UNIVERSITAT DE
BARCELONA

Respostes fotosintètiques de diverses espècies d’interès forestal en front al dèficit hídric, concentració elevada de CO_2 i contaminació per metalls pesants

Jordi Fernàndez Martínez

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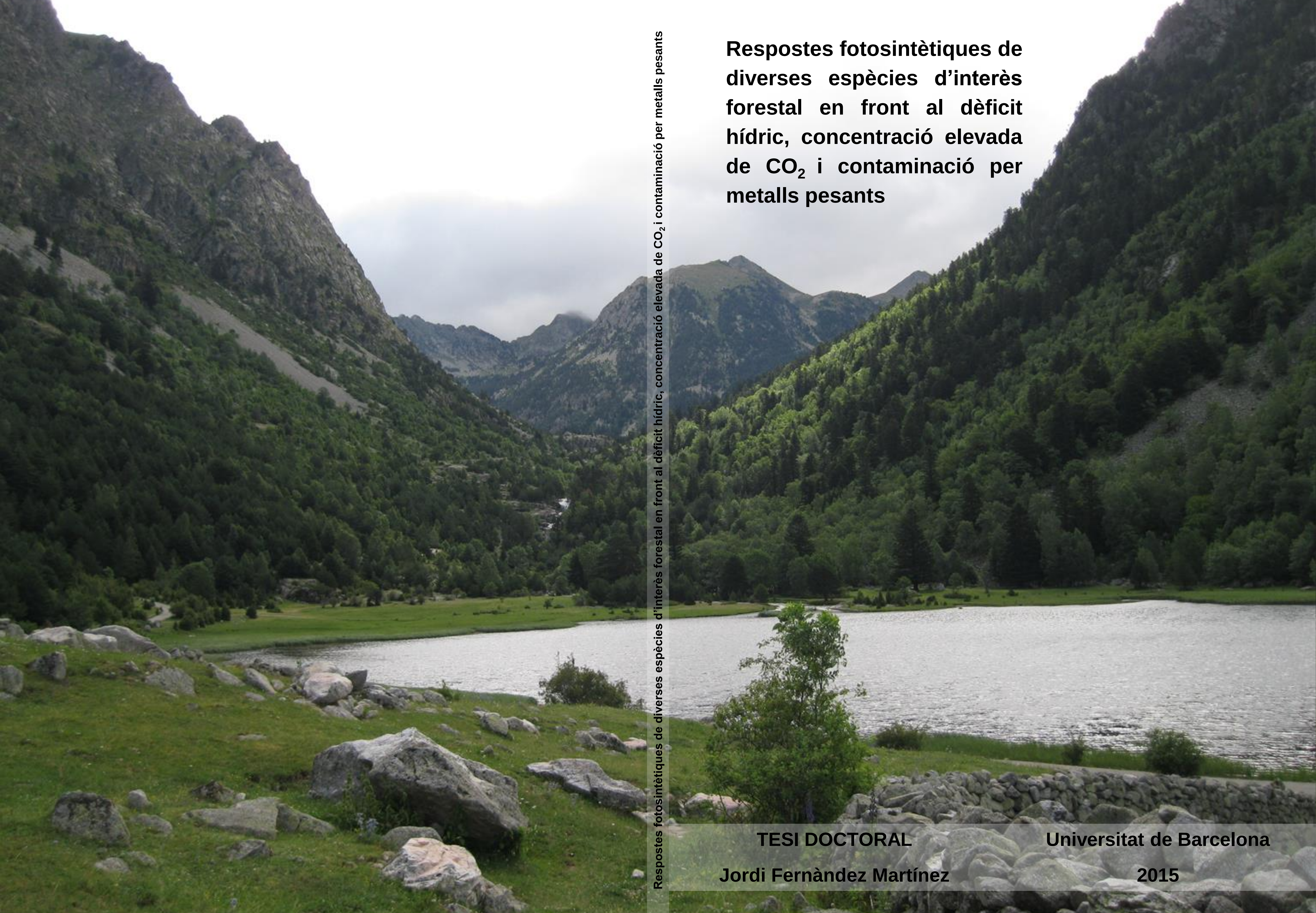

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TESI DOCTORAL

Jordi Fernàndez Martínez

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"El espíritu del valle nunca muere.
Se le llama la hembra misteriosa.
El umbral de la hembra misteriosa
Es la raíz del cielo y de la tierra.
Perseverante, parece existir siempre
y su contenido nunca se agota."
Lao-Tse

Respostes fotosintètiques de diverses espècies d'interès forestal en front al dèficit hídric, concentració elevada de CO_2 i contaminació per metalls pesants

Memòria presentada per Jordi Fernàndez Martínez per optar al títol de Doctor per la Universitat de Barcelona. Aquest treball s'emmarca dins el programa de doctorat EEES "HD206 Biologia Vegetal", corresponent al bienni 2009/2015 del Departament de Biologia Vegetal de la Facultat de Biologia de la Universitat de Barcelona.

Aquest treball ha estat realitzat al Departament de Biologia Vegetal de la Facultat de Biologia de la Universitat de Barcelona, sota la direcció de la Dra. Isabel Fleck Bou.

Doctorand	Directora de la Tesis
Jordi Fernàndez Martínez	Dra. Isabel Fleck Bou

BARCELONA, MARÇ DEL 2015

Universitat de Barcelona

Als meus pares
al meu germà
a l'Àuria

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ÍNDEX

INTRODUCCIÓ

Els boscos estan sotmesos a condicions ambientals fluctuants que poden arribar a tenir una forta influència en el creixement i el desenvolupament de les plantes. Aquestes condicions venen determinades en gran mesura pel clima que pot afectar la vegetació a nivell de planta o fulla; a nivell de sistemes com la comunitat vegetal o el bosc; i a escales més grans com el bioma. Les variables climàtiques exerceixen una influència molt important en la distribució de les poblacions vegetals, en els límits de supervivència i en els estatges altitudinals, mentre que altres factors més localitzats com són el tipus de sòl o la topografia, també hi juguen un paper destacat (Prentice *et al.* 1992).

Les plantes poden estar influenciades per diversos factors ambientals. Per una banda trobem els factors biòtics, que estan constituïts per organismes simbionts, paràsits, patògens, herbívors i competidors i que poden originar situacions d'estrès (Baier *et al.* 2002). Per altra banda hi ha els factors abiòtics, com són, la llum, la disponibilitat d'aigua, la humitat, la temperatura, els nutrients, el CO_2, el vent i els pol·lutants, entre d'altres, i que determinen el creixement de les plantes, (Schulze *et al.* 2005). L'efecte que pot arribar a tenir un factor abiòtic en la planta depèn de la seva quantitat o intensitat. Les plantes presenten un rang òptim de creixement per a cada tipus de factor abiòtic. Qualsevol desviació d'aquest òptim dóna lloc a una situació d'estrès que afecta negativament el seu creixement, desenvolupament i/o productivitat (Bray *et al.* 2000). Els factors d'estrès abiòtic inclouen, per exemple, irradiàncies molt altes o molt baixes, sequera, temperatures extremes, dèficit o excés d'elements minerals en el sòl, entre d'altres (Gleick 2000).

Les plantes han de presentar tolerància als factors d'estrès per tal de mantenir la seva integritat, característica que ve determinada genèticament i que també depèn de l'edat (Nilsen i Orcutt 1996), de l'estadi de desenvolupament (Mittler 2006) i de factors climàtics i edàfics (Skuodene 2005). Si un factor d'estrès supera el llindar de tolerància de la planta, aquesta ha d'utilitzar energia addicional per a poder sobreviure (Mandre 2002).

