\mu L \cdot L^{-1}$) CO_2 concentration (E-plants) showed, when measured at their respective CO_2 growth concentration, differences in water relations, i.e. lower E and g_T in E-plants, implying higher WUE (Figure 1). All the other measured parameters did not show differences at this stage. The observed g_T decline at elevated CO_2 is consistent with results by Curtis *et al*. (1996), Tognetti *et al*. (1998) and Medlyn *et al*. (2001) and could compensate for the effects of drought in a future high CO_2 ambient (Wullschlegel *et al*. 2002). However, E and g_T decline was not always supported in FACE experiments (Ainsworth and Long 2005).

Concerning the resprouts obtained from A- or E-plants and grown for four months at the two CO_2 concentrations (RA and RE respectively), they showed also decreased g_T and E in RE and lower photosynthesis rates (Figure 2). Stomatal density was similar in RA and RE, therefore it is likely that changes in stomatal aperture rather than stomatal density determine the response of g_T to elevated CO_2 (Ainsworth and Rogers 2007, Paoletti *et al* 2007). In spite of the differences in g_T, similar RWC in both kinds of resprouts reflected the conservative use of water in this species (Peña-Rojas *et al*. 2005).

Photosynthesis down-regulation in RE has been reported in earlier works for *Q. ilex* (Aranda *et al*. 2006, Saurer *et al*. 2003) and for other *Quercus* species (Li *et al*. 1999, Faria *et al*. 1996, Bunce 1992), whereas a stimulation of *Q. ilex* photosynthesis has also been described (Scarascia-Mugnozza *et al*. 1996, Paoletti *et al*. 2007). However, F_v/F_m showed no differences between treatments indicating no CO_2 effect on photoinhibition as reported for beech and pine (Hogan *et al*. 1997). RE showed increased growth (higher leaf, stem and root biomass) than RA (Table 1), that can be attributed to enhanced growth and photosynthesis of RE at the initial stages of development after excision (Hättenschwiler *et al*. 1997). However, differences in aboveground/underground biomass ratio were not observed. CO_2 growth concentration did not affect LMA as reported in *Q. ilex* (Staudt *et al*. 2001, Aranda *et al*. 2006).

Down-regulation of photosynthesis in RE can likely have been originated by an accumulation of TNC (26% higher than in RA) (Table 2), mainly due to higher starch content (Körner and Miglietta 1994, Tognetti *et al*. 1998). TNC accumulation is the consequence of an inadequate sink capacity (Ainsworth *et al*. 2004), which is often correlated with the repression of Rubisco gene expression (Moore *et al*. 1999). Photosynthesis acclimation is also attributed to decreases in leaf N (Griffin *et al*. 2000), but in our study, it was similar in both

kinds of resprouts as found in other woody species (Ainsworth and Long 2005). Sink limitations by pot size (Staudt *et al*. 2001, Woodward 2002) were no likely since we observed no growth restriction in the roots of RE (Table 1). Also cellulose, hemicellulose and lignin did not differ in accordance with Blaschke *et al*. (2001). Growth in elevated CO_2 resulted in higher lipid concentration in RE (Table 2) due to changes in the quality of lipid metabolism (Williams *et al*. 1998), as well as higher total phenolic compounds concentration (Fig. 7c) as observed by Tognetti and Johnson (1999). The excess of TNC accumulated in RE may account for their incorporation into C-based secondary compounds as phenolics as predicted in the C-nutrient balance hypothesis and may have consequences in leaf quality for herbivores (Peñuelas and Estiarte 1998).

It is a general evidence that *Q. ilex* leaves experience stress below 15°C and above 35°C (Larcher 2000); in fact our results show stability in gas-exchange and chlorophyll fluorescence parameters from 25ºC to 35ºC and a decline thereafter (Figs. 4 and 5) especially in RA. Net photosynthesis showed a marked drop (around 40%) in both kinds of resprouts. A is particularly sensitive to heat stress and declines as temperature increases due to the relative increase of respiration and photorespiration as reported in *Q. suber* (Faria *et al*. 1999). The observed decline in photosynthesis, likely due to Rubisco deactivation at moderately high temperature (Kozba and Edwards 1987, Salvucci *et al*. 2001, Haldimann and Feller 2004) would protect against high rates of photorespiration especially under elevated CO_2 (Sharkey *et al*. 2005). Results on g_T and E showed that stomata responses of RE were less sensitive to high temperature stress than those of RA (Chaves *et al*. 1995, Faria *et al*. 1996, 1999) (Fig. 4b-c). Thermotolerance above 45ºC was unlikely and leaves did not show a recovery when submitted back to 25ºC in all measured parameters (Peñuelas *et al*. 2005).

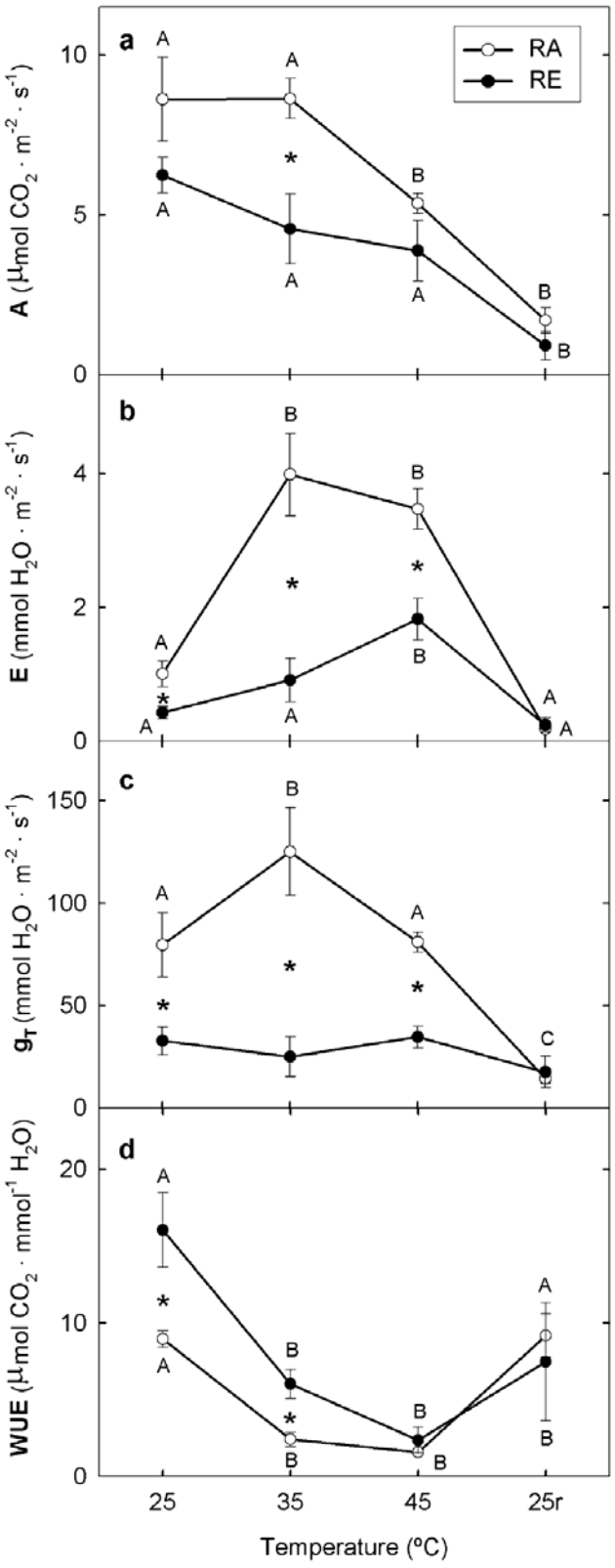

Figure 4. Changes in net CO_2 assimilation rate (A), transpiration (E), total conductance (g_T) and water use eficiency (WUE) in resprouts grown at ambient (RA, open circles) or elevated CO_2 concentration (RE, filled circles) progressively exposed to increasing temperatures (from 25 to 45ºC), followed by a final recovery at 25ºC (25R). Each value represents the mean ± S.E. of five plants per treatment (fluorescence values are calculated from the mean of 3 measurements per plant). Different letters indicate significant differences between temperature treatments and asterisks indicate significant differences between kinds of resprout ($p<0.05$). No letters indicate no significant differences among temperature treatments.

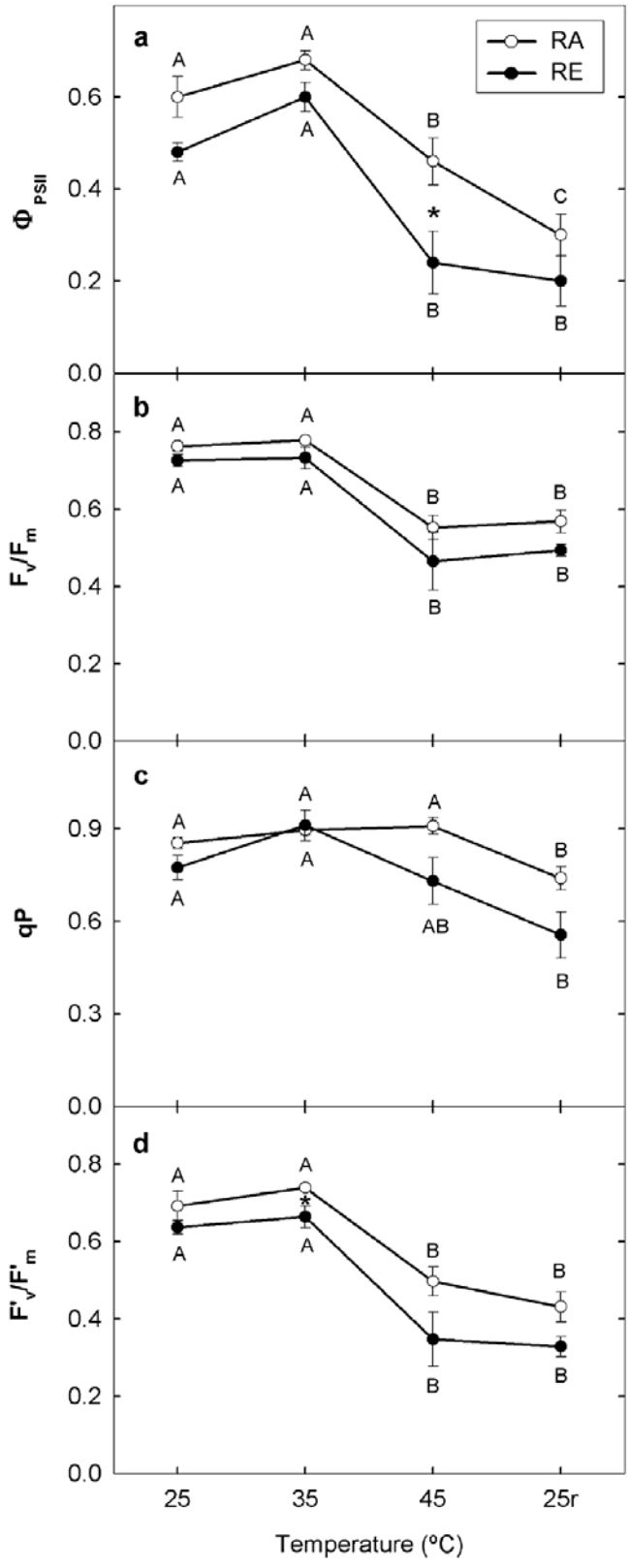

Figure 5. Changes in photochemical PSII efficiency (Φ_{PSII}), potential PSII quantum yield (F_v/F_m), photochemical quenching of fluorescence (qP) and, intrinsic efficiency of open PSII centers (F'_v/F'_m) in resprouts grown at ambient- (RA, open circles) or elevated CO_2 concentration (RE, filled circles) progressively exposed to increasing temperatures (from 25 to 45ºC), followed by a final recovery at 25ºC (25R). Each value represents the mean ± S.E. of five plants per treatment (fluorescence values are calculated from the mean of 3 measurements per plant). Different letters indicate significant differences between temperature treatments and asterisks indicate significant differences between kinds of resprout ($p<0.05$).

The chloroplast pigment and antioxidant compounds content highlight the differences between RE and RA. Enhanced concentration of Asc in RE (Fig. 7d) was remarkable as reported in *Trifolium repens, Dactylis glomerata* (Sanità di Toppi *et al*. 2002) and *Medicago sativa* (Sgherri *et al*. 2000) grown under elevated CO_2. Ascorbate is the most abundant antioxidant in *Q. ilex* leaves (García-Plazaola *et al*. 1999), and acts as scavenger of excess hydrogen peroxide generated in the leaf mesophyll chloroplasts during plant stress (Foyer and Noctor 2000). The higher Asc concentration in RE may provide in future holm-oak resprouts a higher antioxidant protection against any biotic or abiotic stress that act via generation of activated oxygen species. In that direction, under elevated CO_2, Marabottini *et al*. (2001) observed a large ascorbate pool in *Q. ilex* and *Q. pubescens* and Schwanz *et al*. (1996a) in *Cistus aurantium* leaves exposed to high light stress. As reported by Pallanca (1996), Schwanz *et al*. (1996b) and Polle and Eiblmeier (1995) the increase in Asc levels was correlated with carbohydrate accumulation.

RE also showed other photoprotection differences with respect to RA that became especially significant at 35ºC and higher temperature: lower content of lipophilic molecules (carotenoids chlorophylls and tocopherols), increased de-epoxidation index (Z/VAZ) (Fig. 7b), lower epoxidated products such as Lx content (Fig. 6d) and the decrease in F'_v/F'_m (Fig 5d). These results indicate a higher energy dissipation as heat and attributable to the decreased photosynthetic sink for electrons during high temperature stress.

The expected enhanced contribution of photorespiration as electronic sink with temperature increases was not sufficient to mitigate the unbalance between light capture and their utilization especially in RA, since several changes in photoprotective compounds were observed to avoid ROS production. Moreover, hydrogen peroxide is originated as a side reaction of oxygenation of RuBP and increases with increasing temperatures (Kim and Portis 2004). RA showed a significant increase with increasing temperature in their lipophilic components (Figs. 6 and 7): α-Car, Lut, Total Chl and α-Toc, whereas RE showed higher resistance to the heat treatment showing a little response in their antioxidant composition. The greater tolerance to oxidative stress caused by high temperatures in elevated CO_2-grown plants has been reported Bongi and Long (1987), Königer *et al*. (1998), Taub *et al*. (2000) and Naumburg *et al*. (2004) including *Q. ilex* (Chaves *et al*. 1995) and *Q. suber* (Faria *et al*. 1996 and 1999). Lower concentration of some chloroplast molecules in RE can be, partly, a consequence of the inhibition of the transcription of genes under elevated CO_2

(Moore *et al*. 1999) and attributed to the accumulation of soluble sugars (van Oosten *et al*. 1994).