Aquests factors d'estrès es manifesten cada vegada amb major freqüència i de manera més intensa com a conseqüència del canvi climàtic. L'augment de les concentracions de CO_2 i d'altres gasos en l'atmosfera que són producte de l'activitat humana han donat lloc a que les temperatures globals hagin augmentat aproximadament 1ºC al llarg de darrer segle, i ho continuaran fent, i de manera més ràpida, en les properes dècades (IPCC 2014). Els models de canvi climàtic prediuen pels ecosistemes forestals un augment dels nivells de CO_2 atmosfèric de fins a 730-1020 ppm per l'any 2100 (Canadell *et al.* 2007), un augment de les temperatures entre 3ºC i 4ºC, una disminució en les precipitacions i un augment de les exposicions a les altes irradiàncies (Christensen *et al.* 2007, IPCC 2014). Aquestes condicions poden limitar la productivitat de les plantes de manera significativa (Niinemets i Kull 2001, Chaves *et al.* 2009) i poden alterar l'estructura de les comunitats vegetals. Els ecosistemes que es troben a alçades i latituds elevades seran especialment sensibles al canvi climàtic (Inauen *et al.* 2012, Streit *et al.* 2014) i, com a conseqüència de l'increment de les temperatures, les espècies es desplaçaran a alçades i latituds majors (Körner 2012).

La pol·lució és un altre factor d'estrès abiòtic al que les plantes estan cada vegada més exposades degut a l'activitat humana, i que contribueix a la degradació dels ecosistemes. Les causes principals poden ser: causes generals, a nivell planetari, lligades principalment a la contaminació atmosfèrica (pluja àcida, deposició seca, etc.); i causes específiques, localitzades geogràficament, com pot ser el dipòsit de residus (Boixadera i Cortés 2002). La pol·lució pot afectar directament les plantes causant danys en els seus òrgans, o bé indirectament quan s'acumulen en el seu medi de creixement (sòl o aigua) i afecten el seu metabolisme (Mandre 2002).

Les activitats humanes com la industrialització, la urbanització, la mineria o l'agricultura han contribuït a un increment en els nivells de metalls en la biosfera. Degut a aquestes activitats, actualment existeixen àrees molt extenses al Japó, Indonèsia i Xina contaminades amb metalls com el Cd, el Cu o el Zn (Herawati *et al.* 2000). Aquestes alteracions en el medi, poden tenir un efecte important sobre la composició i l'estructura dels boscos (Frelich 2002). La toxicitat per metalls en les plantes varia en funció de l'espècie, del tipus de metall, de la seva concentració, de la composició del sòl i del pH (Nagajyoti *et al.* 2010). L'acumulació de metalls pesants com el zinc (Zn) pot donar lloc a danys molt severs en la vegetació (Ernst i Joosse-van Damme 1983). A més hi ha moltes fonts de contaminació per Zn en els sòls i sovint estan associades a contaminació per altres metalls com són el Plom (Pb), el Coure (Cu), i el Cadmi (Cd) (Pedler *et al.* 2004).

Els estudis d'investigació sobre l'efecte dels factors d'estrès associats al canvi climàtic així com l'efecte de la contaminació per metalls en les espècies vegetals permetran comprendre la seva capacitat per tolerar i desenvolupar-se en aquestes condicions, informació necessària per ajudar a preservar els ecosistemes forestals, per restaurar aquells que estiguin degradats, i seran d'utilitat a l'hora de realitzar prediccions sobre l'efecte del canvi climàtic en la distribució de les poblacions vegetals en el futur.

En aquesta tesi s’han volgut dur a terme diversos estudis sobre l’efecte d’alguns dels factors d’estrès de les plantes més importants que sovint guarden relació amb l’activitat humana. Per una banda, tenint en compte que els ecosistemes d’alta muntanya presenten una especial sensibilitat al canvi climàtic, s’han volgut conèixer les respostes de tres espècies representatives de l’estatge sub-alpí, com són el bedoll, el pi negre i el neret davant algunes condicions associades al canvi climàtic, com són les altes concentracions de CO_2 ambiental, el déficit hídric i les altes irradiàncies. Les diferents característiques d’aquestes tres espècies (un arbre caducifoli, una conífera i un arbust perennifoli respectivament) han permés obtenir diferents respostes fisiològiques davant aquestes condicions, informació que pot ésser d’utilitat a l’hora de realizar prediccions sobre les distribucions de les seves poblacions en un futur.

Les condicions de canvi climàtic poden afectar de manera diferent a les plantes en funció del seu genotip i de l’hàbitat en el que es desenvolupen (entre altres característiques). A més d’un estudi en la vegetació sub-alpina s’ha volgut conèixer les respostes davant condicions ambientals limitants en una espècie adaptada al bosc mediterrani, on les condicions de déficit hídric i d’altes temperatures a l’estiu són més severes. Per això s’ha triat el pollancre, per una banda pel seu alt requeriment hídric i, per una altra per la seva aplicabilitat en tasques de remediació ambiental. D’aquesta manera, realitzant un estudi comparatiu entre 5 clons de pollancre s’han pogut proposar aquells que presenten millors característiques per tolerar les condicions adverses per a ésser utilitzats per a fitoremediació. D’entre aquests cinc clons, se’n va triar un per fer un estudi per analitzar la seva capacitat de tolerar i acumular el Zn, i comparar les seves respostes amb les del clon Eridano (*Populus deltoides* x *maximowiczii*), pollancre que va tenir èxit en altres experiments de fitoremediació.

1. L’ESTRÈS HÍDRIC

Generalment, es produeix estrès hídric en les plantes quan disminueix la disponibilitat d’aigua en el sòl i quan les condicions atmosfèriques causen pèrdues contínues per transpiració. L’estrès hídric es caracteritza per una reducció del contingut hídric foliar, del potencial hídric i per la pèrdua de turgència i disminució de la mida de les cèl·lules (Lawlor i Cornic 2002). Es dóna una reducció del creixement de la planta tot afectant diversos processos fisiològics i bioquímics, com la fotosíntesi, la respiració, el metabolisme nutricional (Farooq *et al*. 2008), i el contingut en clorofil·les (Massacci *et al*. 2008). Quan l’estrès és sever, pot ocasionar finalment la mort de la planta (Jaleel *et al*.

2008).

La disminució de la conductància estomàtica (g_s) és el principal limitant de la fotosíntesi (*A*) en condicions de d'estrès hídric (Cornic 2000, Llorens *et al*. 2004), ja que dóna lloc a una disminució en la concentració interna del CO_2 (C_i) i finalment a una inhibició del metabolisme fotosintètic (Figura 1). El tancament estomàtic es dóna quan hi ha baixa disponibilitat d'aigua en el sòl, a través de senyals entre arrel i fulla (mitjançant l'àcid abscísic ABA); també es dóna degut a la diferència en el dèficit de pressió de vapor (VPD) de la fulla en relació a l'atmosfera; així com per l'estat nutricional de la planta (Oren *et al*. 1999). El dèficit hídric pot produir un tancament estomàtic heterogeni, fet que suposa que diferents àrees de la fulla operin a diferents concentracions de CO_2, tal i com es va observar en un estudi amb *Quercus ilex* en condicions de camp i d'hivernacle en el que va participar el doctorand (Guàrdia *et al*. 2012 veure annex).

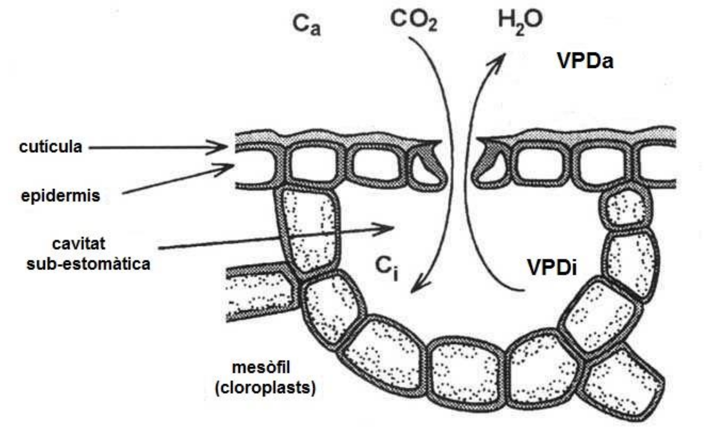

Figura 1. Representació esquemàtica de la secció d'un estoma mostrant l'entrada de CO_2 i l'alliberament de H_2O per transpiració. El Ca i el Ci són el CO_2 ambiental i intern respectivament. El VPDa i VPDi són el dèficit de pressió de vapor a l'exterior i a l'interior de la fulla respectivament.

La taxa de fotosíntesi a les plantes superiors depèn, entre altres factors, de la Ribulosa 1,5-bisfosfat carboxilasa/oxigenasa (RuBisco) així com de la síntesis de RuBP (Parry *et al*. 2002). En condicions de sequera, es dóna una disminució de la síntesis de RuBisco i de la seva activitat, reduint d'aquesta manera la fotosíntesi (Chaitanya *et al*. 2003).

La tolerància a l'estrès per sequera varia en funció de l'espècie, del seu hàbitat (Nakayama *et al*. 2007), de l'estadi de creixement en el que es troba i de la durada del factor d'estrès (Chaves *et al*. 2002). Els estudis sobre les respostes de les plantes en front l'estrès hídric són rellevants, ja que les prediccions del canvi climàtic suggereixen un increment en l'aridesa en moltes regions del planeta (Petit *et al*. 1999). A més dels canvis en els règims de precipitació, amb pluges menys freqüents però més intenses, la neu serà cada vegada menys present en moltes àrees, fet que afectarà els nivells d'humitat del sòl (Anderegg 2013). Els models de canvi climàtic prediuen pels boscos alpins que la precipitació total entre abril i setembre disminuirà entre un 20% i un 30% cap a finals de segle XXI (IPCC 2014), incrementant-se d'aquesta manera els episodis de sequera, poc habituals en alta muntanya. En els boscos mediterranis el clima, caracteritzat per presentar sequeres estivals pot influenciar de manera significativa el creixement de les plantes. Aquestes poden ser susceptibles de patir fotoinhibició durant condicions d'alta irradiància, temperatura i dèficit de pressió de vapor a l'estiu (Demmig-Adams *et al.* 1989). Les espècies adaptades a aquestes condicions solen presentar estructures adaptades com ara fulles escleròfil·les i/ o un major accés a les reserves del subsòl. Els arbres caducifolis com els pollancres solen presentar una alta productivitat, associada amb alts requeriments d'aigua. En el cas d'aquestes espècies, la seva introducció en la regió mediterrània per al seu cultiu per a la producció de biomassa, limita la seva distribució en regions on la disponibilitat d'aigua és gran, com ara les planícies al·luvials (Tschaplinski *et al.* 1994).

1.1. La discriminació isotòpica del ^{13}C (Δ^{13}C) i l'eficiència en l'ús de l'aigua (WUE)

El CO_2 atmosfèric està constituït per dos isòtops estables, el ^{12}C i el ^{13}C, en una proporció del 98.9% i de l'1.1% respectivament (O'Leary 1981). En la fotosíntesi, degut a la discriminació isotòpica que es dóna en els processos físics (p. ex. coeficients de difusió, dissolució, evaporació, etc.) i químics (p. ex. les reaccions enzimàtiques) durant la incorporació del CO_2 a la biomassa vegetal, els isòtops estables del C es distribueixen en quantitats desiguals. Les plantes discriminen en contra del ^{13}C durant el procés fotosintètic (O'Leary 1981), fet que es manifesta en la composició isotòpica dels teixits. El factor que més influència té en la discriminació del carboni en els òrgans fotosintètics de les plantes C3 és la concentració intercel·lular de CO_2 (C_i). A partir de la composició isotòpica (δ^{13}C) atmosfèrica i la δ13C del material orgànic de la planta es calcula la discriminació isotòpica del ^{13}C (Δ^{13}C) (Farquhar i Richards 1984):

$$\delta^{13}\mathrm{C}_{\mathrm{fulla}} = \delta^{13}\mathrm{C}_{\mathrm{aire}} - a - (b - a)\frac{\mathrm{C_i}}{\mathrm{C_a}}$$

on a és el fraccionament del $^{13}CO_2$ en relació al CO_2 durant la difusió a través del’estoma (4.4‰; O’Leary 1981); *b* és el fraccionament causat per la discriminació del $^{13}CO_2$ per part de la Rubisco (27‰; Farquhar i Richards 1984); i C_a és la concentració de CO_2 atmosfèric.

Els canvis en les relacions entre isòtops lleugers i pesants degut als fraccionaments isotòpics poden ésser mesurats amb tècniques d’alta precisió com l’espectrometria de masses (IRMS). La composició isotòpica mesurada mitjançant el IRMS s’expressa en relació a un estàndard, Pee Dee Belemnite (PDB), de la forma següent:

$$\delta^{13}\mathrm{C} = \frac{\mathrm{R_S} - \mathrm{R_{PBD}}}{\mathrm{R_{PBD}}} = (\mathrm{R_S} - \mathrm{R_{PBD}}) - 1$$

on δ és la composició isotòpica i $\mathrm{R_S}$ i $\mathrm{R_{PDB}}$ són les relacions isotòpiques de la mostra i de l’estàndard de PDB, respectivament. L’estàndard PDB és la belemnita fòssil provinent de la formació geològica de Pee Dee a Carolina del Sud, als Estats Units. La PDB és altament enriquida en ^{13}C ($\mathrm{R_{PDB}}$ = 0.0112372) comparada amb gairebé tots els materials orgànics i inorgànics (Ghashghaie i Tcherkez 2013).

La discriminació isotòpica ($\Delta^{13}C$) s’expressa com a diferència entre el valor $\delta^{13}C$ de la font i del producte:

$$\Delta(‰) = \frac{\delta^{13}\mathrm{C_{font}} - \delta^{13}\mathrm{C}_{\mathrm{producte}}}{1 + (\frac{\delta^{13}\mathrm{C_{font}}}{1000})}$$

on $\delta^{13}C$ de la font (atmosfera) generalment està entre 6.5 – 8.0‰, segons la ubicació geogràfica i l’alçada (Francey i Tans 1987).

La distribució natural de $^{13}C/^{12}C$ i el IRMS permeten investigar el comportament del metabolisme primari del C i del flux respiratori (Ghashghaie and Tcherkez 2013).

L’eficiència en l’ús de l’aigua (WUE) definida com la relació entre l’assimilació de CO_2 i la conductància estomàtica juga un paper important en l’aclimatació, la productivitat i en la probabilitat de sobreviure en condicions d’estrès, com és, per exemple, la sequera (Lauteri *et al.* 1997). Les plantes poden presentar una alta WUE degut a unes altes taxes fotosintètiques, degut a una baixa conductància estomàtica, o bé com a conseqüència de les dues. En les plantes C3, la discriminació isotòpica del C ($\Delta^{13}C$) està relacionada amb la WUE a través del control estomàtic del bescanvi de gasos, i del grau de discriminació del ^{13}C, i amb canvis en el rang entre el C_i i el C_a (Farquhar *et al.* 1982). Normalment, les plantes amb un estat hídric favorable presenten un alta relació C_i/C_a i tenen un baix contingut en ^{13}C, mentre que les plantes que presenten estrès hídric tenen una baixa relació C_i/C_a i estan enriquides en ^{13}C, tot reflectint la compensació entre A i g_s.