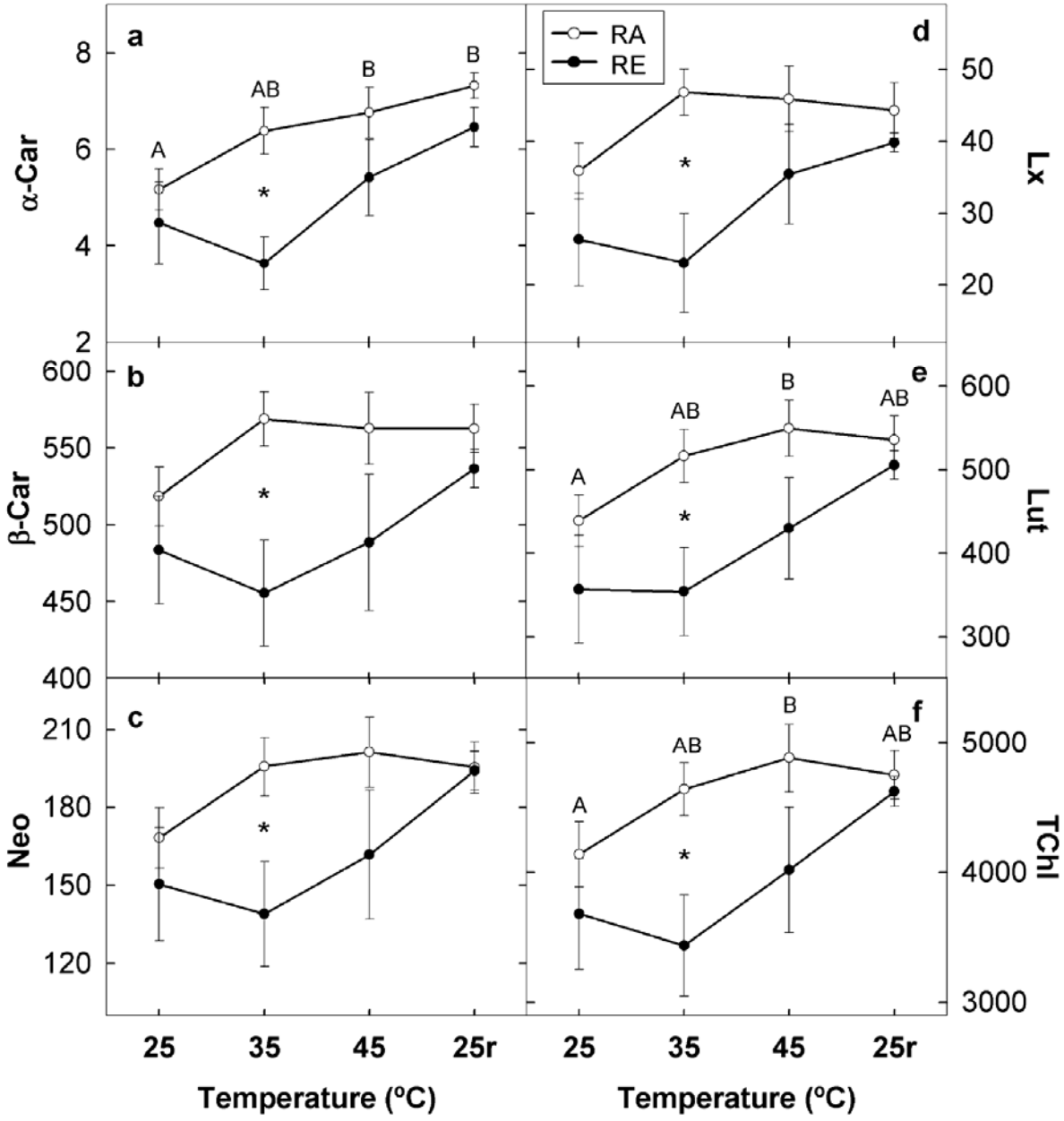

Figure 6. α- and β-carotene (α-Car, β-Car), lutein (Lut), lutein epoxide (Lx), neoxanthin (Neo) and total chlorophyll (Total Car/Chl) content in *Q. ilex* resprouts grown at ambient (RA, open circles) or elevated CO_2 concentration (RE, filled circles), progressively exposed to increasing temperatures (from 25 to 45ºC), followed by a final recovery at 25ºC. Each value represents the mean ± S.E. of five plants per treatment. Different letters indicate significant differences among temperature treatments and asterisks indicate significant differences between kinds of resprout ($p<0.05$). No letters indicate no significant differences among temperature treatments. Results are expressed as nmol·g^{-1}DW.

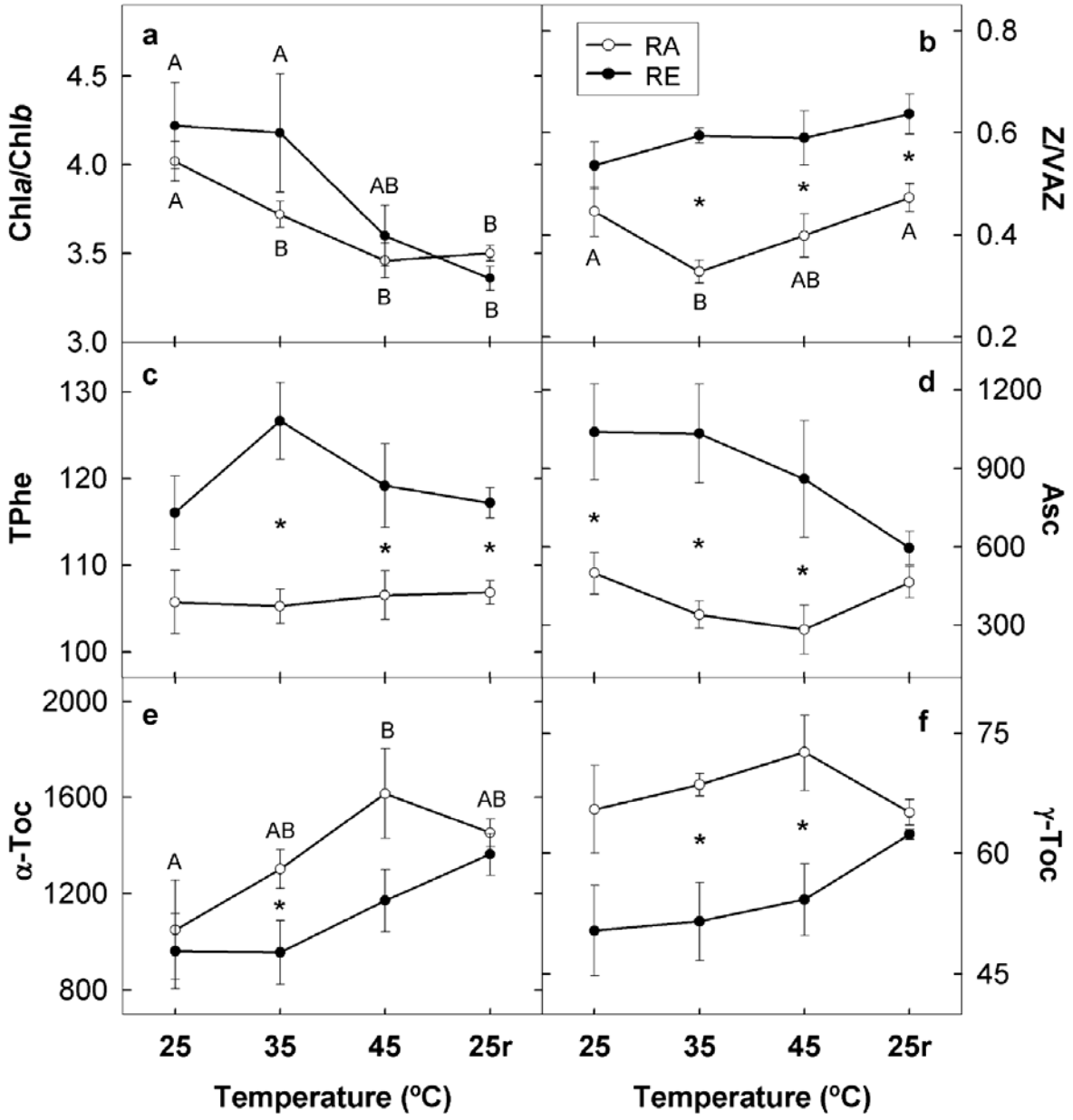

Figure 7. Chlorophyll*a*/chlorophyll*b* ratio (Chl*a*/*b*), the de-epoxidation state (Z/VAZ), total phenolicsv (TPhe), ascorbate (Asc), α-tocopherol (α-Toc) and γ-tocopherol (γ-Toc) in *Q. ilex* resprouts grown at ambient (RA, open circles) or elevated CO_2 concentration (RE, filled circles), progressively exposed to increasing temperatures (from 25 to 45ºC), followed by a final recovery at 25ºC. Each value represents the mean ± S.E. of five plants per treatment. Different letters indicate significant differences among temperature treatments and asterisks indicate significant differences between kinds of resprout ($p<0.05$). No letters indicate no significant differences among temperature treatments. Total phenolics are expressed as mg gallic acid equivalents·g^{-1}DW, ascorbate is expressed as µmol Ascorbic Acid·g^{-1}DW and α- and γ-tocopherol as nmol·g^{-1}DW.

In conclusion, our results indicate that higher antioxidant content and increased resistance to high temperature stress in resprouts grown under elevated CO_2 will contribute to the protection and regeneration of *Q. ilex* after disturbances in a future high CO_2 atmosphere.

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CAPÍTOL 4.

SEASONAL AND DIURNAL MONITORING OF LEAF POLYAMINE CONTENT IN *QUERCUS ILEX* L. RESPROUTS AFTER FIRE IN RELATION TO CHANGES IN IRRADIATION AND PHOTOSYNTHETIC PARAMETERS

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Variacions estacionals i diàries del contingut en poliamines en fulles de rebrot de *Quercus ilex* L. després d'incendi en relació a canvis d'irradiància i de paràmetres fotosintètics

Tant en alzines no perturbades (controls, C) com en els rebrots originats després d'incendi (R) i al llarg de les dues estacions que impliquen un estat d'estrès més destacat (estiu i hivern) van caracteritzar-se, en fulles de la mateixa edat, les variacions estacionals en el contingut de putrescina, espermidina i espermina lliures, així com en paràmetres de bescanvi gasós i de fluorescència de les clorofil·les. Observàrem una tendència general de descens en el contingut en putrescina en augmentar la intensitat lumínica. Així, el contingut en putrescina disminuí de forma notable d'hivern a estiu i especialment en els R, els quals es trobaven en una ubicació amb una disponibilitat lluminosa molt més elevada. En quant a les variacions diàries, la putrescina mostrà un descens del seu contingut del matí al migdia, el qual fou també més destacat en els R crescuts després d'incendi. Les mesures dels paràmetres del bescanvi gasós i de la fluorescència de les clorofil·les mostraren diferències notables entre ambdos tractaments (C i R) en les seves respectives condicions naturals. En els paràmetres de bescanvi gasós com la taxa d'assimilació de CO_2 (A), la conductància estomàtica (g_s) i la transpiració (E), els rebrots sempre tingueren uns valors superiors als dels controls (essent significativament superiors a l'estiu). En quant als indicadors de l'estat fotoquímic dels fotosistemes, els rebrots mostraren valors inferiors del rendiment quàntic relatiu del PSII (Φ_{PSII}), de la fracció de centres oberts del PSII (quenching fotoquímic, qP) i de l'eficiència intrínseca dels centres oberts del PSII (F'_v/F'_m). En la corba de resposta a la llum, Φ_{PSII} /PPFD, els rebrots posserien, amb la mateixa intensitat de llum, millor capacitat per la resposta fotoquímica (Φ_{PSII} més alt) que els C, fet que s'accentuà a intensitats més elevades. Malgrat l'augment d'irradiància de l'hivern a l'estiu, i especialment en les arees cremades, els paràmetres de fluorescència de les clorofil·les estudiats van mantenir els seus valors, indicant l'adaptació de l'aparell fotosintètic. Els resultats derivats de les corbes de resposta A/C_i i A/PPFD indicaren, en els rebrots al llarg de l'estrès estival, una capacitat fotosintètica superior i una baixa limitació no-estomàtica de la fotosíntesi. Es discuteix la contribució del descens de la putrescina lliure en el procés de fotoadaptació de l'aparell fotosintètic d'espècies forestals en el seu hàbitat natural.

Paraules clau: Bosc mediterrani; estrès abiòtic; fotosíntesi; paràmetres de fluorescència; putrescina.

Seasonal and diurnal monitoring of leaf polyamine content in *Quercus ilex* L. resprouts after fire in relation to changes in irradiation and photosynthetic parameters

Seasonal variations in free putrescine, spermidine and spermine content, gas-exchange and chlorophyll fluorescence parameters were followed during winter and summer on leaves of a similar age from undisturbed holm oak trees (control, C) and resprouts (R) originated after fire. We observed a general trend of putrescine content decrease with increasing irradiance. Putrescine content decreased markedly from winter to summer, especially in R, which were located on a site with much higher irradiation. Daily summer variations in putrescine showed a decline at midday from morning values, and they were also more accentuated in R. Measurement of gas-exchange and chlorophyll fluorescence parameters showed marked differences between C and R under their respective light conditions. R showed lower values of PSII quantum yield (Φ_{PSII}), photochemical quenching (qP) and intrinsic efficiency of open PSII centres (F'_v/F'_m). The Φ_{PSII}/PPFD response curve showed that under the same irradiance, Φ_{PSII} was enhanced in R and mainly under high light conditions. In spite of increasing irradiance from winter to summer, and especially in burned areas, the mentioned chlorophyll fluorescence parameters were maintained indicating the adaptation of the photosynthetic apparatus. Results derived from A/C_i and A/PPFD response curves showed enhanced photosynthetic capacity and lower non-stomatal limitation of photosynthesis in R during summer stress. The contribution of putrescine decline in the photoadaptation of the photosynthetic apparatus of species growing in natural forest habitats is considered.

Keywords: Abiotic stress; fluorescence parameters; Mediterranean forest; photosynthesis; putrescine.

4.1 Introduction

During their development, evergreens growing in Mediterranean forests are submitted to environmental constraints, which affect their physiology. The evergreen holm oak (*Quercus ilex* L.) is a deep-rooted dominant species of these forests that shows a great resprouting capacity after perturbations (fire, clear-cut, grazing). Resprouts show stimulated photosynthesis and rapid growth (Fleck *et al*. 1998) because of greater water and nutrient availability with respect to the original plants, as the pre-existing root system is associated with a much smaller aerial biomass (Kruger and Reich 1997). Holm oak is often exposed to high irradiance and also to other environmental stress factors (Savé *et al*. 1999). During the summer, drought induces reductions in leaf stomatal conductance, thereby restricting the availability of CO_2 in chloroplasts, resulting in a midday depression of photosynthesis (Tenhunen *et al*. 1987); in winter, low temperatures reduce CO_2 fixation (Baker 1994). In both seasons, energy originated by light absorption can exceed that used for carbon assimilation, thereby leading to a high production of reactive oxygen species (ROS) affecting the structural integrity and promoting cellular death (Baier and Dietz 1999). Resprouting vegetation after disturbances can be especially affected since ambient light intensity is increased with respect to a mature forest with its self-shading effect.

Several defence systems protect the photosynthetic apparatus of *Q. ilex* under conditions of excess excitation energy; these include dissipation as heat both by the xanthophyll cycle (Fleck *et al*. 1998; Peñuelas *et al*. 1998) and by the lutein epoxide cycle (Llorens *et al*. 2002; García-Plazaola *et al*. 2003). Moreover, the contribution of antioxidant systems and the expression of low molecular weight heat-shock proteins (sHsps) are also enhanced in this species under summer stress conditions (Verdaguer *et al*. 2003).

Polyamines (PAs) are small polycations that are present in all living organisms, from bacteria to animals. Putrescine (Put), spermidine (Spd) and spermine (Spm) are the most abundant PAs in these organisms. PAs are involved in various plant growth and developmental processes ranging from promotion of cell growth and differentiation (Evans and Malmberg 1989), inhibition of senescence (Borrell *et al*. 1997), and induction of antioxidative defence (Løvaas 1997; de Agazio and Zacchini 2001) to responses to environmental stress (Kumar *et al*. 1997; Bouchereau *et al*. 1999).

The contribution of PAs to the protection of the photosynthetic apparatus in colonial green algae during certain kinds of stress, namely elevated CO_2 treatment (Logothetis *et al*. 2004), UV-B radiation (Sfichi *et al*. 2004), and ozone pollution (Navakoudis *et al*. 2003) has attracted considerable interest. Beigbeder *et al*. (1995) described the relationship between PA levels and the rates of chlorophyll biosynthesis and photosynthesis during the development of pro-plastids into chloroplasts. Besford *et al*. (1993) observed that the addition of PAs inhibits the destruction of thylakoids and prevents loss of pigment during senescence. In spinach, Put, Spd and Spm are associated with thylakoid membranes and with various photosynthetic sub-complexes, while the PSII core and the reaction centre of this system contain mainly Spm (Kotzabasis *et al*. 1993).

Little information is available on variations in the PA content in forest trees during natural stress in function of plant photosynthetic performance during the growth cycle. In this study, we monitored the free PA content in leaves from undisturbed holm oak plants and from resprouts after fire in the two main stressing seasons, winter and summer, and throughout the day in summer. Concomitantly we monitored climatic conditions and performed leaf gas-exchange and fluorescence measurements on the same leaves. The aim of the study was to ascertain if changes in the polyamine content in holm oak occurred during the adaptation to climatic changes. *Q. ilex* is endangered by increased drought and forest fire frequency and intensity (Davis and Michaelsen 1995), phenomena associated with global change (Houghton *et al*. 2001). The characterization of the physiological responses of this tree to environmental stress is crucial in order to predict its survival rate and resprouting capacity under more arid conditions and therefore of the maintenance of ecosystem diversity.

4.2 Material and methods

Experimental site and plant material

Studies were carried out in Sant Medir, Serra de Collserola forest, Barcelona, Spain; 41º26’45’’N, 2º07’28’’E at an elevation of 160 m and oriented north-northwest. The climate is Mediterranean, with cold winters, cool wet springs and autumns, and hot dry summers. The area registers a mean annual temperature of 13–14ºC, and an annual rainfall of 500–700 mm. Climatic data during the study were recorded at the Fabra Observatory meteorological station,

located 5 km from the study site (Table 1). The days on which measurements were taken and samples were harvested are considered representative of winter and summer days. In July 2003, a wildfire burnt 100 ha of the forest. In winter 2004, we set out two plots: one intact (10 m × 50 m) (control) and one burned (15 m × 25 m), both with the same orientation and soil characteristics. The pre-fire vegetation was a 30-year-old forest dominated by *Pinus halepensis* and *Q. ilex.* Seven *Q. ilex* plants per site were randomly selected; plants of the unburned area were designated C (control) and resprouts originated after the fire R.

	Winter (03.01.04–03.02.04)	Summer (11.07.04–11.08.04)
Mean daily temperature (°C)	8.62 ± 0.43	23.44 ± 0.47
Mean daily maximum temperature (°C)	14.06 ± 0.43	29.68 ± 0.58
Mean daily minimum temperature (°C)	4.58 ± 0.54	18.00 ± 0.43
Mean daily relative humidity (%)	72.13 ± 1.86	67.19 ± 1.99
Mean daily atmospheric pressure (HPa)	971.03 ± 1.08	970.13 ± 0.47
Mean daily precipitation (mm)	0.06 ± 0.04	1.07 ± 0.88
Solar irradiance (W m^{-2})	88.91 ± 5.34	329.56 ± 18.92

Table 1. Climatological data. Data were obtained from the meteorological station nearest to the study site for 1 month before the days that samples and measurements were taken.

Samples for PA content analysis were harvested at midday in winter and in the morning, midday and evening in summer. Two fully developed, current-year leaves from three to five plants randomly selected in each plot (C, R) were immediately frozen in liquid nitrogen, stored at -80ºC and lyophilised.

Analysis of polyamine content

Lyophilised samples, to which five volumes (FW/v) of a 5% (w/v) cold perchloric acid solution were added, were maintained at 4ºC for 24 h and then centrifuged at 15,000×g for 10 min. Aliquots of the supernatant were dansylated (Goren *et al*. 1982) and separated by high-performance liquid chromatography (HPLC Beckman-Coulter, Fullerton, CA, USA). Samples were injected on a C18 reverse-phase column (250 mm×4.6 mm, 5 µmpore size, Alltech, Deerfield, IL, USA) and quantified using a Jasco (mod. FP 2020 Plus, Tokyo, Japan, excitation 360 nm, emission 510 nm) fluorescence detector, following standard curves. Elution was performed with a multi step linear gradient of aqueous acetonitrile: 50–30% water in 3 min, 30–20% in 4 min, 20–0% in 6 min, 0% for 3 min, 0–50% in 2 min, at a flow rate of 1 ml min^{-1} (Zacchini and de Agazio 2004). Chromatograms were recorded and integrated by the 32 KaratTM Software v 5.0 (Beckman-Coulter, Fullerton, CA, USA).

Leaf gas-exchange and chlorophyll fluorescence measurements

Simultaneous measurements of gas-exchange and chlorophyll fluorescence were performed with a gas-exchange system (LI-6400 Li-Cor, Lincoln, NE, USA) equipped with a 6400-40 Leaf Chamber Fluorometer. Inside the chamber, the following conditions were maintained during the measurements for plotting A/PPFD (light response curves response curves of CO_2 assimilation): C_a (ambient CO_2 concentration): 400 µl l^{-1}, PPFD (photosynthetic photon flux density): 0, 50, 100, 200, 400, 600, 800, 900, 1000, 1500 µmol m^{-2} s^{-1}. For plotting A/C_i (CO_2 response curves of CO_2 assimilation), PPFD was established at 900 µmol m^{-2} s^{-1}, which was saturating for holm oak photosynthesis (Fig. 3b) and C_i (intercellular CO_2 concentration): 0–730 µl l^{-1}. PPFD (A/PPFD) and CO_2 (A/C_i) response curves of CO_2 assimilation were obtained from data one attached, fully developed, current-year leaf from 3 to 5 plants randomly selected in each plot (C, R). Data were recorded at all levels of the response curve when the parameters measured were stable (3–9 min). The following conditions were established for the two response curves: in the winter, water mole fraction: 8±0.3 molH_2O molair^{-1}, leaf temperature: 15ºC and air flux: 200 µmol s^{-1}; and in the summer, water mole fraction: 21±0.4 mol H_2O mol air^{-1}, leaf temperature: 32ºC and air flux: 100 µmol s^{-1}.

Analyses of A/PPFD curves allowed the determination of A_{sat}, the light-saturated rate of net CO_2 assimilation at ambient CO_2 concentration. Analyses of the A/C_i curves allowed the determination of the changes in net CO_2 assimilation at saturating C_i (A_{max}), maximum carboxylation velocity of Rubisco (Vc_{max}) and maximum potential rate of electron transport contributing to RuBP regeneration (J_{max}) for the distinct leaves. Vc_{max} and J_{max} were calculated by fitting a maximum likelihood regression below and above the inflexion of the A/C_i response using Kc, Ko (Michaelis-Menten constants for CO_2 and O_2), as described by Mc-Murtrie and Wang (1993).

Steady-state modulated chlorophyll fluorescence was determined simultaneously with the gas-exchange measurements while plotting A/PPFD and A/C_i response curves. Leaves were dark-adapted to obtain minimum fluorescence yield (F_o), maximum fluorescence yield (F_m) and maximum quantum yield of PSII photochemistry (F_v/F_m) (equivalent to $(F_m-F_o)/F_m$). Adaptation took at least 20 min, after which F_v/F_m values reach about 95% of the predawn values in *Q. ilex* (Fleck *et al*. 1998). The light adapted components of chlorophyll fluorescence (steadystate yield (F), maximum fluorescence yield (F'_m) and quantum yield of photosystem II (PSII) photochemistry (Φ_{PSII}; equivalent to $(F'_m-F)/F'_m$)) (Genty *et al*. 1989) were measured simultaneously

during every plot of each curve. Parameters F'_o, minimum fluorescence yield in lightadapted state; qP, photochemical quenching (equivalent to $(F'_m-F)/(F'_m-F'_o)$), and F'_v/F'_m, intrinsic efficiency of open PSII centres during illumination (equivalent to $(F'_m-F'_o)/F'_m$) were estimated following Oxborough and Baker (1997). The relative electron transport rate ETR, was calculated according to Krall and Edwards (1992) as $ETR=\Phi_{PSII}\times PPFD\times 0.5\times 0.82$, where 0.5 is a factor that assumes equal distribution of energy between the two photosystems and 0.82 the light absorptance we obtained on *Q. ilex* leaves using an integrating sphere.

During summer gas-exchange measurements, leaf and air temperature, PPFD and vapour pressure deficit (VPD) were recorded in control (C) and burned stands (R) in the morning (8–9 h), midday (12.30–13.30 h) and evening (17–18 h) (Table 2).

	Control	Resprouts
Morning		
PPFD (μmol m^{-2} s^{-1})	28.50 ± 0.50	331.50 ± 106.50
VPD (kPa)	1.24 ± 0.04	1.66 ± 0.46
T air (°C)	32.23 ± 1.13	35.59 ± 1.45
Midday		
PPFD (μmol m^{-2} s^{-1})	62.75 ± 11.29	1045.80 ± 29.34
VPD (kPa)	2.89 ± 0.42	3.06 ± 0.60
T air (°C)	31.45 ± 1.15	31.40 ± 1.11
Evening		
PPFD (μmol m^{-2} s^{-1})	6.00 ± 2.00	54.00 ± 15.00
VPD (kPa)	3.04 ± 0.62	2.75 ± 0.51
T air (°C)	27.18 ± 0.41	28.02 ± 0.78

Table 2. Climatological data. Data were recorded at the forest sites: unburned (control) and burned (resprouts) in the summer during the gas-exchange measurements in the morning (8–9 h), midday (12.30–13.30 h) and evening (17–18 h)

Statistical analyses

All statistical procedures were performed using SPSS for Windows (SPSS forWindows v. 11.0, SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to test the main effects and interactions, against appropriate error terms, of kinds of leaf (R, C) and season (winter, summer) on results of PA content, gas-exchange or chlorophyll fluorescence; time of day (morning, midday and evening) was also included for summer PA content analysis. Scheffe post-hoc test was applied when it was appropriate. Logarithmical regressions were calculated to test the relationship between parameters in the A/C_i; and A/PPFD response curves. Statistical significance was set at $p\leq 0.05$.

4.3 Results and discussion

Seasonal and diurnal variations of free Put, Spd and Spm content were observed in holm oak leaves of a similar age collected in the forest of undisturbed trees (C) and resprouts (R) originated after fire. Free total PA content on fresh weight basis was higher in winter than in summer in C and R (twofold and threefold, respectively) (Table 3). Results on dry weight or area basis (data not shown) presented the same trends as those reported for PAs on fresh weight basis. Moreover, neither treatment showed differences in their total free PA content in the winter, whereas in the summer, R showed lower values than C. In winter for both kinds of leaf and in summer for R, the contribution of Put, Spd and Spm to the total amount of free PAs was around 40, 50 and 10%, respectively, while, in C leaves in summer, the contribution of these PAs was 60, 33 and 7%, respectively. The decrease in Put content in R accounted for the lower total PA content of leaves in summer with respect to C.

	Control	Resprouts
Putrescine (nmol g FW^{-1})		
Winter	50.42 ± 2.57 aA	54.70 ± 2.69 aA
Summer	38.88 ± 5.33 aA	16.55 ± 1.83 bB
Spermidine (nmol g FW^{-1})		
Winter	69.79 ± 3.94 aA	57.81 ± 2.73 bA
Summer	21.42 ± 2.74 aB	19.38 ± 1.06 aB
Spermine (nmol g FW^{-1})		
Winter	15.35 ± 1.37 aA	12.36 ± 0.94 aA
Summer	4.21 ± 0.41 aB	4.66 ± 0.57 aB
Total PAs (nmol g FW^{-1})		
Winter	135.56 ± 2.63 aA	124.87 ± 2.12 aA
Summer	64.51 ± 2.82 aB	40.58 ± 1.16 bB

Table 3 Seasonal variations in free polyamines. Putrescine, spermidine and spermine content, and total free polyamine content (Total PAs, Put+Spd+Spm) in *Q. ilex* control leaves and resprouts. Each value represents the mean ± SE of three to five plants randomly selected per kind of leaf (control, resprouts). In rows, different letters indicate significant differences ($p<0.05$)between kinds of leaf (a, b) or between seasons (A, B)

A higher level of Put content in winter than in summer was also observed in Scots pine needles (Sarjala and Savonen 1994), with a positive correlation with frost resistance (Sarjala *et al*. 1997). In addition, during the summer, Put concentration in Picea abies needles reaches a minimum level (Tenter and Wild 1991). The higher level of Spd in winter (earlier stages of development) than in summer in C and R is consistent with the data reported by Fujihara and Yoneyama (2001) in cucumber plants and by Mad-Arif *et al*. (1994), who found that the expression of the gene coding S-adenosylmethionine

decarboxylase, a rate limiting enzyme in Spd and Spm synthesis, was high in young and actively dividing tissues.

The PA content of C and R leaves was also analysed during the day in summer (Fig. 1). In C, Put and Spm decreased throughout the day, whereas in R, they decreased until midday and increased thereafter. Spd values were maintained during the day and showed no differences between the two kinds of leaf. A daily decrease in Put from the morning to the night in upper leaves of *Cucumis sativus* in greenhouse has been reported (Fujihara and Yoneyama 2001).

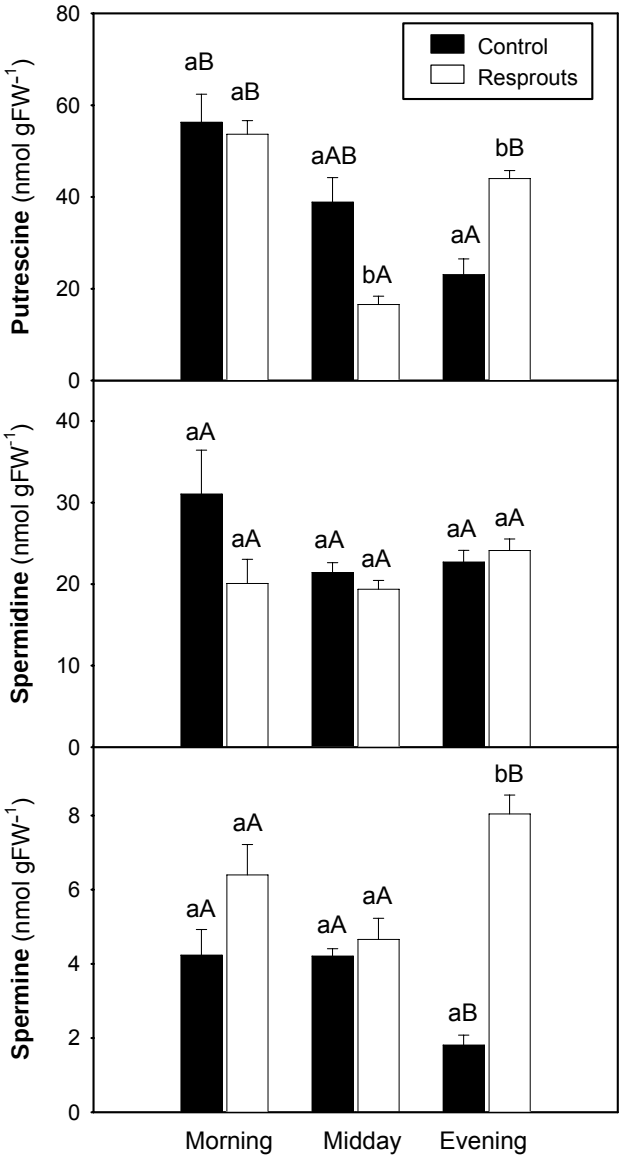

Fig 1. Summer daily variations in free putrescine, spermidine and spermine content in *Q. ilex* control leaves and resprouts. Each value represents the mean ± SE of 3-5 plants randomly selected per kind of leaf (Control, Resprouts) in the morning (8-9h), midday (12.30-13.30h) and evening (17-18h). Different letters indicate significant differences ($p<0.05$) between kinds of leaf (a, b) or among the different time of the day (A, B)).

Measurements of several leaf gas-exchange and chlorophyll fluorescence parameters gave a picture of the different structure and function of the photosynthetic apparatus of the two leaf groups under their respective light conditions. Photosynthesis (A), stomatal conductance (g_s) and transpiration (E) at midday were higher in R than in C (Fig. 2a–c), as reported in earlier studies

(Fleck *et al*. 1998). In C, g_s decreased significantly from winter to summer. The induction of stomatal closure to maintain operational relative water content (RWC) (Gulías *et al*. 2002) demonstrates the conservative use of water in this species (Tognetti *et al*. 1998). In contrast, in R, higher nutrient and water availability (Fleck *et al*. 1998; Peña-Rojas *et al*. 2005), because of the high root/shoot ratio, accounts for the maintenance of high stomatal conductance and photosynthesis rates in spite of high transpiration (E) in the summer (Fig. 2c).