En la següent equació es pot veure la correlació entre WUE i $\Delta^{13}C$ (Farquhar i Richards 1984; Masle i Farquhar 1988; Condon i Richards 1992). Amb aquesta aproximació, es pot obtenir informació de la WUE a partir dels valors obtinguts de $\Delta^{13}C$:

$$\mathrm{WUE} = \frac{\mathrm{A}}{\mathrm{g_s}} = \frac{\left(\frac{\mathrm{C_a}}{1.6}\right)(b' - \Delta^{13}\mathrm{C})}{b' - a}$$

on A és la taxa d'assimilació de CO_2 de la fulla, g_s és la conductància estomàtica, C_a és el CO_2 atmosfèric, el factor 1.6 correspon al quocient entre la difusivitat de vapor d'aigua i és una constant associada a la conductància estomàtica.

2. ESTRÈS PER ALTA IRRADIÀNCIA

L'energia provinent de la llum és utilitzada per produir ATP i NADPH en la fase fotoquímica de la fotosíntesi que s'utilitzarà per a l'assimilació del CO_2. Davant d'altes irradiàncies, el fotosistema II (PSII) pot mostrar sensibilitat i experimentar una disminució de la seva activitat. Com a conseqüència l'energia provinent de la llum incident s'acumula en excés en els centres de reacció del PSII i es produeix un desequilibri entre la seva activitat fotoquímica i el requeriment d'electrons necessaris per dur a terme la fotosíntesi (Foyer i Noctor 2000), fet que pot donar lloc a processos de fotoinhibició i danys oxidatius en la maquinària fotosintètica (Adir *et al.* 2003). Aquest processos ocasionen una disminució de l'activitat fotosintètica i, en el cas de que perdurin en el temps, del creixement i de la productivitat de la planta (Aro *et al.* 2005).

2.1. Fluorescència de les clorofil·les

Com ja s'ha comentat anteriorment, part de l'energia lumínica absorbida pels pigments fotosintètics és transferida en forma d'energia d'excitació al centre de reacció, on una part és utilitzada per fer treball químicament útil, i l'altra part és dissipada principalment en forma de calor i, en menor grau, en forma d'energia lluminosa de major longitud d'ona (fluorescència). Aquesta distribució de l'energia en els tres processos es dóna simultàniament, de tal manera que l'increment en l'eficiència d'un d'ells resultarà en la disminució dels altres dos. Per tant, a través de la mesura del rendiment de la fluorescència de les clorofil·les es pot obtenir informació de l'eficiència fotoquímica i de la dissipació tèrmica de l'energia absorbida. Només entre l'1 i el 2% del total de llum absorbida per les clorofil·les és re-emesa en forma de fluorescència (Maxwell i Johnson 2000).

Actualment existeix un ús generalitzat de la fluorescència de les clorofil·les en estudis fisiològics i ecofisiològics per fer un seguiment dels processos fotosintètics i de l'estrès en les plantes mitjançant fluorímetres modulats. La mesura de la fluorescència de les clorofil·les és una tècnica que aporta informació sobre diverses reaccions de transferència d'electrons específiques del PSII, és una mesura no destructiva, ràpida i fàcil de manipular (Baker 2008). Els fluorímetres modulats permeten analitzar les alteracions dels processos metabòlics que no estan directament involucrats amb el metabolisme fotosintètic i que sovint indueixen canvis en els paràmetres de fluorescencia. La mesura de la fluorescència de la fulla per imatges és una tècnica molt útil que aporta informació sobre la heterogeneïtat espacial dels processos fotosintètics de la fulla (Figura 3). Mitjançant aquesta tècnica s'ha observat heterogeneïtat fotosintètica, a l'inici de la senescència foliar (Wingler *et al.* 2005), en condicions d'estrès hídric (Guàrdia *et al.* 2012), sota estrès per metalls pesants (Pietrini *et al.* 2014) i per infeccions per fongs (Elena *et al.* 2014). Tant el fluorímetre modulat com les mesures de fluorescència mitjançant imatges s'han utilitzat en aquesta tesi per analitzar l'efecte de la contaminació per Zn i de patògens foliars en la fotoquímica de fulles de diferents espècies.

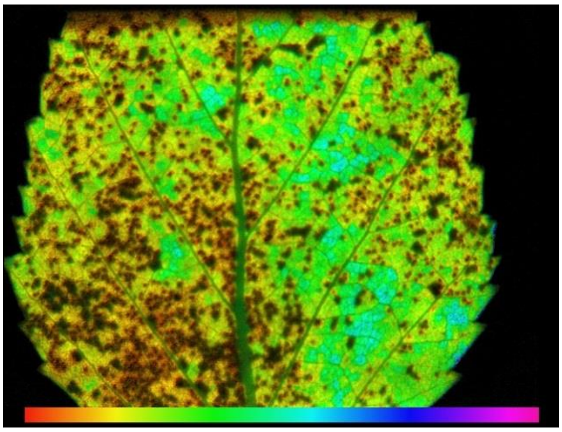

Figura 2. Imatge de fluorescência de les clorofil·les d'una fulla de bedoll infectada per rovell obtinguda mitjançant el fluorimetre modulat Imaging Pam (Walz). El paràmetre que s'ha mesurat és el quenching fotoquímic (qP). Els resultats varien en funció del color que va desde 0 (negre) fins a 1 (lila).

Quan el PSII absorbeix energia lumínica i la cedeix en forma d'electró a la plastoquinona Q_A, aquesta no podrà acceptar-ne més fins que no l'hagi cedit a la plastoquinona Q_B. Fins llavors el centre de reacció restarà tancat (reduït). Si es produeix el tancament d'un cert nombre de centres de reacció fotosintètics, es pot donar una disminució de l'eficiència fotosintètica (que es manifesta amb un augment de la fluorescència) i que es pot evitar mitjançant dues vies: el quenching o atenuació fotoquímica (qP), que consisteix en l'augment de la cessió d'electrons per part del PSII a la cadena de transport electrònic; i el quenching no fotoquímic (NPQ), que consisteix en la dissipació tèrmica de l'excés d'energia d'excitació (Demmig-Adams *et al.* 1996; Fleck *et al.* 1998; Maxwell i Johnson 2000).

Una fulla que s'ha adaptat a la foscor presenta tots els centres de reacció oberts (oxidats) (qP=1) i s'enregistra el valor de fluorescència mínima (F_o) (Figura 3). Si a continuació s'il·lumina la fulla a intensitats de llum saturants, al cap d'aproximadament un segon tots els centres de reacció esdevenen tancats (reduïts) (qP=0) i es dóna l'emissió de la fluorescència màxima (F_m). La diferència entre Fo i F_m constitueix la fluorescència variable, F_v. La relació F_v/F_m es pot utilitzar per estimar el rendiment quàntic màxim de la fotoquímica del PSII. Les plantes exposades a la llum i que pateixen estressos biòtics i abiòtics presenten amb freqüència una disminució en el F_v/F_m, fet que indica danys per fotoinhibició. Les plantes no estressades presenten valors de F_v/F_m = 0.85 (Björkman i Demmig 1987). Les mesures de F_v/F_m suposen una manera ràpida i simple per monitoritzar l'estrès (Adams i Demmig-Adams 2004):