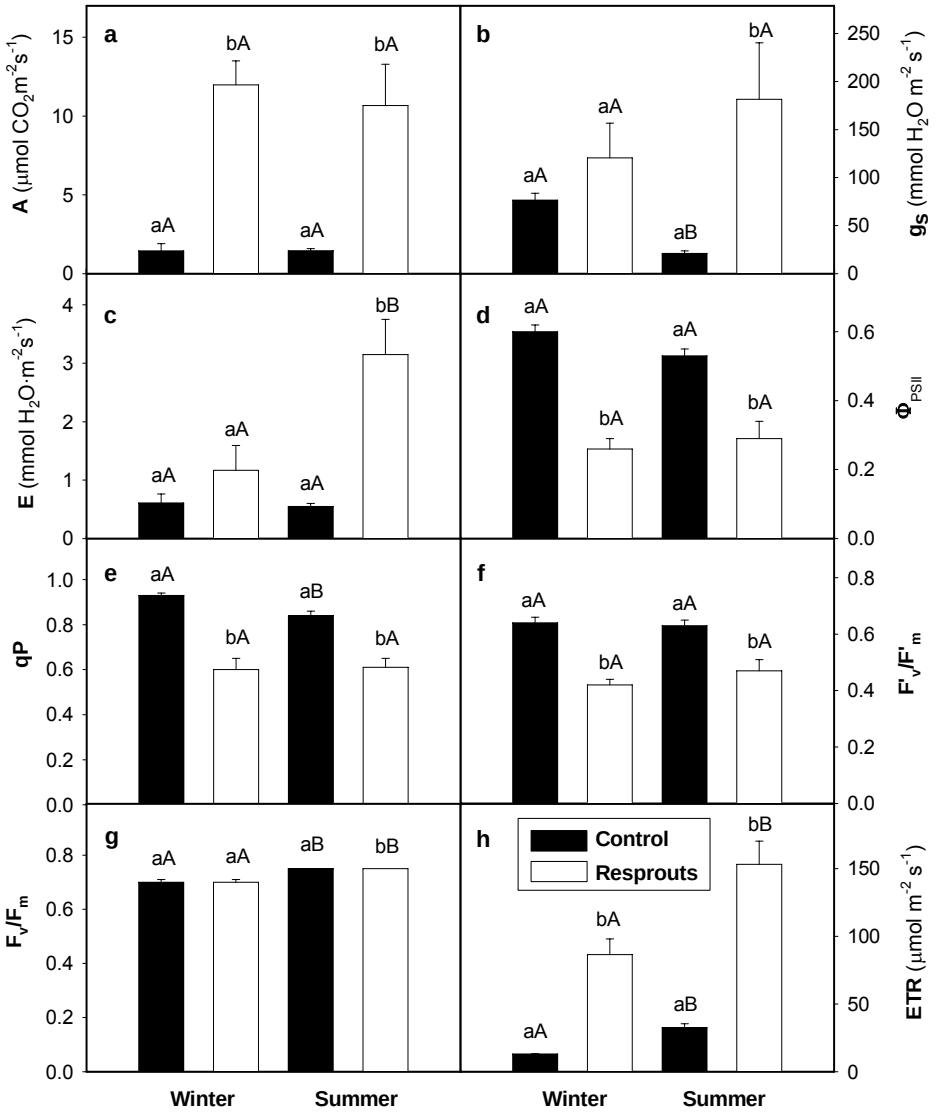

Fig. 2. Net photosynthesis (A), stomatal conductance (g_s), transpiration (E), PSII quantum yield (Φ_{PSII}), photochemical quenching of fluorescence (qP), intrinsic efficiency of open PSII centres during illumination (F'_v/F'_m), maximum quantum yield of PSII photochemistry (F_v/F_m) and relative electron transport rate (ETR) in *Q. ilex* control leaves and resprouts calculated from the A/PPFD curves considering the incident irradiance received at each site ($PPFD_{winter}$ = 50 μmol m^{-2} s^{-1} (control) and 800 μmol m^{-2} s^{-1} (resprouts); $PPFD_{summer}$ = 150 μmol m^{-2} s^{-1} (control) and 1300 μmol m^{-2} s^{-1} (resprouts)). Each value represents the mean ± SE of 3-5 measurements per kind of leaf (Control, Resprouts). Different letters indicate significant differences ($p<0.05$) between kinds of leaf (a, b) or between seasons (A, B)).

During the winter, photosynthetic parameters derived from the analyses of A/C_i curves (Fig. 3a) showed no significant differences between C and R in A_{max}, Vc_{max} or J_{max}. In contrast, A_{sat} showed a twofold increase in R (Table 4). During the summer, all these parameters showed differences between C and R with higher values in the latter. A_{sat} was achieved at PPFD=1500 μmol m^{-2} s^{-1} in R in both seasons and in C in the winter, while for C in summer A_{sat} was achieved at PPFD=1000 μmol m^{-2} s^{-1} (Fig. 3b). Our results show an increased photosynthetic capacity and lower non-stomatal limitation of photosynthesis in R during summer stress with respect to the undisturbed vegetation.

μmol m^{-2} s^{-1}	Winter Control	Winter Resprouts	Summer Controls	Summer Resprouts
A_{max}	8.90 ± 0.57 aA	16.80 ± 2.85 aA	4.98 ± 0.30 aB	15.01 ± 5.20 bA
Vc_{max}	60.85 ± 2.51 aA	62.39 ± 3.02 aA	20.18 ± 1.24 aB	58.93 ± 8.05 bA
J_{max}	194.87 ± 24.60 aA	174.46 ± 10.30 aA	35.69 ± 2.33 aB	101.09 ± 29.26 bA
A_{sat}	5.13 ± 0.73 aA	12.07 ± 2.13 bA	1.98 ± 0.18 aB	8.77 ± 3.01 bA

Table 4. Photosynthetic parameters. A_{max}, net CO_2 assimilation at saturating C_i; Vc_{max}, maximum carboxylation velocity of Rubisco; J_{max}, maximum potential rate of electron transport contributing to RuBP regeneration; and Asat, light-saturated rate of net CO_2 assimilation at ambient CO_2 concentration of *Q. ilex* control leaves and resprouts in winter and summer measurements. Each value represents the mean ± SE from of three to five measurements per kind of leaf (control, resprouts). In rows, different letters indicate ignificant differences ($p<0.05$) between kinds of leaf (a, b) or between seasons (A, B).

In unburned sites, midday PPFD increased from winter to summer from 50 to 155 μmol m^{-2} s^{-1} whereas in burned sites, it increased from 800 to 1400 μmol m^{-2} s^{-1}, respectively. Leaf chlorophyll fluorescence analyses reflected these different light conditions with lower values of Φ_{PSII}, qP and F'_v/F'_m in R than in C in both seasons. The Φ_{PSII}/PPFD curve (Fig. 3c) showed also Φ_{PSII} enhancement in R with respect to C especially under high PPFD. However, in spite of irradiance increase from winter to summer, the values of the parameters above did not change in either leaf group (Fig. 2d–f) indicating the adaptation of the photosynthetic apparatus. Photoprotective mechanisms were effective since F_v/F_m values were maintained. Seasonal and daily changes in PA content, especially in Put, indicate the influence of an external factor that affects R and C in a distinct manner. Variations in irradiance may account for the PA results obtained. In fact, we detected a general trend of Put content decrease with increasing PPFD. Put decreased in C and R from winter to summer, but especially in the latter, which were located in a site with much greater solar radiation (Tables 1 and 2). Moreover, in the summer, the Put content of C and R showed a decline from morning values; again, this effect being more accentuated in the latter.

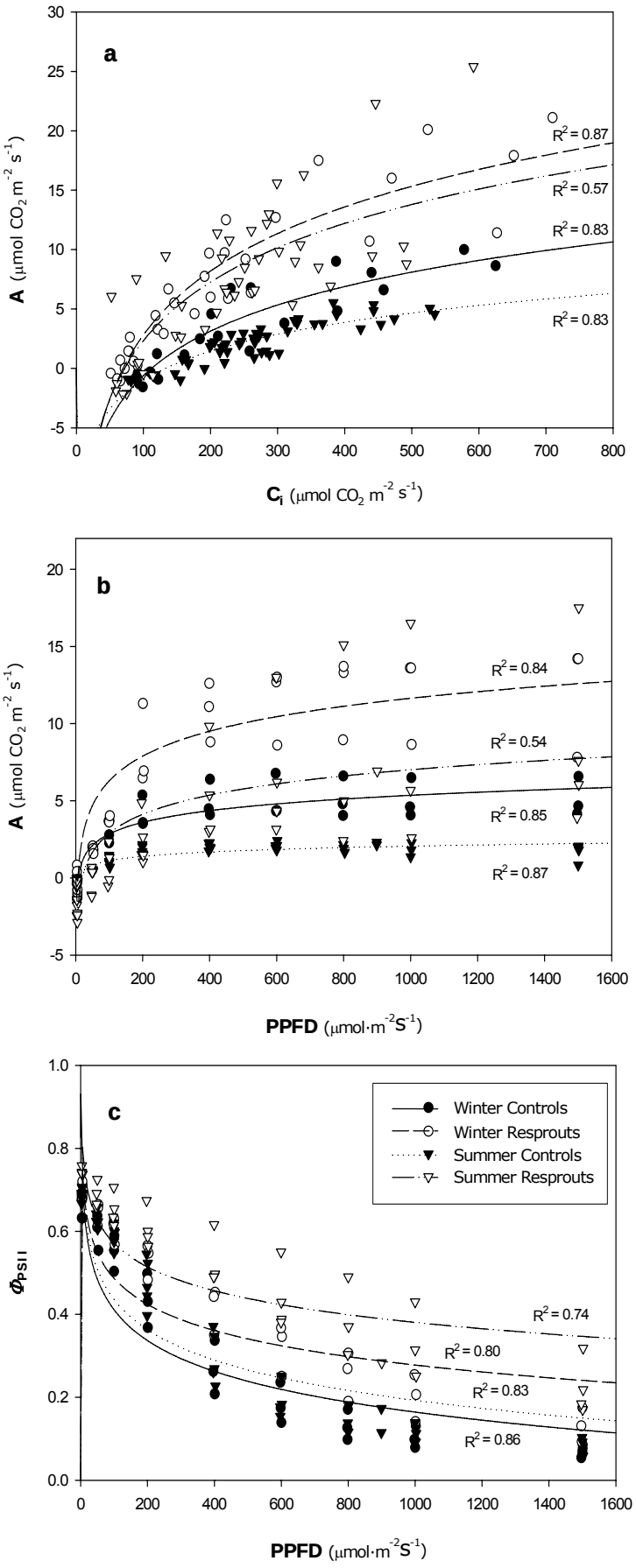

Fig. 3. a) CO_2 response curves of CO_2 assimilation (A/C_i), b) light response curves of CO_2 assimilation (A/PPFD) and c) light response curve of PSII quantum yield (Φ_{PSII}/PPFD) for *Q. ilex* control leaves and resprouts of 3-5 plants in winter and summer.

The decrease of free-PA, especially Put with increasing PPFD could be due to a binding of Put to thylacoid membranes that leads to a photoadaptation of the photosynthetic apparatus to high light conditions as proposed by other authors but under controlled stress conditions (e.g. Besford *et al*. 1993 in excised oat leaves, and Logothetis *et al*. 2004; Sfichi *et al*. 2004; Kotzabasis *et al*. 1999 in cultures of the unicellular green alga *Scenedesmus obliquus*). Our next goal is to establish in *Q. ilex* the relation of the observed free Put decline under high light stress with increased thylakoid-bound forms and thylakoid transglutaminase enzyme activity. Data reported by Della Mea *et al*. (2004) in *Zea mays* and by Dondini *et al*. (2003) in *Helianthus tuberosum* indicate that a transglutaminase, localised close to or associated with the LHCII, catalyses the production of mono and bis glutamyl-polyamines and is affected by light.

We can conclude that the seasonal and daily fluctuation in free Put content observed reflects the variation in light availability in undisturbed holm oak leaves and resprouts after fire. Putrescine involvement in the photoadaptation of photosynthetic apparatus under high light stress in the forest can be relevant in resprouting vegetation contributing to enhanced photosynthesis and rapid growth after disturbance.

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CAPÍTOL 5.

RESPONSE OF TRANSGLUMINASE ACTIVITY AND BOUND PUTRESCINE TO CHANGES IN LIGHT INTENSITY UNDER NATURAL OR CONTROLLED CONDITIONS IN *QUERCUS ILEX* LEAVES

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Resposta de l'activitat transglutaminasa i de la putrescina lligada als canvis d'intensitat lumínica en fulles de *Quercus ilex* en condicions naturals i controlades

Amb l'objectiu d'aprofundir en la relació observada previament entre el contingut en poliamines (PAs) i els canvis d'irradiància, s'estudiaren en les fulles d'alzina (*Quercus ilex* L.) les concentracions en poliamines (lliures i lligades), l'activitat de l'enzim transglutaminasa (TGasa, EC 2.3.2.13) i els paràmetres de fluorescència de les clorofil·les en resposta a diferents intensitats lumíniques i quantitats absolutes de llum rebuda. En individus forestals sota condicions naturals al bosc s'observà una tendència diürna de la putrescina lliure i de la lligada a proteïnes (F-Put i B-Put, respectivament), així com de l'activitat TGasa on els valors més alts corresponguereen sempre al màxim d'intensitat lumínica i quantitat de llum rebuda per les fulles. En un disseny experimental complementari, alzines crescudes en testos a l'entorn dels camps experimentals van ser sotmeses a diferents períodes de llum fotosintètica natural (PPFD) mitjançant el cobriment total dels individus. En les fulles sotmeses a un fotoperíode natural (no cobertes) el contingut en B-Put i l'activitat TGasa seguiren el mateix patró que el de la PPFD, arribant al màxim en el moment de major incidència lluminosa. Prèviament a aquest màxim, el contingut en PAs lliures mostraren un augment significatiu. Els individus que romangueren coberts fins a migdia i sobtadament exposats a altes intensitats de llum mostraren una activitat de la TGasa superior, fet que resultà en una acumulació màxima de B-Put. Es discuteix la implicació de l'acumulació de B-Put (reflectida en els canvis en l'índex B-Put/B-Spd) en els processos de fotoprotecció de plantes forestals sotmeses a estrès per alta irradiància en funció dels resultats de fluorescència de les clorofil·les obtinguts.

Response of transgluminase activity and bound putrescine to changes in light intensity under natural or controlled conditions in *Quercus ilex* leaves

In order to further study a previously observed relationship between polyamine content and changes in irradiation, we examined the level of free (F-PAs) and bound (B-PAs) polyamines, the activity of transglutaminase (TGase EC 2.3.2.13) and chlorophyll fluorescence in holm oak (*Quercus ilex* L.) leaves in response to different levels of light intensity and amount. A diurnal trend of free and bound putrescine (F-Put and B-Put, respectively) and TGase activity was observed in plants under natural conditions in the forest, with the highest value corresponding to the maximum light intensity and amount of light received by the leaves. In another set of experiments, potted *Q. ilex* plants in experimental fields were subjected to a range of periods of natural PPFD by covering or not covering the whole trees. Under a natural photoperiod (uncovered leaves) B-Put content and TGase activity paralleled the diurnal PPFD pattern, reaching a maximum at the highest PPFD; prior to this maximum, F-PA showed a significant rise. Plants that were in darkness until midday and suddenly exposed to high light intensity showed enhanced TGase activity, resulting in the maximum accumulation of B-Put. The involvement of the accumulation of B-Put reflected in the changes of the bound putrescine/bound spermidine ratio during the photoprotective responses to high light stress in forest plants is discussed in relation to the chlorophyll fluorescence parameters observed.

Abbreviations - B-Put, bound putrescine; B-Put/B-Spd, bound putrescine/bound spermidine; B-Spd, bound spermidine; ETR, relative rate of electron transport; F'_m, F'_v maximal and variable fluorescence yields in a light-adapted state; F_v/F_m, maximum quantum yield of PSII photochemistry (equivalent to $(F_m-F_o)/F_m$); F'_v/F'_m, intrinsic efficiency of open PSII centers during illumination (equivalent to $(F'_m-F'_o)/F'_m$); F-Put, free putrescine; F-Spd, free spermidine; LHCII, PSII light-harvesting complex; NPQ, non-photochemical quenching (equivalent to $(F_m-F'_m)/F'_m$); PPFD, photosynthetic photon flux density; PSII photosystem II; Put, putrescine; qP, photochemical quenching of fluorescence; Φ_{PSII}, photochemical PSII efficiency; Spd, spermidine; Spm, spermine; TGase, transglutaminase.

5.1 Introduction

Quercus ilex L. is a deep-rooted dominant species of Mediterranean forests that is endangered by increased drought and forest fires associated with global climatic change (Houghton *et al*. 2001). The effects of environmental stress on the photosynthetic process of *Q. ilex* have been extensively studied by our group. We have described several defence systems that protect the photosynthetic apparatus of vegetation under conditions of excitation excess. These include dissipation as heat both by the xanthophyll (Fleck *et al*. 1998) and the lutein epoxide cycles (Llorens *et al*. 2002), the contribution of antioxidant systems (El Omari *et al*. 2003) and the expression of low molecular weight heat-shock proteins (Hsps) (Verdaguer *et al*. 2003).

Polyamines (PAs) are low molecular weight aliphatic amines that are involved in a wide range of biological processes in all living organisms. Because of their chemical structure, PAs occur in plant cells as free molecules, conjugated with organic acids or bound to negatively charged macromolecules and to many types of proteins with enzymatic activity. These biochemical interactions are crucial for the regulatory effect of these PAs on various growth and developmental processes (Tiburcio *et al*. 1997, Martin-Tanguy 2001, Bais and Ravishankar 2002) and for plant response to environmental stress (Kumar *et al*. 1997, Bouchereau *et al*. 1999).

Moreover, PAs, especially those bound to thylakoid membranes, have been reported to protect the photosynthetic apparatus in colonial green algae by regulating the size of the PSII light-harvesting complex (LHCII) during stress such as ozone pollution (Navakoudis *et al*. 2003), elevated CO_2 treatment (Logothethis *et al*. 2004) and UV-B radiation (Sfichi *et al*. 2004). PAs are also synthesized and oxidized in chloroplasts (Kotzabasis *et al*. 1993), and the addition of PAs inhibits the destruction of thylakoids and prevents loss of pigment during senescence (Besford *et al*. 1993). Endogenous PA levels are also related to chlorophyll biosynthesis and rate of photosynthesis during the light-dependent development of pro-plastids into chloroplasts (Beigbeder *et al*. 1995). In addition, endogenous PA might be involved in the assembly of photosynthetic membrane complexes (Dörnemann *et al*. 1996).

Transglutaminases (TGases. EC 2.3.2.13) are enzymes that catalyse the covalent binding of PAs to γ-carboxamide groups of protein endo-glutamine residues (Folk 1980, Lorand and Graham 2003). TGases are widely distributed in bacteria, animals and plants. However, research on these enzymes in plants is

less developed than in animal systems, in which they were detected for the first time (see Folk 1980, Lorand and Conrad 1984 for reviews). Although TGases are found in several organs in lower and higher plants, chloroplast TGases have received the most attention and were the first to be cloned in plants (Torné *et al*. 2002). Apoproteins of the LHCII as endogenous substrates of TGase in chloroplasts of *Helianthus tuberosum* leaves have been described by using polyclonal antibodies (Del Duca *et al*. 1994). In *H. tuberosum* (Dondini *et al*. 2003) and *Zea mays* (Della Mea *et al*. 2004) TGase, localised close to or associated with the LHCII, catalyses the production of mono and bis glutamyl-PAs and is affected by light. Moreover, a TGase detected in green maize meristematic calli and their chloroplasts was observed to be light-sensitive and showed a daily rhythm (E. Bernet 1997. Thesis, Univ. of Barcelona, Barcelona, Spain, Bernet *et al*. 1999). In mature leaves this enzyme was preferentially present in the grana-appressed thylakoids (Villalobos *et al*. 2001), the abundance depending on the degree of grana development. Furthermore, these authors reported the activity of this enzyme to be light-dependent.

Recently, we described the influence of light intensity on seasonal changes of free PA content in leaves from undisturbed holm oak forest vegetation and resprouts originated after fire (Pintó-Marijuan *et al*. 2006). Specifically, the content of free putrescine (Put) decreased with increasing irradiance, especially in resprouts at sites with a high PPFD. These results suggest that Put is involved in the photoprotection of photosynthetic apparatus of species growing in high light stress in natural forest habitats.

Here we studied the relation between the decrease in Put under high light stress and the increase in B-Put mediated by TGase activity in leaves of the evergreen holm oak (*Quercus ilex* L.). Plants in two locations were used: forest plants under natural conditions and potted plants grown in experimental fields. In the forest, we compared the responses to light intensity of undisturbed (control) individuals and resprouts originated from the root-crown region after a fire in the two main stressing seasons, winter and summer. Resprouts showed stimulated photosynthesis and rapid growth (Fleck *et al*. 1998) because of greater water and nutrient availability than for the original plants, as the pre-existing root system is associated with a much smaller aerial biomass (Kruger and Reich 1997). Moreover, resprouting vegetation is located in sites with greater solar radiation.

Potted plants in the experimental fields were exposed to a range of photoperiods of natural irradiance in order to ascertain whether changes in PA content were due to a) variations in light intensity, b) changes in the duration of

light exposure or c) changes in the amount of light received during the last 2 h before sampling. We also measured several chlorophyll fluorescence parameters in order to establish the mechanisms of photoprotection in *Q. ilex* leaves.

5.2 Material and methods

Experimental site and plant material

a.Experimental fields

Five *Q. ilex* plants (6 years old) were grown in 9-l pots filled with a mixture of soil from the area (40%) (by volume), peat (20%), vermiculite (20%) and perlite (20%) watered with 500 ml of Hoagland solution per day at the Experimental Fields of the Faculty of Biology, University of Barcelona, NE Spain (41°22'59" N, 02°06'44" E, 60 m above sea level).

Initial work was done in the winter on 5 randomly selected plants. Five fully-developed, current-year leaves per plant were sampled under dark conditions at 8.00 h (local time) and at 14.00 h under high light intensity (around 1000 μmol m^{-2} s^{-1}). Leaves were immediately frozen in liquid nitrogen and stored at -80ºC until analyses of PA content and TGase activity.

On three spring days, samples were taken during the day (Fig. 1) on 5 randomly selected plants and after a range of exposure to light (hours of illumination) in order to detect the effect of these factors on the parameters of interest (measurements in the summer would include drought stress and therefore were not done). On each experimental day, the first sampling was taken at 8.00 h (local time) after 11 h of absolute darkness. Plants maintained in darkness until 12.30 h, and thereafter uncovered and exposed to solar light are described as "Covered plants (CV)"; (Fig. 1c): these plants were sampled at 14.30 h and 16.30 h. The covers of other plants (Fig. 1a-b), described as "Uncovered plants (UCV)", were removed at 8.00 h and sampling was done at 14.30 h and 16.30 h (Day 1) or at 10.30 h, 12.30 h 14.30 h and 16.30 h (Day 2), respectively. Daily light intensity curves were established from the data recorded by the PPFD sensor of a gas-exchange system LI-6200 (Li-Cor, Lincoln, NE, USA) (Fig. 1) at leaf level. By integrating these curves we obtained them to calculate the amount of light received by the leaves during the last 2 h before each sampling. At each sampling point, 5 - 8 fully-developed, current-year leaves per plant were frozen in liquid nitrogen. Thereafter, all frozen leaves from

each sampling point were ground with liquid nitrogen and stored at -80ºC until analyses.

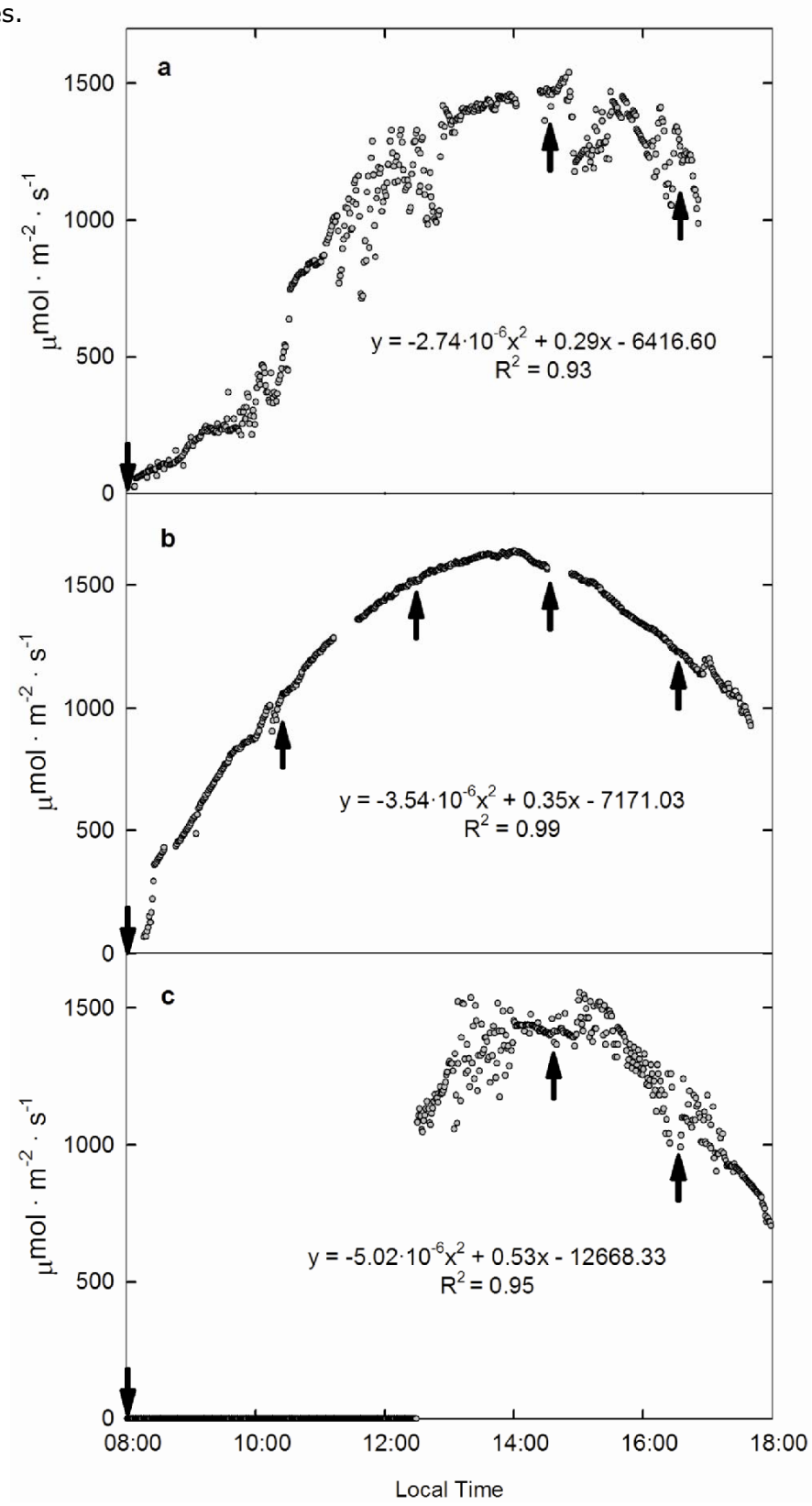

Fig. 1. Daily light intensity curves established from the data recorded by the Photosynthetic Photon Flux Density (PPFD) (µmol m^{-2} s^{-1}) sensor of a gas-exchange system LI-6200 (Li-Cor, Lincoln, NE, USA) during measurement and sampling days of potted plants in the Experimental Fields during spring. Sampling points are indicated by arrows. Curve equation and regression coefficient (R^2) are indicated for each day.

b.Forest

Studies were carried out in Castellbisbal forest (Serra de les Forques), Barcelona, Spain; 41º48'45" N, 01º96'35" E at an elevation of 144 m and oriented N-NW. The climate is Mediterranean, with cold winters, cool wet springs and autumns, and hot dry summers. The area has a mean annual temperature of 13-14ºC and an annual rainfall of 500-700 mm. Climatological data measured during the sampling are shown in Table 1. On two winter and two summer days, 7 *Q. ilex* plants were randomly selected per treatment: 25-year-old undisturbed individuals (Control) and resprouts originated after a previous summer fire (Resprouts). Six fully developed, current-year (same age) leaves from each plant were harvested at each sampling point: morning (winter 9.00-11.30 h local time; summer 9.00-11.30 h), midday (winter 14.00-16.30 h; summer 13.30-16.00 h) and evening (winter 18.30-20.00 h; summer 18.00-20.00 h). Leaves were immediately frozen in liquid nitrogen and stored at -80ºC until PA content and TGase activity analyses.

Table 1. Climatological data at the forest sites (Unburned (control) and burned (resprouts)) recorded during the winter and summer sampling. Different letters indicate significant differences during the day at $p<0.05$.

	PPFD (μmol m^{-2} s^{-1})	Temperature (°C)
Winter		
Control		
Morning	30 ± 2^a	10.6 ± 0.6^a
Midday	144 ± 21^b	19.1 ± 0.9^b
Evening	6 ± 1^a	13.0 ± 0.5^c
Resprouts		
Morning	42 ± 4^a	7.0 ± 0.9^a
Midday	595 ± 110^b	20.4 ± 1.4^b
Evening	23 ± 2^a	13.7 ± 0.9^c
Summer		
Control		
Morning	67 ± 8^a	19.8 ± 0.3^a
Midday	710 ± 187^b	27.6 ± 1.2^b
Evening	45 ± 5^a	22.3 ± 0.5^c
Resprouts		
Morning	73 ± 9^a	17.1 ± 0.4^a
Midday	801 ± 132^b	25.3 ± 0.8^b
Evening	46 ± 12^a	21.3 ± 0.4^c

Analysis of free and bound PAs

Frozen leaf samples were ground to powder with liquid nitrogen in a pre-chilled mortar and pestle and homogenized with 5 vols of a 5% (v/v) cold PCA solution by Polytron (Kinematica AG, Switzerland). The homogenates were kept for 1 h in ice and centrifuged at 14 000 *g* for 15 min. The supernatant was set aside for free PA analysis while the pellet was re-suspended and washed with the same volume of PCA previously used. After centrifugation at 14 000 *g* for 15 min, the pellet was re-suspended with a volume of 1 M NaOH equal to that used for PCA

extraction. Aliquots of the re-suspended pellet were hydrolyzed for 18 h at 110°C after adding an equal volume of 12 M HCl. The hydrolyzed samples were centrifuged (14 000 *g* for 20 min) and the supernatants dried at 80°C under a flow of nitrogen before being re-dissolved in the original PCA volume. Each of the fractions (supernatant and hydrolyzed pellet) was dansylated (de Agazio and Zacchini 2001) and free (S, supernatant fraction) and bound (PH, hydrolyzed pellet) PAs were separated by HPLC (Beckman-Coulter, Fullerton, CA, USA). Samples were injected on a C18 reverse phase column (250x4.6 mm, 5 µ pore size, Alltech, Deerfield, IL, USA) and measured by a Jasco fluorescence detector (model FP 2020 Plus, Tokyo, Japan, excitation 350 nm, emission 530 nm), following standard curves. Elution was performed with a multi-step linear gradient of aqueous acetonitrile: 50 to 30% water in 3 min, 30 to 20% in 4 min, 20 to 0% in 6 min, 0% for 3 min, 0 to 50% in 2 min, at a flow rate of 1 ml min^{-1}. Chromatograms were recorded and integrated by the 32 Karat™ Software v 5.0 (Beckman-Coulter, Fullerton, CA, USA).

TGase activity assays

Radiolabelled putrescine method

Protein extracts from soluble fractions of the expression experiments were used to determine TGase activity in the presence of 3 µl of [1,4(n)-3H] Put (specific activity 962 GBq $mmol^{-1}$). The pH of the incubation mixture was adjusted to 8.0. The enzymatic mixture was as described previously (Bernet *et al*. 1999). After 30 min of incubation at 30ºC, the reaction was blocked by adding 10% TCA (trichloroacetic acid) containing 2 mM unlabelled Put. Samples were repeatedly precipitated and the radioactivity was measured in a scintillation counter as described by Villalobos *et al*. (2004). The dark and light assays were performed on in vivo plants under the same light conditions.