$$F_v/F_m = \frac{F_m - F_o}{F_m}$$

Les fulles que estan exposades a la llum actínica (fotosintètica) presenten un nivell de fluorescència anomenat F, que pot augmentar fins arribar a la màxima fluorescència (F'_m) quan la fulla és exposada a un puls de llum saturant i que provoca la reducció de la Q_A. Els valors de F'm i F s'utilitzen en els paràmetres d'atenuació (quenching) fotoquímica. El paràmetre més utilitzat és l'eficiència operacional del PSII (Φ_{PSII}) i expressa l'eficiència amb la que la clorofil·la associada al PSII absorbeix un fotó per després alliberar un electró en la cadena de transport electrònic (Genty *et al.* 1989):

$$\Phi_{PSII} = \frac{F'_m - F}{F'_m}$$

El Φ_{PSII} es pot utilitzar per calcular el transport electrònic linear (ETR). Quan s'oxida Q_A tot cedint un electró a Q_B s'inicia el transport d'electrons que condueix a la fixació de CO_2 (Genty *et al.* 1989):

$$ETR = \Phi_{PSII} \cdot PPFD \cdot 0.5 \cdot 0.82$$

0.5 és el factor que assumeix que es dóna la mateixa distribució de l'energia entre els dos fotosistemes, i 0.82 és el factor d'absorbància de la llum per la fulla.

El Φ_{PSII} s'obté a partir del producte de dos paràmetres de fluorescència: el F'_v/F'_m i el qP. En una fulla adaptada a la llum, el F'_v/F'_m estima el rendiment quàntic màxim de la fotoquímica del PSII que es pot arribar a assolir quan la Q_A es troba en el seu estat màxim d'oxidació (Genty *et al*. 1989):

$$F'_v/F'_m = \frac{F'_m - F'_o}{F'_m}$$

F'_o és la fluorescència mínima que es pot obtenir en presència de llum actínica.

Aquest paràmetre es pot utilitzar per obtenir informació sobre les contribucions del quenching no-fotoquímic als canvis que es donen en el Φ_{PSII}.

El qP aporta informació sobre la quantitat de centres de reacció oberts, és a dir, sobre l'habilitat de l'aparell fotosintètic per mantenir el Q_A en estat oxidat (Genty *et al*. 1989):

$$F'_v/F'_m = \frac{F'_m - F}{F'_m - F'_o}$$

Els paràmetres de F'_v/F'_m i qP permeten saber si els canvis en Φ_{PSII} són conseqüència de variacions en el quenching no fotoquímic o bé de l'habilitat d'un centre de reacció del PSII en estat excitat per cedir un electró a la cadena de transport electrònic. El Φ_{PSII} d'una fulla disminueix quan el PPFD augmenta degut a una disminució en F'_v/F'_m i qP. De totes maneres, la contribució d'aquests dos paràmetres pot variar amb l'increment de PPFD (Baker 2008).

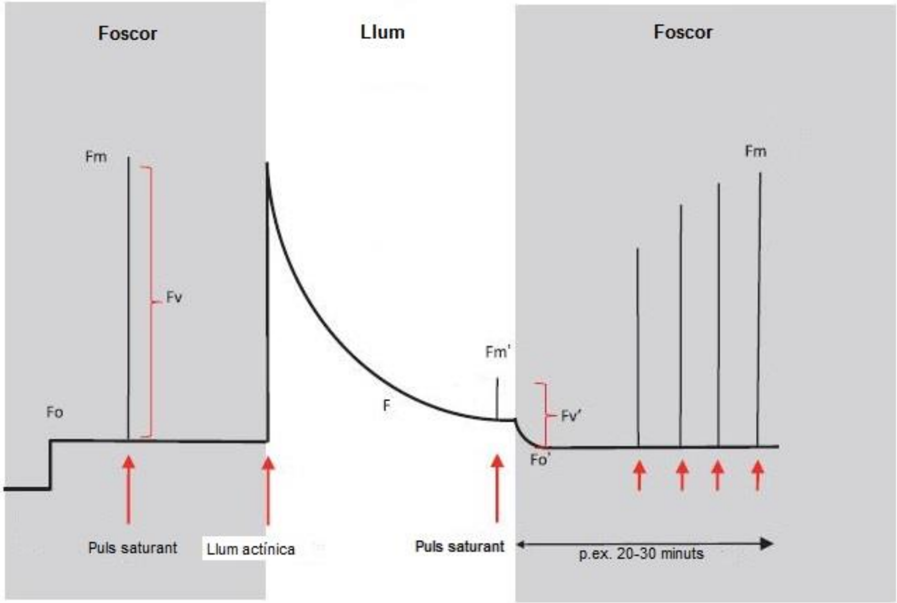

Figura 3. Fluorescència de les clorofil·les d'una fulla després d'ésser adaptada a la foscor i de la qual se'n poden obtenir els paràmetres fotoquímics i no-fotoquímics. Aquesta resposta es dóna després d'aplicar llum d'intensitats ≥ 500 µmol·m^{-2}·s^{-1}.

Tot i que el F'_v/F'_m es pot utilitzar per avaluar la contribució dels canvis en el quenching no-fotoquímic als canvis en el Φ_{PSII}, sovint s'utilitza el NPQ per determinar els nivells de quenching no-fotoquímic (Krause i Jahns 2004):

$$NPQ = \frac{F_m - F'_m}{F'_m}$$

En aquest treball s'ha emprat la F'_v/F'_m per avaluar la contribució de la dissipació tèrmica, i s'ha expressat com a 1- F'_v/F'_m.

2.2. Fotoprotecció i sistemes antioxidants

Existeixen diferents respostes de les plantes davant altes irradiàncies en funció de les seves característiques. Així, per exemple, les plantes adaptades a intensitats de llum altes presenten una alta transferència de l'energia provinent de la llum cap a la cadena de transport electrònic, una alta capacitat fotosintètica, un menor nombre de proteïnes de captació de la llum (LHCII), i un menor apilament dels tilacoides en comparació amb les plantes adaptades a l'ombra (Lichtenthaler *et al*. 1984).

Davant d'una situació d'estrès, el PSII pot veure's afectat negativament i disminuir la seva activitat (Anderson *et al*. 2001), mostrant-se més susceptible a patir fotoinhibició davant altes irradiàncies. Quan l'excés d'energia d'excitació no pot ésser utilitzada per a la fixació de CO_2 i tampoc pot ésser dissipada de manera eficient, l'O_2

actua com a acceptor final d'electrons donant lloc a la producció d'espècies reactives d'oxigen (ROS). La reducció de l'O_2 en el fotosistema I (PSI) per part de la cadena de transport electrònic origina la formació del radical superòxid (O^{2-}), que pot convertir-se en peròxid d'hidrogen (H_2O_2); i del radical hidroxil (OH^-) (Asada 1999). Per altra banda, la transferència d'energia d'excitació des de les clorofil·les al O_2 en el complex d'absorció de la llum (light-harvesting complex) produeix la formació de l'Oxigen singlet ($^1O_2$) (Zolla i Rinalducci 2002). Les ROS poden causar efectes nocius en les estructures biològiques, com ara el DNA, les proteïnes i els lípids (Asada 1999).

Per evitar la fotoinhibició i els danys oxidatius causats per les ROS, les plantes han desenvolupat diferents mecanismes de fotoprotecció, com ara evitar la irradiància directa de la llum mitjançant el moviment de les fulles i dels cloroplasts, la dissipació de l'excés d'energia d'excitació en forma de calor i la fotorespiració, entre d'altres; i la captura o detoxificació de ROS mitjançant el sistema d'antioxidants (Takahashi i Badger 2001). El sistema de defensa antioxidant de les plantes és altament eficient per contrarestar la toxicitat per ROS, i està constituïda per antioxidants enzimàtics i no enzimàtics. En aquesta tesi s'ha treballat amb antioxidants no enzimàtics com l'ascorbat, el glutatió, el α-tocoferol i el β-carotè, que cooperen pel manteniment de la integritat de les membranes fotosintètiques (Smirnoff i Wheeler 2000).

El **cicle de les xantofil·les** és present en totes les plantes vasculars i és especialment important en estar involucrat en la dissipació de l'excés d'energia d'excitació en forma de calor. L'absorció de la llum, la seva utilització en la fotòlisi de l'H_2O i el transport d'electrons genera una variació en el pH del lumen del cloroplast, que possibilita la síntesi d'ATP. Aquesta disminució en el pH indueix la protonació de les proteïnes del PSII i l'activació de l'enzim violaxantina de-epoxidasa (VDE) tot fent servir àcid ascòrbic com a substrat per a la de-epoxidació de la violaxantina (V) en anteraxantina (A) i, finalment, en zeaxantina (Z) (Ort 2001). La protonació a la que es fa referència es tracta de la unió de les les proteïnes PsbS dels complexes antena a protons, que generen un canvi conformacional en el LHCII al mateix temps que l'enzim VDE s'activa i catalitza la conversió de V a Z. La unió de Z a les proteïnes PsbS constitueix el centre dissipador d'energia (Li *et al.* 2002). El PSII passarà a un estat d'atenuació, que fa que la clorofil·la excitada tingui un curt període de vida, un baix rendiment de la fluorescència i que augmenti la dissipació de l'energia d'excitació (Gilmore 1997). Quan les condicions d'excés de llum desapareixen, l'enzim zeaxantina epoxidasa (ZE) realitza la reacció inversa de transformació en A i després en V tot utilitzant NADPH o ferredoxina com a cofactors (Eskling *et al*. 1997).

L'**àcid ascòrbic (AscA)** és l'antioxidant hidrosoluble més abundant en les plantes (Sies i Stahl 1995). Actua com a cofactor de molts enzims relacionats amb la fotosíntesi, en la biosíntesi d'hormones i en la regeneració d'altres antioxidants com el α-tocoferol (Noctor *et al.* 2000). El AscA pot ésser reciclat a través de diferents mecanismes. El monodehidroascorbat (MDHA), que es produeix amb l'oxidació del AscA, pot ésser reciclat mitjançant la reducció de la ferredoxina o de la monodehidroascorbat reductasa (MDAR). El MDHA també pot donar lloc al dehidroascorbat (DHA) i a AscA. El DHA pot ésser reciclat a AscA mitjançant el dehidroascorbat reductasa (DHAR) tot utilitzant el glutatió reduït (GSH) com a reductant, en cas de no fer-ho serà hidrolitzat de manera irreversible (Figura 4). A través del seu reciclatge es mantenen el contingut en AscA i el seu estat d'oxido-reducció, fet important en condicions d'alta demanda de AscA, com ara en condicions d'alta irradiància o d'altres condicions d'estrès, com les causades pel dèficit hídric o per metalls pesants, que incrementen la producció de ROS. Així, davant la presència de ROS com $^-O_2$, $O_2^{\cdot -}$, OH^- i H_2O_2, el AscA és oxidat a MDHA que a continuació pot donar lloc a la producció de DHA i AscA. Alternativament, la MDAR pot reduir el MDHA a AscA tot utilitzant NADPH com a reductant.

El AscA pot ésser utilitzat com a part de la reacció de Mehler-peroxidasa per mantenir el flux d'electrons a través del PSI per així prevenir la sobreexcitació del PSII i el dany oxidatiu (Asada 1999). En la reacció de Mehler, el $O_2^{\cdot -}$ es genera a través de la transferència d'electrons des del PSI al O_2, i la superòxid dismutasa (SOD) donarà lloc a la formació d'O_2 i H_2O_2. L'ascorbat peroxidasa (APX) catalitza la transferència d'electrons des de dues molècules de AscA a H_2O_2 per la formació de H_2O i de dues molècules de MDHA en l'estroma.

L'AscA pot cedir directament un electró al PSI i al PSII en condicions on el sistema primari donador d'electrons està inhibit degut, per exemple, a altes intensitats de llum (Mano *et al*. 2004).

El **glutatió** (g-Glu-Cys-Gly) és un metabòlit multifuncional en les plantes. És un reservori important de sulfur i té una funció crucial en la defensa i protecció de la cèl·lula. El Glutatió reacciona químicament amb un ampli rang de ROS. El glutatió reduït (GSH) protegeix les proteïnes evitant la seva desnaturalització causada per la oxidació del grup tiol durant l'estrès. Les reaccions on intervé el GSH donen lloc a la seva oxidació, obtenint-se el glutatió disulfit (GSSG), la seva forma oxidada. La relació GSH/GSSG es manté gràcies a l'activitat de la glutatió reductasa (GR), que utilitza NADPH per reduir el GSSG a GSH (Noctor *et al*. 2002) (Figura 4).

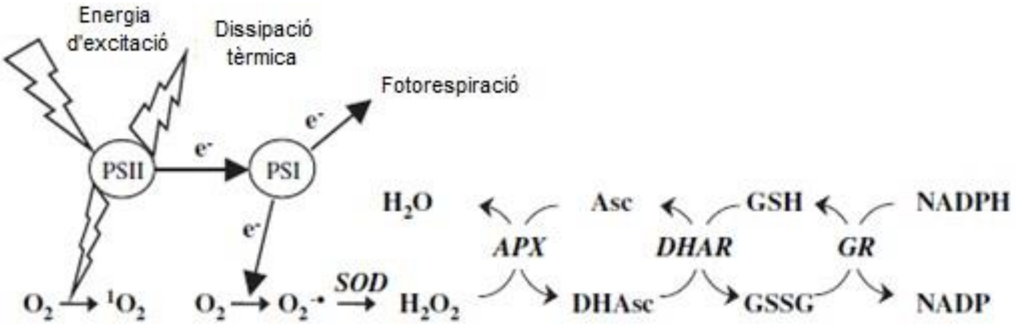

Figura 4. Cicle de l'ascorbat-glutatió on s'observen els diferents enzims que hi participen així com la reacció de Mehler per tal d'evitar la sobreexcitació del PSII i el dany oxidatiu.

La capacitat del cicle ascorbat-glutatió depèn de les concentracions d'antioxidants i de les activitats dels enzims relacionats (Tausz *et al*. 2004). La resposta del sistema glutatió a l'estrès foto-oxidatiu presenta diferents fases: (i) Quan la dissipació de l'excés d'energia d'excitació no és suficient, l'augment en la producció de ROS augmenta l'activitat del cicle ascorbat-glutatió. La relació GSH/GSSG passarà a un estat lleugerament oxidat. Aquesta fase correspon a una reacció inicial davant l'estrès. (ii) En una reacció d'aclimatació, es dóna un augment de les concentracions d'antioxidants i dels enzims associats, fet que depèn de l'estat d'oxidoreducció de GSH/GSSG (Baena-Gonzalez *et al*. 2001) i/o de la presencia de ROS com H_2O_2 (Neill *et al*. 2003) donant lloc a una major capacitat del cicle ascorbat-glutatió per detoxificar ROS. En aquest estadi s'observarien altes concentracions d'ascorbat i glutatió i s'obtindrien altes relacions de GSH/GSSG indicant una adaptació a l'estrès per part de la planta. (iii) Si la planta té una baixa capacitat d'aclimatació, l'estrès oxidatiu causaria una disminució en el sistema antioxidant, i es donaria una progressiva oxidació i degradació del contingut en ascorbat i glutatió, arribant-se a ocasionar processos de senescència i, finalment, la mort (Tausz *et al*. 2004).