Chlorophyll fluorescence measurements

A portable modulated fluorimeter (Mini-Pam Photosynthesis Yield Analyser, Walz, Effeltrich, Germany) was used to measure chlorophyll fluorescence in plants at the Experimental Fields and in the forest at each sampling point. The light-adapted components of chlorophyll fluorescence (steady-state yield (F), maximum fluorescence yield (F'_m) and quantum yield of photosystem II (PSII) photochemistry (Φ_{PSII}; equivalent to (F'_m-F)/F'_m)) (Genty *et al*. 1989) were measured. Parameters F'_o, minimum fluorescence yield in light-adapted state; qP, photochemical quenching (equivalent to (F'_m-F)/(F'_m-

F'_o)), and F'_v/F'_m, intrinsic efficiency of open PSII centers during illumination (equivalent to $(F'_m-F'_o)/F'_m$) were estimated following Oxborough and Baker (1997). NPQ, non-photochemical quenching (equivalent to $(F_m-F'_m)/F'_m$), was calculated. The relative electron transport rate (ETR) was calculated following Krall and Edwards (1992) as ETR = Φ_{PSII} x PPFD x 0.5 x 0.82, where 0.5 is a factor that assumes equal distribution of energy between the two photosystems and 0.82 the light absorbance we obtained on *Q. ilex* leaves using an integrating sphere (Aranda *et al*. 2006).

To obtain minimum fluorescence yield (F_o), maximum fluorescence yield (F_m) and maximum quantum yield of PSII photochemistry (F_v/F_m) (equivalent to $(F_m-F_o)/F_m$), leaves were dark-adapted. In the Experimental Fields, leaves were covered from the afternoon to the next morning (for at least 11 h), and in the forest, leaves were covered for at least 20 min, after which F_v/F_m values reach about 95% of the pre-dawn values in *Q. ilex* (Fleck *et al*. 1998). All data were corrected for changes in the sensitivity of the fluorescence detector induced by temperature variation of the Mini-Pam.

Statistical analyses

All statistical procedures were done using SPSS for Windows (SPSS for Windows v. 11.0, SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to test the main effects and interactions, against appropriate error terms, of time of the day (8.00, 10.30, 12.30, 14.30 and 16.30 h) and hours of illumination (0, 2, 2.5, 4, 4.5, 6.5 and 8.5) in TGase activity, PA content and chlorophyll fluorescence parameters on the experimental field results. ANOVA was also used to test the main effects and interactions, against appropriate error terms, of time of the day (Morning, Midday and Evening) in TGase activity, PA content and chlorophyll fluorescence parameters in both seasons (Winter and Summer) on the forest results. The post-hoc Duncan test was applied when appropriate. Statistical significance was set at $p \leq 0.05$. The number of replicates is indicated in the table and figure legends. Quadratic polynomial curves were fitted to the PPFD graphs and equations were calculated by Sigmaplot 8.0 (SYSAT Software, Inc.501 Canal Blvd, Point Richmond, CA, USA).

5.3 Results and discussion

PA content and TGase activity

Preliminary results (Fig. 2) indicate that when *Q. ilex* potted plants in winter were changed from dark to light conditions, a decline in free PAs and an increase in bound PAs concomitant with an enhancement of TGase activity occurred.

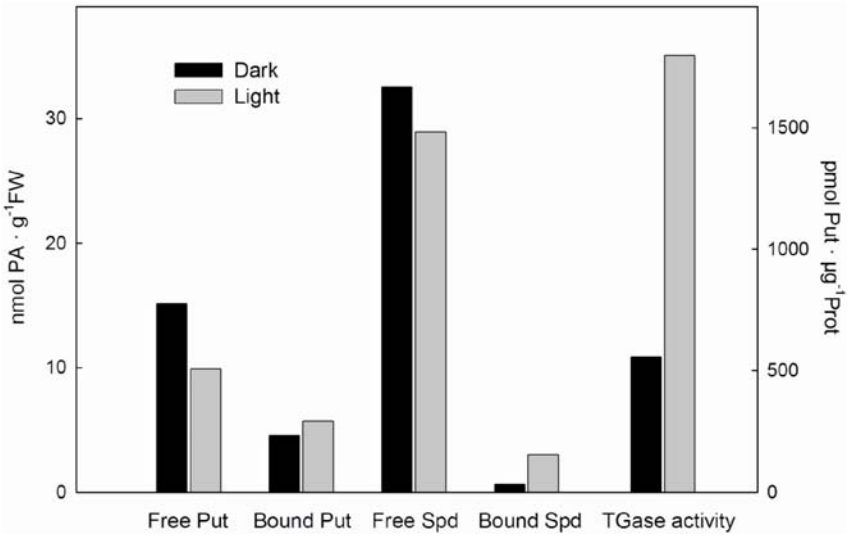

Fig. 2. Bound and free putrescine and spermidine content (left axis: nmolsPA g^{-1}FW) and TGase activity (right axis: pmolsPut μg^{-1}Prot) in *Q. ilex* leaves of potted plants at the Experimental Fields under dark (7.00 h) or light (14.00 h) conditions in the winter. Each value corresponds to the measurement of a pool composed of 25 leaves (5 leaves from 5 plants).

In order to investigate the previously observed relationships, experiments on plants (control and resprouts after fire) in their natural environment and on potted plants in Experimental Fields were carried out. Results obtained in plants placed in their natural environment (*Q. ilex* forest) also showed the same trends. In winter and summer, both control and resprouting holm oak leaves showed an increase in F-Put and B-Put at midday, which declined thereafter (Table 2).

	Winter		Summer	
	Control	Resprouts	Control	Resprouts
Free				
Put				
Morning	78.0 ± 2.3^{a}	137.7 ± 8.4^{a}	37.4 ± 6.2^{a}	32.7 ± 3.4^{a}
Midday	96.2 ± 2.3^{b}	166.2 ± 10.7^{b}	43.5 ± 3.3^{a}	41.8 ± 0.6^{b}
Evening	79.9 ± 4.0^{a}	162.6 ± 7.3^{ab}	41.5 ± 2.7^{a}	26.3 ± 0.6^{a}
Spd				
Morning	38.6 ± 4.9^{a}	58.9 ± 13.8^{a}	13.3 ± 0.5^{a}	15.1 ± 1.3^{a}
Midday	40.6 ± 3.5^{a}	69.9 ± 13.8^{a}	13.3 ± 2.4^{a}	14.0 ± 0.6^{a}
Evening	35.3 ± 2.7^{a}	52.2 ± 3.4^{a}	15.5 ± 1.9^{a}	13.7 ± 0.5^{a}
Bound				
Put				
Morning	4.4 ± 0.4^{a}	4.1 ± 1.0^{a}	3.2 ± 0.5^{a}	2.3 ± 0.2^{a}
Midday	12.4 ± 2.4^{b}	5.3 ± 0.3^{a}	4.8 ± 0.3^{b}	5.1 ± 0.8^{b}
Evening	4.8 ± 0.8^{a}	4.7 ± 0.4^{a}	3.7 ± 0.3^{ab}	2.7 ± 0.4^{ab}
Spd				
Morning	1.7 ± 0.2^{a}	4.0 ± 0.8^{a}	2.3 ± 0.2^{a}	2.2 ± 0.4^{a}
Midday	2.3 ± 0.2^{a}	2.0 ± 0.4^{b}	1.9 ± 0.2^{a}	2.1 ± 0.2^{a}
Evening	2.3 ± 0.8^{a}	2.2 ± 0.4^{b}	1.7 ± 0.3^{a}	1.5 ± 0.2^{a}

Table 2. Free polyamine (Put, Spd) and bound polyamine content (Put, Spd) expressed as pmol PA · µg $prot^{-1}$ in *Q. ilex* control leaves and resprouts from *Serra de les Forques* forest at 3 times on 2 representative days in each season (winter, summer). Different letters indicate significant differences during the day at $p<0.05$. Values are mean ± S.E. of 3 replicates per day from pools composed of 42 leaves (6 leaves from 7 plants).

Neither the F-Spd nor the B-Spd content varied significantly during the day. Spermine could not be detected by HPLC in any of the analysed leaves due to their low levels. Moreover, B-Put/B-Spd ratio and TGase activity (Fig. 3) also followed the same pattern increasing in parallel with irradiance (Table 1), reaching a marked maximum at midday.

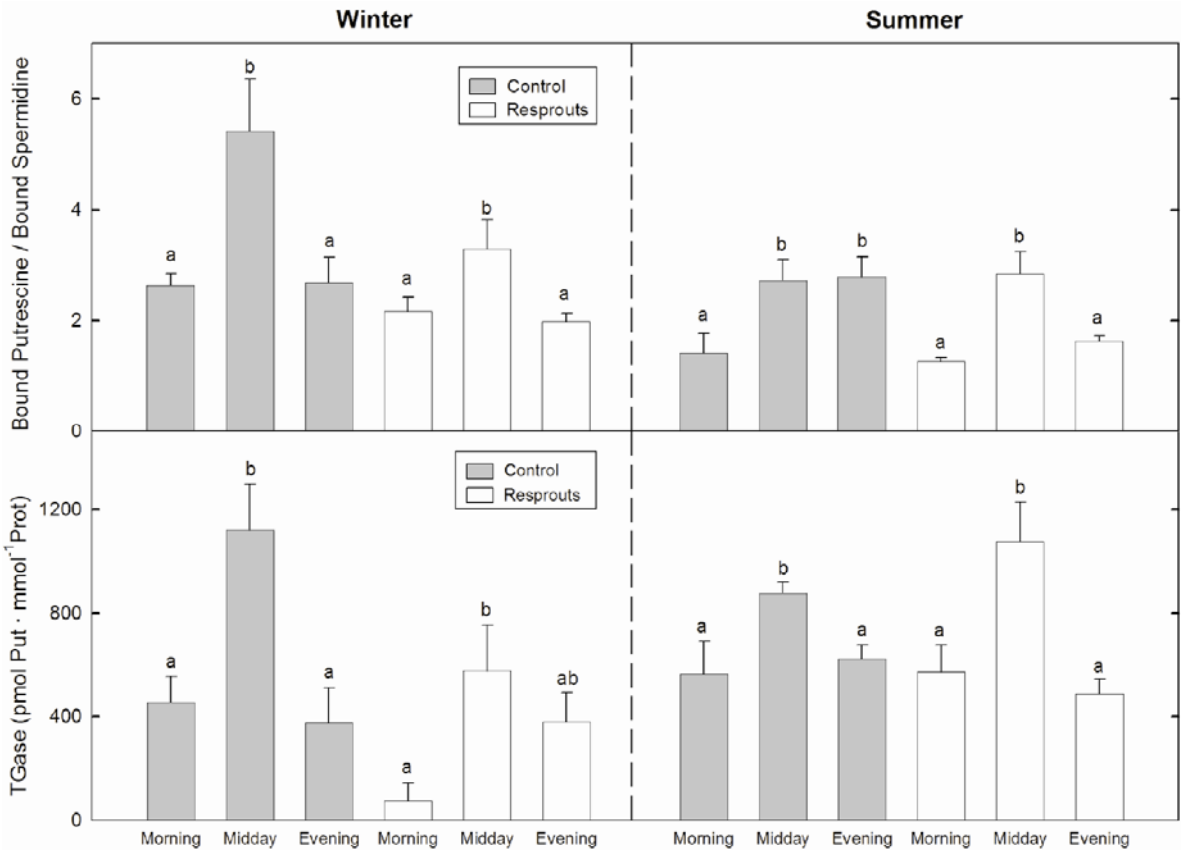

Fig. 3. Bound putrescine/bound spermidine ratio and TGase activity of *Q. ilex* control leaves and resprouts from *Serra de les Forques* forest at three times of the day in two representative days in each season (winter, summer). Different letters indicate significant differences at $p<0.05$. Values are mean ± S.E. of 3 replicates per day from pools composed of 42 leaves (6 leaves from 7 plants).

The increase in both B-Put and B-Put/B-Spd ratio observed in our study during exposure of plants to increasing light at midday might be involved in PSII photoprotection, as described by Kotzabasis *et al*. (1999) in green algae. In a previous study in the forest, we already observed a decrease in free PAs, especially Put from winter to summer in *Q. ilex* leaves, with increasing PPFD. The binding of Put, especially to thylakoid proteins, could be responsible for this decrease (Pintó-Marijuan *et al*. 2006).

TGase activity occurs in green cells, isolated chloroplasts and isolated thylakoids (Margosiak *et al*. 1990, Del Duca *et al*. 1994, Lilley *et al*. 1998, Bernet *et al*. 1999). A peculiar characteristic of chloroplast TGase is that its activity increases after exposure to light during the assay (Villalobos *et al*. 2001,

2004, Dondini *et al*. 2003). The present study also indicates that TGase is stimulated by increasing irradiance in *Q. ilex* leaves in the forest.

In *Zea mays*, TGase (detected by immunolocalization) localizes close to LHCII in the the grana-appressed thylakoids of the mesophyll cell chloroplasts (Villalobos *et al*. 2001). Della Mea *et al*. (2004) also have suggested that TGase associates with the major antenna complex of PSII in *Zea mays* chloroplasts.

A significant increase in RNA expression was observed in maize plantlets after 2 h of light exposition. A putative chloroplast signal peptide has also been identified in the maize TGase sequences. These data and the effect of specific inhibitors of TGase activity on purified grana maize proteins seem to indicate that chloroplast TGase plays a role in polyamine conjugation to photosystem antenna proteins and can contribute to the efficient distribution of light energy between PSI and PSII complexes (Villalobos *et al*. 2004).

TGase may affect the regulation of the ratio of stacked to unstacked thylakoids via polyamine conjugation to photosystem antenna proteins and may favour the transfer of excitation energy between the photosystems (Mullet 1983, Allen *et al*. 1981). Moreover, D1, D2, cytochrome f and the large Rubisco subunit are all stabilized by PAs (Besford *et al*. 1993).

To our knowledge, the present study is the first report on the daily pattern of TGase activity in *Q. ilex* trees in their natural environment.

Another set of experiments was done in the spring in Experimental Fields on potted plants, involving different duration and intensity of light exposure in order to ascertain the effect of light on the binding of PAs to proteins and the characteristics of TGase activation by light. Results obtained for uncovered plants (UCV) with a gradual PPFD increase showed a daily peak of F-Put and F-Spd at 12.30 h, decreasing thereafter (Fig. 4a). B-Put and TGase activity (Fig. 4b-c) increased in CV and UCV plants, achieving a maximum at 14.30 h. Daily changes in B-Spd were not significant but showed the same trend. The B-Put/B-Spd ratio in UCV plants showed a maximum at 10.30 h, decreasing thereafter. In CV plants the maximum ratio was observed at 14.30 h (Fig. 4d). The irradiance pattern (intensity and amount of light) (Fig. 4e) shows that changes in light intensity paralleled the changes in TGase activity and bound PA content.

The TGase-induced binding of free Put or Spd to proteins by covalent linkage would account for the decrease observed in free PAs, as reported by Folk (1980) and Serafini-Fracassini *et al*. (1995). The increase in free PAs before the maximum in bound PA (E. Bernet 1997. Thesis, Univ. of Barcelona, Barcelona, Spain) indicates an accumulation of free molecules before their binding to

proteins. Although not significant, free or bound Spd showed similar trends to those of Put observed in the forest.

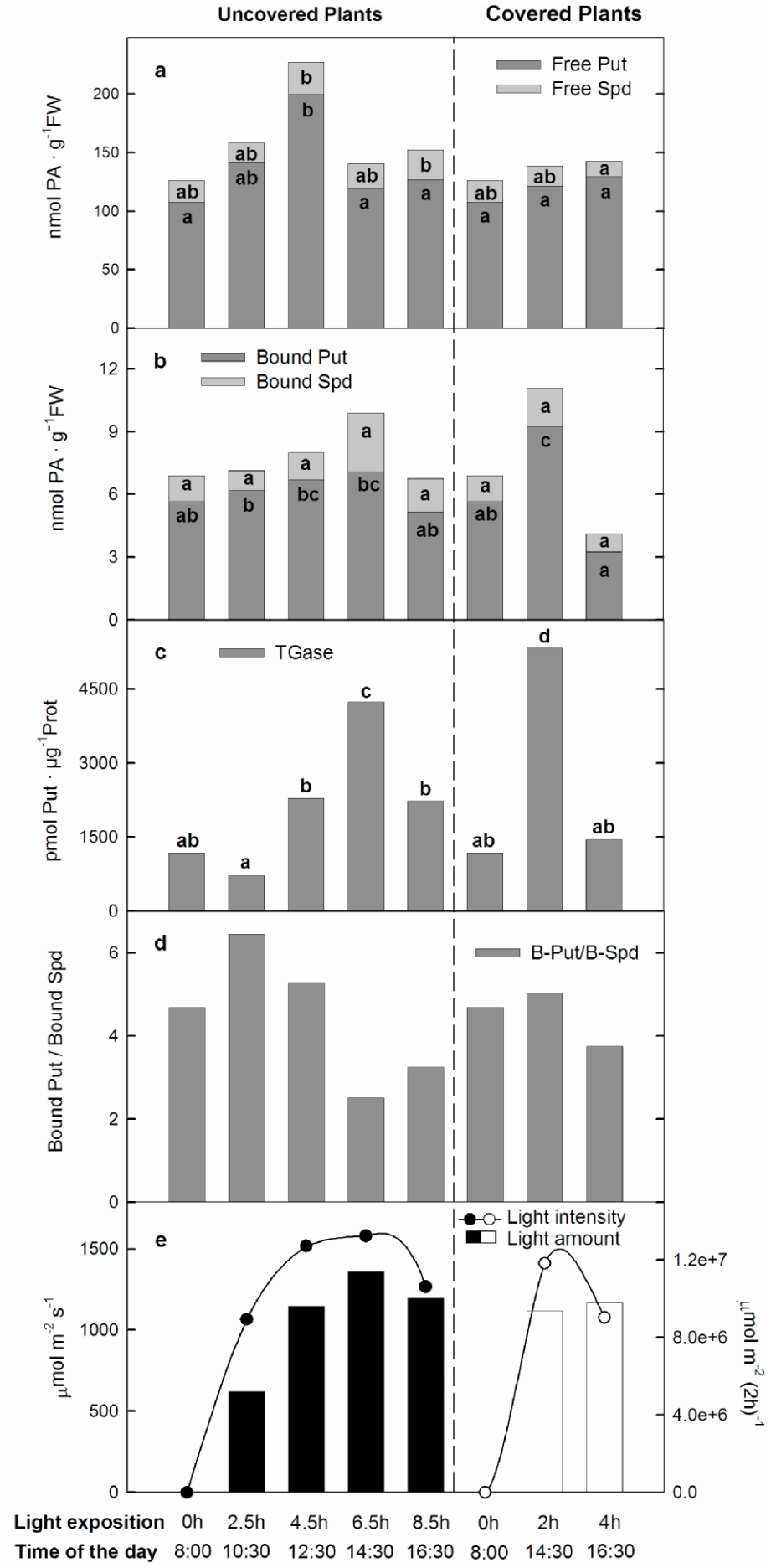

Fig. 4. a) Free polyamine content (Put, Clear; Spd, Dark); b) Bound polyamine content (Put, Clear; Spd, Dark); c) TGase activity; d) Bound put/bound spd ratio and e) Light intensity (left axis: µmol m^{-2} s^{-1}) and amount of light received by leaves during the 2 h (right axis: µmol m^{-2} $(2h)^{-1}$) before sampling and measurements of *Q. ilex* leaves of potted plants at the Experimental Fields in spring taken several times of the day and after a range of lengths of exposure to light. Different letters indicate significant differences at $p<0.05$ among all measurements. In a), b) and c) each value is the mean of 3 replicates from pools composed of circa 35 leaves (5-8 leaves from 5 plants).

In contrast, results for covered plants (CV) (i.e. plants subjected to absolute darkness until midday) showed that a sudden exposure to high light intensity strongly activated TGase and accounted for the activity being higher than for UCV plants. Results for B-Put were related to the TGase activity response. In fact, in UCV plants at the highest light exposure (PPFD = 1578 μmol m^{-2} s^{-1}) and with the highest amount of light received during the 2 h before sampling, TGase activity was 20% lower than for CV plants which received a similar but sudden light intensity (PPFD = 1410 μmol m^{-2} s^{-1}) after the cover had been removed. During a progressive increase in irradiance, the TGase activity increased gradually as did B-Put, but after an abrupt exposure to high intensity light, the TGase activity as well as the B-Put reached a marked maximum. We assume therefore that maximum values in TGase activity and bound PAs (especially B-Put) are obtained when plants receive an abrupt change in light intensity.

Chlorophyll fluorescence

Increases in TGase activity, B-Put and in the B-Put/B-Spd ratio observed after exposure of plants to greater irradiance were paralleled by changes in several parameters of chlorophyll fluorescence. In fact, both in the forest and in the Experimental Fields, leaves showed a decline in Φ_{PSII} with increasing PPFD at the time of maximum TGase activation. In the forest (Table 3), both in winter and in summer, Φ_{PSII}, qP and F'_v/F'_m declined markedly from morning to midday, but then increased. qP values around 0.6, as observed at winter midday in controls and at summer midday in both kinds of leaf, were reported to accelerate photoinhibition (Öquist *et al*. 1992). However, in the early morning, the high qP values obtained in both sites showed that photoprotective mechanisms enabled the photosynthetic apparatus to recover. Increased irradiance in burned sites in the winter was reflected in higher ETR and NPQ, especially at midday. Increased NPQ indicates higher dissipation of thermal energy by the xanthophyll cycle (Demmig-Adams and Adams 1996), thereby contributing to the photoprotection of the photosynthetic apparatus in *Q. ilex* (Fleck *et al*. 2000).

In the summer, light availability was similar between sites, and differences at midday in NPQ reflect the photosynthetic differences between different kinds of vegetation. In fact, in resprouts, higher nutrient and water availability, because of the high root/shoot ratio, accounts for the maintenance of high stomatal conductance and photosynthesis rates in spite of the high

transpiration in summer (Fleck *et al*. 1998, Peña-Rojas *et al*. 2005). The higher photochemical sink caused by increased photosynthesis in resprouts diminished the need for thermal energy dissipation, as indicated by the NPQ values being lower than in the controls.

	Φ_{PSII}	qP	F'_v/F'_m	ETR (μmol m^{-2} s^{-1})	NPQ
Winter					
Control					
Morning	0.67 ± 0.01^a	0.86 ± 0.02^a	0.79 ± 0.01^a	8.3 ± 0.6^a	0.33 ± 0.07^{ab}
Midday	0.49 ± 0.03^b	0.68 ± 0.04^b	0.72 ± 0.01^b	25.8 ± 2.6^b	0.49 ± 0.10^a
Evening	0.72 ± 0.02^a	0.95 ± 0.01^c	0.75 ± 0.01^b	1.6 ± 0.4^c	0.26 ± 0.05^b
Resprouts					
Morning	0.61 ± 0.01^a	0.93 ± 0.01^a	0.65 ± 0.01^a	10.9 ± 1.0^a	0.75 ± 0.10^a
Midday	0.40 ± 0.03^b	0.78 ± 0.04^b	0.54 ± 0.02^b	75.4 ± 10.9^b	2.06 ± 0.43^b
Evening	0.67 ± 0.01^a	0.93 ± 0.02^a	0.72 ± 0.01^c	6.3 ± 0.5^a	0.28 ± 0.04^a
Summer					
Control					
Morning	0.68 ± 0.02^a	0.84 ± 0.04^a	0.82 ± 0.02^a	18.4 ± 2.0^a	0.34 ± 0.07^a
Midday	0.40 ± 0.06^b	0.61 ± 0.07^b	0.61 ± 0.04^b	67.4 ± 15.3^b	2.11 ± 0.52^b
Evening	0.73 ± 0.01^a	0.94 ± 0.01^a	0.79 ± 0.01^a	13.7 ± 1.5^a	0.16 ± 0.03^a
Resprouts					
Morning	0.73 ± 0.01^a	0.97 ± 0.02^a	0.75 ± 0.01^a	21.7 ± 2.6^a	0.21 ± 0.03^a
Midday	0.34 ± 0.04^b	0.60 ± 0.05^b	0.54 ± 0.03^b	87.0 ± 8.0^b	1.39 ± 0.29^b
Evening	0.70 ± 0.01^a	0.99 ± 0.01^a	0.71 ± 0.01^a	13.0 ± 3.2^a	0.14 ± 0.02^a

Table 3. Fluorescence parameters in *Q. ilex* control leaves and resprouts from *Serra de les Forques* forest at three times of the day on two representative days in each season (winter, summer). Φ_{PSII}, quantum yield of PSII; qP, photochemical quenching; F'_v/F'_m , intrinsic efficiency of open PSII centers; ETR, relative rate of electron transport; NPQ, non-photochemical quenching. Each value represents the mean ± S.E. of 3 measurements per plant (seven plants per treatment). Different letters indicate significant differences during the day at $p<0.05$.

Maximum quantum yield values (F_v/F_m) in the winter were 0.776 ± 0.003 in control leaves and 0.754 ± 0.006 in resprouts. In the summer, values were 0.806± 0.004 in controls and 0.717± 0.008 in resprouts. These values were within the range 0.75-0.85, which is normal for well-watered plants (Björkman and Demmig 1987) even if they showed slight photoinhibition.

In the Experimental Fields, potted UCV plants in spring showed a 46% Φ_{PSII} decline from the morning to the afternoon before increasing again (Table 4). The decrease in Φ_{PSII} was due to a lower proportion of open PSII reaction centres (qP), whereas the intrinsic efficiency of the open PSII centres did not change significantly. qP values obtained in potted plants were lower than in the forest, indicating a very high energy pressure of PSII and thus a strong effect of ambient stressing conditions. The ETR showed no significant variations and NPQ was highest between 12.30 and 14.30 h. The pre-dawn F_v/F_m was 0.802 ± 0.002, indicating that the photosynthetic apparatus was completely recovered after the strong light exposure during the day. In CV plants, the results for the fluorescence parameters between 14.30 and 16.30 h also showed the lowest Φ_{PSII} and qP and the highest NPQ at 14.30h. The pre-dawn F_v/F_m values (0.810

± 0.001) indicate that photoprotective mechanisms were effective, since values were similar to those reported for unstressed *Q. ilex* plants (Fleck *et al.*1998).

	PPFD (μmol m^{-2} s^{-1})	Φ_{PSII}	qP	F'_v/F'_m	ETR (μmol m^{-2} s^{-1})	NPQ
UCV plants (h)						
10:30	713 ± 3^{a}	0.25 ± 0.05^{ab}	0.49 ± 0.09^{ab}	0.51 ± 0.01^{a}	65 ± 20^{a}	2.7 ± 0.1^{a}
12:30	1240 ± 33^{b}	0.12 ± 0.02^{a}	0.28 ± 0.05^{a}	0.46 ± 0.03^{a}	63 ± 8^{a}	3.6 ± 0.5^{ab}
14:30	1263 ± 70^{b}	0.14 ± 0.02^{a}	0.34 ± 0.06^{ab}	0.42 ± 0.01^{a}	73 ± 13^{a}	4.4 ± 0.2^{b}
16:30	783 ± 89^{a}	0.32 ± 0.04^{b}	0.57 ± 0.05^{b}	0.52 ± 0.02^{a}	81 ± 6^{a}	3.0 ± 0.3^{ab}
CV plants (h)						
14:30	1238 ± 29^{a}	0.18 ± 0.00^{a}	0.37 ± 0.01^{a}	0.48 ± 0.01^{a}	90 ± 3^{a}	3.7 ± 0.2^{a}
16:30	1016 ± 43^{b}	0.21 ± 0.03^{a}	0.39 ± 0.04^{a}	0.54 ± 0.02^{a}	89 ± 13^{a}	2.8 ± 0.3^{b}

Table 4. Fluorescence parameters in *Q. ilex* leaves from Experimental fields in spring at several times of the day in each light exposure condition: UCV (Uncovered Plants) and CV (Covered Plants). PPFD, Photosynthetic photon flux density; Φ_{PSII}, quantum yield of PSII; qP, photochemical quenching; F'_v/F'_m , intrinsic efficiency of open PSII centers; ETR, relative rate of electron transport; NPQ, non-photochemical quenching. Each value represents the mean ± S.E. of 5-8 measurements per five plants. Different letters indicate significant differences during the day at $p<0.05$.

On the basis of our data for chlorophyll fluorescence parameters, the highest TGase activity and B-Put content coincided with the period of the highest photoprotective need. It has been reported that the ratio Put/Spd in thylakoids is related to the status of the photosynthetic apparatus (Logothetis *et al*. 2004, Sfichi *et al*. 2004, Sfakianaki *et al*. 2006). In the forest, the increase in the B-Put /B-Spd ratio from morning to midday and its decrease in the evening to about the initial values, indicate photoadaptation of the photosynthetic apparatus to the highest PPFD at midday. The higher B-Put/B-Spd in the summer than in the winter also reflects an increased need for photoprotection as indicated by the high NPQ values. In the spring experiments on uncovered potted plants, we observed an increase in the B-Put/B-Spd ratio until 10.30 h. The maintenance of high PPFD from 12.30 h to 14.30 h (around 1300 µmol m^{-2} s^{-1}) (Table 2) would account for the decline of the B-Put/B-Spd ratio despite the increase in TGase activity, indicating the onset of severe photoinhibition (qP values were around 0.30).

In conclusion, the results obtained under natural conditions support the hypothesis that the relationship between PA concentration (free and bound) and TGase activity is modulated by light intensity in *Q. ilex* leaves and that PAs are involved in the photoprotection of the photosynthetic apparatus.

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RESUM DELS RESULTATS I DISCUSSIÓ

L'objectiu general d'aquest treball fou aprofundir en el coneixement de sistemes de fotoprotecció en fulles de *Quercus ilex*. En aquest sentit, es va voler facilitar una base de dades gràcies a la qual amb una sola lectura mitjançant espectroscòpia de reflectància al vermell proper (NIRS), es pogués aconseguir la predicció de les concentracions de molècules amb caràcter fotoprotector o antioxidant emprades amb més freqüència en estudis ecofisiològics.

La utilització d'aquesta tècnica precisava utilitzar material vegetal dessecat, ja que l'aplicació del NIRS a material fresc ha de tenir en compte els effectes que provoca l'aigua en bandes molt àmplies del rang de longituds d'ona que s'analitzen. En estudis anteriors, Lacaze i Joffre (1994), intentaren correlacionar els espectres de diversos materials en fresc (entre els que es trobaven fulles d'alzina), amb les concentracions bioquímiques de nitrogen i de molècules estructurals com la lignina o la cel·lulosa i comprovaren que l'ús de les derivades millorava molt més utilitzant les mostres seques i triturades que utilitzant les de material fresc. Posteriorment, Méthy *et al*. (1998), comprovaren que en fulles d'alzina, els resultats eren variables degut entre altres causes a la heterogeneitat del material vegetal fresc i a la dificultat en poder reproduir acuradament la posició de la fulla durant les rèpliques necessaries per cada anàlisi. Així doncs, s'adoptà com a primer objectiu l'estandardització d'un protocol per assegurar la conservació del material vegetal en sec, evitant la degradació de les molècules implicades en la fotoprotecció, durant el seu magatzematge, transport i anàlisi. La liofilització (duta a terme als Serveis Científico-Tècnics de la Universitat de Barcelona) fou l'eina escollida per testar tots els requisits.

En primer lloc, es realità un experiment amb diferents tipus de liofilització (**Capítol 1**) utilitzant fulles de dues espècies amb característiques estructurals diferents: *Taraxacum officinale* Weber (herbàcia) i *Quercus ilex* L. (esclerofil·le).

Amb l'objectiu de millorar el procés de liofilització simple, van desenvolupar-se paral·lelament cinc protocols de liofilització per als dos tipus de fulles. Cadascún es caracteritzà per l'addició d'un conservant diferent amb propietats de molècules antioxidants o biocides (2,6-ditertbutil-4-metilfenol, cisteamina, 1,4-ditiotreitol i 2-butilamino etanol). S'aplicà com a criteri per a definir el grau de conservació del material vegetal la comparació dels resultats obtinguts en les anàlisi d'un ampli rang de pigments cloroplàstics (neoxantina, violaxantina, zeaxantina, anteraxantina, α i ß-carotè, luteina, epoxiluteina, clorofil·la *a* i *b*), α- i γ-tocoferol, així com el càlcul dels índexs d'isomerització i l'estat de de-epoxidació de les xantofil·les (Z/VAZ). Les anàlisi es dugueren a

terme conjuntament amb la col·laboració del Departament de Biologia Vegetal i Ecologia de la Universitat del País Basc (UPV) a les instal·lacions ubicades a Bilbao. La comparació dels resultats obtinguts a cada protocol (un control amb fulles liofilitzades sense l'addició de cap conservant i liofilitzacions amb addició de diferents tipus de conservants) resultà en una inducció de canvis en la composició de pigments (formació de feofitina, reducció de violaxantina, isomerització de la luteina) en tots els tractaments excepte en el que contingué 2-butilamino etanol (BAE), el qual protegí efectivament la majoria dels compostos en ambdues espècies i mantingué l'estat de de-epoxidació del cicle de les xantofil·les.