Els **tocoferols** són components constituïts per un grup cromanol en un extrem i una cadena prenyl. En la membrana lipídica, la cadena prenyl constitueix la part hidrofòbica i està associada als lípids, i el grup cromanol és polar i està exposat a la superfície de la membrana. El **α-tocoferol** predomina a les fulles de les plantes vasculars, on es troba en l'embolcall i en les membranes dels tilacoides en els cloroplasts (Grusak i Della Penna

1999). En la seva activitat antioxidant, el α-tocoferol actua contra radicals lliures d'àcid grassos poliinsaturats, acabant així amb l'oxidació lipídica, desactivant el $^1O_2$ i reduint el O_2^- (Polle i Rennenberg 1994). Davant de condicions ambientals que indueixen a l'estrès oxidatiu el contingut en tocoferols presents als tilacoides augmenta, participant d'aquesta manera en la fotoprotecció de les plantes (Munné-Bosch i Alegre 2000).

El **β-Caroté** és un carotenoide localitzat a les membranes del tilacoide amb capacitat d'atenuar l'estat excitat del triplet de les molècules de les clorofil·les que poden interaccionar amb l'O_2 i formar $^1O_2$. Per altra banda, els carotenoides són atenuants eficients del $^1O_2$ (Young 1991) i poden jugar un paper important com a antioxidant en les membranes tilacoidals (Müller *et al.* 2001). Davant de situacions d'estrès, les seves concentracions poden variar, així s'ha vist que en condicions de sequera els seus nivells augmenten, incrementant la capacitat fotoprotectora (Demmig- Adams i Adams 1994). En casos d'estrès més sever, aquestes substàncies poden ser degradades (Munné-Bosch i Alegre 2000), i es pot donar peroxidació lipídica i degradacions de pigments degut al dany oxidatiu.

Els **fenols** són components químics caracteritzats per almenys un anell aromàtic (C6) que presenta un o més grups hidroxil (Sakihama *et al.* 2002). Els estressos biòtics i abiòtics, com ara l'atac per insectes patògens, les altes irradiàncies, la llum ultraviolada (UV), les baixes temperatures i els baix contingut en nutrients, entre altres, donen lloc a una acumulació de fenols en les plantes (Dixon i Paiva 1995). Els fenols, com els flavonoids, els fenilpropanoids i els àcids fenòlics tenen un paper important en l'activitat antioxidant (Rice-Evans *et al.* 1997). En la seva activitat antioxidant, els fenols poden segrestar ROS com ara el O_2^-, el H_2O_2, el OH^- i el $^1O_2$. Sota condicions d'estrès sever, la capacitat del cloroplast per segrestar ROS es veu superada quan el contingut en ascorbat esdevé oxidat (Grace i Logan 2000). Es creu que els fenols realitzen una funció de suport al sistema de detoxificació primari ascorbat-depenent en les plantes superiors (Yamasaki *et al.* 1999).

3. EFECTE DEL CO_2 ELEVAT

Al llarg dels darrers 800.000 anys la concentració de CO_2 atmosfèric ha fluctuat entre 180-200 ppm en períodes glacials, i 250-280 ppm en períodes interglacials (Lüthi *et al.* 2008). Actualment ens trobem en un període interglacial on els nivells de CO_2 van oscil.lar al voltant de 280 ppm durant els darrers 10.000 anys, i van superar aquests nivells des de la revolució industrial iniciada al voltant de l'any 1800. Des de llavors els nivells de CO_2 atmosfèric han anat augmentant fins a l'actualitat situant-se en 400 ppm. Aquest increment tan acusat dels nivells de CO_2 atmosfèric és el factor més influent en l'escalfament global (IPCC 2013). L'augment dels nivells de CO_2, i també de les temperatures, suposen un problema seriós pel medi ambient i, particularment, per les plantes ja que moltes d'elles estan adaptades a nivells de CO_2 per sota dels 300 ppm i tenen dificultats per adaptar-se a l'increment ràpid de les concentracions de CO_2 atmosfèric (Terashima *et al*. 2014)

Un increment en les concentracions de CO_2 atmosfèric dóna lloc a un augment en les taxes fotosintètiques de les plantes. Aquestes respostes són més importants en plantes C3 que en plantes C4 degut als mecanismes de concentració de CO_2 propis d'aquestes últimes (Wang *et al*. 2012). L'assimilació de CO_2 per unitat d'àrea foliar depèn de la relació entre el CO_2 i el O_2 atmosfèric i de l'activitat de l'enzim ribulosa-1,5-bisfosfat carboxilasa-oxigenasa (RuBisco), quan es dóna un augment dels nivells de CO_2 per damunt dels nivells actuals la fotosíntesi s'incrementa i la fotorespiració disminueix (Sage i Monson 1999).

L'augment de la fotosíntesi com a conseqüència de la major disponibilitat de CO_2 dóna lloc a un increment en la taxa de producció de la biomassa, com succeeix, per exemple, en la relació tija-arrel. En alguns estudis s'ha observat que la biomassa de les parts aèries d'algunes espècies augmenta fins a més del doble de la que solen presentar en condicions de CO_2 ambiental actuals, fet que succeeix sobretot en les coníferes (increment del 130%) en comparació amb les plantes caducifòlies (increment del 49%) (Ceulemans i Mousseau 1994). Els individus juvenils tenen una taxa de creixement més alta que els individus adults a condicions de [CO_2] elevat (Norby *et al.* 1996). Paràmetres estructurals com la mida de la fulla i la seva anatomia sovint són alterades per l'augment en els nivells de CO_2 ambiental (Tsutsumi *et al*. 2014) observant-se en algunes espècies un augment en la seva àrea foliar, en l'àrea foliar total de la planta i en el gruix foliar (Pritchard *et al*. 1999).

Quan les plantes estan exposades a altes concentracions de CO_2 atmosfèric poden presentar diferents respostes en funció de la durada d'aquesta exposició, del seu genotip o de la disponibilitat de recursos (Poorter i Pérez-Soba 2001). Quan el període d'exposició de la planta als alts nivells de CO_2 és curt, es dóna una estimulació de la seva assimilació per part de la RuBisco, no obstant, quan l'exposició es dóna durant un període de temps llarg, aquest efecte pot disminuir degut a una aclimatació de la fotosíntesi i a una regulació a la baixa d'aquesta (Nogués i Azcón-Bieto 2013), observant-se una disminució en la velocitat de carboxilació de la RuBisco ($V_{c,max}$) i de la taxa de transport electrònic que permet la regeneració de la Ribulosa-1,5-bisfosfat (RUBP) (J_{max}) (Ainsworth i Rogers 2007, Aljazairi *et al*. 2014). Aquesta regulació a la baixa de la fotosíntesi pot estar relacionada amb una disminució de l'activitat i de la producció de la RuBisco deguda a una reducció dels nivells de N (Luo *et al*. 2004) o bé, a una sobreproducció d'assimilats i, per tant, amb una acumulació de carbohidrats no estructurals en les fulles (van Oosten i Besford 1996). La producció de biomassa varia en funció de l'espècie ja que existeixen diferències interespecífiques en la taxa de creixement o en les taxes fotosintètiques, per tant, l'augment en la biomassa serà menor, per exemple, en espècies de creixement lent en comparació amb les espècies de creixement ràpid (Kirschbaum 2011). L'augment en la capçada degut a l'augment del CO_2 ambiental serà diferent entre espècies, podent-se arribar a donar canvis en la competència interespecífica en els boscos (Bazzaz *et al*. 1993).