Finalment es compararen els resultats obtinguts amb el millor mètode de liofilització de les fulles (BAE) amb els de les fulles únicament congelades i sense liofilitzar per comprovar que el grau de modificació dels compostos estudiats no era significatiu.

Tanmateix, en aquesta direcció i en col·laboració amb grups científics d'organismes diversos es portà a terme un disseny experimental per caracteritzar, les modificacions que podien sofrir cadascun dels fotoprotectors des de la recol·lecció de les fulles fins al seu anàlisi. L'objectiu d'aquest nou treball fou la recerca del millor mètode per conservar material vegetal sense alteracions químiques en el seu contingut, facilitant el seu transport des d'ubicacions allunyades fins a un laboratori ben equipat (**Annex I**). S'analitzaren els extractes de fulles de *Olea europaea* i de *Taraxacum officinale* controls i conservats mitjançant múltiples metodologies: liofilitzats, extractes *in situ* amb acetona, extracció passiva amb acetona, conservació en dimetil sulfòxid (DMSO), assecat amb silica gel i magatzematge *in vivo*. Amb els resultats obtinguts es realitzà una taula de doble entrada per a l'ajut en la decisió de quin seria el millor mètode en funció del tipus de molècula a analitzar i de les facilitats de les que es disposi en el punt de mostreig.

Com a conclusió d'aquests estudis s'obtingué un protocol estandarditzat de liofilització de fulles vegetals per aconseguir la conservació màxima dels compostos a analitzar posteriorment mitjançant el NIRS. El fet de tenir el material vegetal sec i homogenitzat garanteix la conservació de les mostres vegetals durant el seu magatzematge i transport.

Mitjançant aquest nou protocol de preparació de mostres vegetals seques, homogenitzades i inalterades bioquímicament, es procedí a l'execució del segon objectiu del treball: la realització d'una base de dades per a la quantificació de pigments cloroplàstics i antioxidants fotoprotectors (**Capítol 2**). Aquesta base de dades es generà correlacionant els resultats de les anàlisi

bioquímiques amb els obtinguts a través de l'anàlisi per NIRS del material vegetal. La nova base de dades inclogué la possibilitat de la quantificació ràpida i econòmica de les següents molècules: neoxantina, violaxantina, zeaxantina, anteraxantina, α i β-carotè, luteïna, luteïna epòxid, clorofil·les *a* i *b* i els antioxidants α, β i γ-tocoferol, ascorbat i fenols totals. S'estableixen correlacions entre la concentració bioquímica de cada molècula i els espectres del NIRS a cada mostra de fulles de *Q. ilex* liofilitzades. S'observà clarament la teoria de la trompeta de Horwitz que s'estandarditzà a pincipis dels anys vuitanta (Horwitz *et al*. 1980), on els analits que es troben en menys concentració mostraren una desviació estàndard relativa en les repeticions majors, fet que implica una incertesa superior en la mesura. Així doncs, s'obtingueren unes equacions de cal·libració per als analits més abundants amb uns coeficients de correlació molt propers a 1, mentre que altres molècules com (α-Car, Lx, A, Z i (β+γ)-Toc) han donat un coeficient de correlació més baix, indicant valors de les mesures una mica més dispersos.

Tal i com s'observà, alguns pigments com la zeaxantina, l'anteraxantina, l'α-carotè i la luteïna epòxid o amb l'antioxidant (β+γ)-tocoferol presentaren concentracions baixes a les fulles de *Q. ilex*; aquest fet, juntament amb una dispersió del conjunt de les mosatres no adequada, implicà uns valors més baixos dels coeficients de correlació (R^2) en les equacions de regressió i per tant una precisió de predicció inferior dins la base de dades per l'anàlisi en NIRS (veure el **Capítol 2**).

La nova base de dades NIRS per a la descripció de les respostes dels sistemes antioxidants es va aplicar en dos estudis:

En primer lloc, i com a comprovació de la validesa de la base de dades, es caracteritzaren fulles d'alzines adultes i de rebrots després de tala durant les dues estacions més estressants (estiu i hivern) (**Capítol 2**). Els resultats obtinguts foren coherents amb la bibliografia ja publicada i per tant suportaren la validesa de la nova base de dades. A l'**annex II** es descriuen els diferents compostos fenòlics que integren el conjunt de fenols totals (TPhe) mesurats mitjançant la base de dades NIRS.

En segon lloc, es caracteritzaren respostes fotosintètiques i antioxidants de fulles d'alzines i dels seus rebrots originats sota diferents concentracions de CO_2: ambiental (350 $\mu L \cdot L^{-1}$) (RA) i elevada (750 $\mu L \cdot L^{-1}$) (RE) (**Capítol 3**). En quant a l'anàlisi de les dades de bescanvi de gasos i fluorescència de les clorofil·les, s'observaren diferències significatives en paràmetres hídrics (la conductància total i la transpiració foren sempre inferiors en els RE). Tanmateix s'observà una regulació a la baixa de la fotosíntesi descrita prèviament a *Q. ilex*

(Aranda *et al*. 2006) i en d'altres espècies de *Quercus* (Faria *et al*. 1996) i que es relacionà amb una acumulació de carbohidrats no estructurals (TNC), que foren molt superiors a RE. L'acumulació de TNC està lligada amb la inhibició de determinats gens que intervenen en el procés assimilatori (Ainsworth *et al*. 2004). Segons Moore *et al*. (1999) es regula l'expressió dels gens que codifiquen l'enzim Rubisco, així com dels que codifiquen les clorofil·les, fet que també s'observà en les nostres dades. Els RE compensaren la baixa fotosíntesi amb una major participació dels cicles de les xantofil·les (major Z/VAZ) i de la luteina epòxid (menor Lx), els quals són responsables de la dissipació tèrmica de l'excés d'energia d'excitació (Demmig-Adams i Adams 1996b, García-Plazaola *et al*. 2007). Els RE mostraren també un major contingut en ascorbat (el qual es considera el responsable de la major part de l'activitat antioxidant a *Q. ilex* (García-Plazaola *et al*. 1999)) i un menor contingut en el compostots lipofílics (clorofil·les, carotenoids i tocoferols). Els RE mostraren una major capacitat de resistència a l'estrès tèrmic per altes temperatures.

Així doncs, la base de dades del NIRS ens va permetre la descripció de diferents respostes fisiològiques de les alzines en front a diverses situacions d'estrès.

Donada la implicació de les poliamines (PAs), en la resposta als canvis ambientals (Bouchereau *et al*. 1999) i en la protecció de l'aparell fotosintètic (Sfichi *et al*. 2003), es va preveure la inclusió de les PAs en la base de dades del NIRS. La metodologia emprada habitualment per a l'anàlisi de PAs consisteix en la derivació d'aquestes i una posterior separació i quantificació per cromatografia de líquids d'alta resolució (HPLC). Les PAs es troben a les cèl·lules vegetals en baixes concentracions, de manera que és necessària la seva derivació. La molècula derivatitzant, en el nostre cas el dansil, s'uneix també a tots els altres grups amino (com els d'amines o amides), fet que provocà certes dificultats: 1) l'anàlisi per NIRS no permet fer una separació prèvia de la mostra (com l'HPLC) de manera que només en el cas de que s'acoblessin les dues tècniques seria possible la quantificació de PAs amb el NIRS (tal i com han descrit en eritròcits humans Fu *et al*. (2007) mitjançant la combinació de l'electroforesi capil·lar amb el NIRS); 2) el fet de la derivació de la mostra implica un tractament del material vegetal diferent del requerit per la resta d'anàlisi, per tant limita les anàlisi amb NIRS a lectures exclusives d'amines, dificultant la quantificació de la resta de compostos fotoprotectors que es volien incloure a la base de dades NIRS. Així doncs, cap dels tipus de PAs es pogué incloure en la base de dades NIRS per a la quantificació de pigments cloroplàstics i compostos fotoprotectors.

Malgrat aquest impediment en la ampliació de la base de dades NIRS, es volgué aprofundir en el paper de les poliamines en la fotoprotecció de les fulles d'alzina en condicions naturals (veure els **Capítols 4 i 5**). El primer pas fou la caracterització de les variacions estacionals en el contingut de putrescina (Put), espermidina (Spd) i espermina lliures, en alzines no perturbades (controls) i en rebrots originats després d'incendi al llarg de les dues estacions que impliquen situacions d'estrès majors (estiu i hivern) (veure el **Capítol 4**). Aquestes anàlisi es realitzaren en col·laboració amb L'Institut de Biologia Agro-ambiental i Forestal (IBAF) al Centre Nacional de Recerca (CNR) de Roma. En augmentar la intensitat lumínica observàrem una tendència general de descens en el contingut en Put lliure (considerada la PA més important en quant a la implicació en la fotoprotecció (Navakoudis *et al.* 2003)). Aquest fet s'observà tant en la comparació entre estacions a ambdós poblacions com en la comparació al llarg del dia. La disminució de Put fou també més destacada en els rebrots crescuts després d'incendi (els quals es trobaven en una ubicació amb una disponibilitat lluminosa (PPFD) molt més elevada sense capçades que els interceptessin la llum). Al mateix temps, l'anàlisi dels resultats obtinguts dels indicadors de l'estat fotoquímic dels fotosistemes (mitjançant la fluorescència de les clorofil·les) indicà valors inferiors en els rebrots del Φ_{PSII}, de la qP i major dissipació tèrmica. Aquestes dades descrigueren una necessitat fotoprotectora superior en els rebrots.

La disminució de les PAs lliures (i en especial la Put) en augmentar la PPFD s'atribueix a una necessitat superior de lligar aquestes molècules als tilacoides per ajudar a la fotoprotecció de l'aparell fotosintètic en front a situació d'estrès tal i com suggeriren altres autors: e.g. Besford *et al*. 1993 en extractes de fulles de civada, i Logothetis *et al*. 2004, Sfichi *et al*. 2004 o Kotzabasis *et al*. 1999 en cultius de *Scenedesmus obliquus* (algues verdes unicel·lulars).

Amb l'objectiu d'aprofundir en la relació observada entre el contingut en PAs i els canvis d'irradiància i la fotoprotecció, es quantificaren les poliamines lligades a proteïnes (B-PAs) juntament amb l'activitat de l'enzim transglutaminasa (TGasa, EC 2.3.2.13). Les anàlisi de l'activitat de l'enzim TGasa, mitjançant Put tritiada, es realitzaren en col·laboració amb el Departament de Genètica Molecular de l'Institut de Biologia Molecular de Barcelona (CSIC-IRTA). En tabac es trobà que les B-PAs eren les PAs que tenien un paper més destacat en la protecció de l'aparell fotosintètic (Navakoudis *et al.* 2003). La TGasa és l'enzim responsable de la unió de les PAs a les proteïnes i pot afectar la regulació de l'apilament dels tilacoides mitjançant la unió de PAs a proteïnes de l'antena dels fotosistemes o la unió de les LHCII entre elles

mateixes, així com també poden afavorir la transferència de l'energia d'excitació entre fotosistemes (Mullet 1983, Allen *et al*. 1981, Serafini-Fracassini i Del Duca 2008). El paper de les TGases a les cèlul·les vegetals, mitjançant la sobreexpressió de l'enzim, s'ha estudiat i descrit a l'**Annex III**. S'observà que l'efecte d'una elevada concentració en TGasa provocava un augment en l'apilament dels tilacoids i una conseqüent pèrdua de funció dels mateixos fins al punt d'arribar a una fotoinhibició greu i finalment la mort cel·lular.

En aquest estudi (**Capítol 5**) amb individus sota condicions naturals al bosc s'observà una tendència diürna on els valors més alts de la Put lligada a proteïnes (B-Put) i de l'activitat TGasa, correspongueren sempre al màxim d'intensitat lumínica i dosi de llum rebuda per les fulles. Comparant entre estacions, l'index B-Put/B-Spd, indicatiu de l'estat de l'aparell fotosintètic (Logothetis *et al.* 2004, Sfichi *et al.* 2004, Sfakianaki *et al.* 2006) estigué relacionat amb els resultats obtinguts dels paràmetres de fluorescència.

Per conèixer millor els mecanismes d'activació de la síntesi de PAs i la seva unió a proteïnes mitjançant l'activitat TGasa, es dissenyà un experiment complementari, amb alzines crescudes en testos als camps experimentals de la Universitat de Barcelona. Aquests individus van ser sotmesos a diferents períodes d'irradiància mitjançant el cobriment temporal dels mateixos. En les fulles sotmeses a un fotoperíode natural (plantes descobertes) el contingut en B-Put i l'activitat TGasa seguiren el mateix patró que el de la PPFD (tant d'intensitat com de dosi acumulada), assolint el màxim en el moment de major incidència lluminosa. Els individus que romangueren coberts fins a migdia i foren exposats sobtadament a altes intensitats de llum (i no amb un augment gradual de llum natural) i mostraren una activitat TGasa superior. Així doncs, una exposició abrupta a altes intensitats lumíniques provoca una resposta en les fulles d'alzina d'un augment de l'activitat TGasa i una conseqüent acumulació màxima de B-Put. L'anàlisi de la fluorescència de les clorofil·les permeté la relació entre tots els paràmetres estudiats i la detecció de la necessitat de fotoprotecció a les fulles de *Q. ilex*.

Aquests dos treballs indiquen la participació de les poliamines, i especialment la Put, en la fotoprotecció de l'aparell fotosintètic. En concret, l'activitat TGasa, la quantitat de B-Put i l'índex B-Put/B-Spd són uns clars indicadors de la magnitud d'estrès per excés de llum de la planta.

1. Les condicions òptimes per la conservació de mostres de fulles d'alzina, durant el seu magatzematge i transport fins al moment de l'anàlisi de compostos fotoprotectors, són la seva liofilització amb la prèvia addició de 2-butilamino etanol.

2. La base de dades, realitzada en fulles d'alzina, correlacionà les mesures per espectroscòpia de reflectància al vermell proper (NIRS) amb els resultats de les anàlisi bioquímiques dels compostos antioxidants i pigments cloroplàstics (neoxantina, violaxantina, zeaxantina, anteraxantina, α i ß-carotè, luteïna, luteïna epòxid, clorofil·les *a* i *b*, α, β i γ-tocoferol, ascorbat i fenols totals) demostrant la seva fiabilitat en la seva aplicació en un estudi de bosc.

3. Per primera vegada s'ha creat una base de dades mitjançant NIRS que permet la caracterització, amb una sola mesura de compostos antioxidants i pigments cloroplàstics, constituint una eina adequada per a estudis ecofisiològics, amb els avantatges d'estalvi de temps i diners.

4. L'aplicació de la base de dades mitjançant NIRS, en un estudi en rebrots d'alzina originats sota condicions de CO_2 elevat, mostrà en aquests una major concentració en ascorbat i participació de mecanismes de dissipació tèrmica d'energia. Així mateix mostraren una major resistència a l'estrès per altes temperatures.

5. S'ha demostrat la participació de les poliamines, especialment la putrescina, en fotoprotecció en estudis que relacionaren els nivells de PPFD amb les variacions diàries i estacionals de l'activitat transglutaminasa.

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ALTERNATIVE METHODS FOR SAMPLING AND PRESERVATION OF PHOTOSYNTHETIC PIGMENTS AND TOCOPHEROLS IN PLANT MATERIAL FROM REMOTE LOCATIONS

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ANNEX II

LEAF FLAVONOID CONTENT IN *QUERCUS ILEX* L. RESPROUTS AND ITS SEASONAL VARIATION

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Trees: Structure and Function (2009) 23: 401-408

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REMODELING OF TOBACCO THYLAKOIDS BY OVER-EXPRESSION OF MAIZE PLASTIDIAL TRANSGLUTAMINASE

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Biochimica et Biophysica Acta (BBA) – Bioenergetics (2009) 1787: 1215-1222

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