El creixement també serà major en espècies amb alta disponibilitat de nutrients en comparació amb espècies que creixen en regions amb limitació de nutrients (Kirschbaum 2011). S'ha vist en molts estudis que la disponibilitat de nutrients, sobretot de N, pot modificar en gran mesura les respostes de les plantes davant d'un increment en els nivells de CO_2 atmosfèric (Reich *et al*. 2006). Per exemple, quan la planta presenta una suficient disponibilitat de N, no es donen alteracions ni en els nivells de RuBisco ni en l'increment de la fotosíntesi davant l'augment del CO_2 ambiental (Tsutsumi *et al*. 2014). Les plantes, però, acumulen una major quantitat de sucres en les seves estructures i el seu contingut en N disminueix, donant lloc a una relació C/N elevada. (Takatani *et al*. 2014). En el cas de que la planta presenti dèficit nutricional o de que el substrat sigui poc fèrtil, un increment en els nivells de CO_2 agreujarà encara més la limitació nutricional en la planta (Kirschbaum 2011).

En termes generals, moltes espècies presenten una disminució de la conductància estomàtica (g_s) en resposta als alts nivells de CO_2 ambiental (Leakey *et al*. 2009). S'han observat, per exemple, una disminució de fins el 25% de la g_s en arbres que es van desenvolupar en cambres de creixement (Curtis i Wang 1998), mentre que en experiments realitzats en FACE (enriquiment de CO_2 a l'aire lliure) van presentar una disminució entre el 16% i el 23% (Ainsworth i Rogers 2007, Leakey *et al.* 2009). Hi ha una variabilitat significativa entre grups funcionals de la resposta de la g_s a alts nivells de CO_2. Les respostes estomàtiques solen però ser menys sensibles en arbres i arbusts i que en plantes herbàcies (Nowak *et al.* 2004). Hi ha, però, excepcions a la norma general de que la g_S disminueix a altes concentracions de CO_2, tal i com s'ha vist en *Pinus taeda,* on la g_S es mostra insensible a l'increment de CO_2 (Ainsworth i Rogers 2007). La magnitud amb la que la g_S respon a l'augment de CO_2 varia en funció d'altres factors ambientals. Així, l'efecte del CO_2 elevat en la g_S és reduït durant períodes de sequera (Leakey *et al.* 2006) o quan el dèficit de pressió de vapor (VPD) és elevat (Herrick *et al.* 2004). El CO_2 elevat dóna lloc a un augment en la WUE, en part degut a l'augment de la fotosíntesi, en part degut a la disminució de la conductància estomàtica i de la transpiració (Conley *et al.* 2001).

S'ha vist que en les plantes C3, la fotosíntesi augmenta a alts nivells de CO_2 dins d'un rang ampli de temperatures, fins i tot en condicions d'estrès per altes temperatures, al contrari que en les plantes C4, que presenten un efecte negatiu en la fotosíntesi. Les altes concentracions de CO_2 ambiental poden tenir un efecte atenuant de l'estrès causat per les altes temperatures degut a que es dóna una disminució en la fotorespiració que ajuda a compensar les disminucions en F_v/F_m, activitat de la RuBisco, g_s o N foliar (Wang *et al.* 2012).

4. ESTRÈS PER ALTES CONCENTRACIONS DE METALLS

La contaminació del sòl per metalls suposa un problema mediambiental molt important degut als seus efectes negatius a curt i llarg termini en les plantes. La seva toxicitat pot donar lloc a una inhibició dels processos de creixement de les parts aèries i subterrànies de la planta, així com la disminució de l'activitat de l'aparell fotosintètic, sovint correlacionada amb un avançament en els processos de senescència (Lin *et al.* 2005). Normalment es dóna un menor desenvolupament de les arrels, que esdevenen més curtes i fines (Casella *et al.* 1988). Els metalls pesants poden afectar negativament les proteïnes, lípids i components de la membrana dels tilacoides, indispensables pel manteniment de l'activitat fotosintètica, provocant un desajust en els centres de reacció del PSII i/o en el transport electrònic (Nouari *et al.* 2006). Alguns metalls pesants poden substituir el Mg de la molècula de clorofil·la (Kowalewska *et al.* 1987) o donar lloc a canvis conformacionals en el LHCII (Ahmed i Tajmir-Riahi 1993) comportant una disminució de l'activitat fotosintètica (Linger *et al.* 2005). Aquest efectes són considerats efectes directes dels metalls pesants en l'aparell fotosintètic, mentre que alguns efectes indirectes consisteixen en alteracions en el balanç de nutrients i les seves conseqüències en el creixement de la planta (Siedlecka 1995).

4.1. La toxicitat per Zinc

El Zn és un micronutrient essencial en les plantes i pot actuar com un cofactor de regulació, funcional i/o estructural de molts enzims (Barak i Helmke 1993) com la RuBisco (Brown *et al*. 1993). L'excés de Zn pot afectar directament el creixement de la planta, les relacions hídriques, l'activitat fotosintètica i el metabolisme (Apel i Hirt 2004). La toxicitat per Zn pot afectar les relacions hídriques de moltes maneres: disminuint el transport de l'aigua des del substrat cap a les arrels i també a través d'elles (Poschenrieder i Barceló 1999), disminuint el contingut hídric (Bonnet *et al*. 2000) o la conductància estomàtica (g_s) que, a la vegada, limita les taxes de transpiració i de fotosíntesi (Sagardoy *et al*. 2010). L'excés de Zn inhibeix la fotosíntesi afectant diferents mecanismes. El Zn té un rol específic en el cicle de Calvin (Chaney 1993) i en les activitats fotosintètiques (van Assche i Clijsters 1986). Com a conseqüència de l'exposició a les altes concentracions del metall, les alteracions en el transport electrònic poden donar lloc a la formació d'espècies reactives d'oxigen (ROS) (Asada 1994).

4.2. L'ús d'espècies vegetals en la descontaminació dels sòls: la fitoremediació

En els darrers anys s'ha utilitzat la capacitat de moltes plantes per acumular altes concentracions de metalls provinents del medi de creixement per a la descontaminació de sòls i de sistemes aquàtics. Aquesta tècnica, anomenada fitoremediació, permet la disminució o la completa remobilització dels metalls del sòl i de l'aigua. Aquesta tècnica pot substituir d'altres que s'estan fent servir habitualment i que suposen un impacte més agressiu en el medi ambient, com són les excavacions, els lixiviats amb àcids, separacions físiques de l'agent contaminant o mitjançant processos electroquímics, entre d'altres (Cunningham i Ow 1996). Algunes plantes poden absorbir els metalls del sòl i concentrar-los en les parts aèries (Fitoextracció) (Kumar *et al*. 1995), poden estar associades a microorganismes que degraden els pol·lutants orgànics (Fitodegradació) (Burken i Schnoor 1997), poden absorbir els metalls a través de les arrels que tenen accés als rius contaminats (Rizofiltració) (Dushenkov *et al*. 1995), poden reduir la mobilitat i la biodisponibilitat dels pol·lutants en el medi mitjançant la immobilització o evitant-ne la seva migració (Fitoestabilització) (Vangronsveld *et al*.

1995; Smith i Bradshaw 1972), o bé poden volatilitzar els pol·lutants cap a l'atmosfera a través de la transpiració (Burken i Schnoor 1999).

És important obtenir informació sobre el potencial d'espècies llenyoses per a ésser utilitzades en la restauració d'àrees contaminades (Romeo *et al.* 2014). Les plantes acumuladores i tolerants al Zn poden ésser utilitzades per remobilitzar aquest metall del sòl o d'aigües contaminades i acumular-lo en les parts aèries, de millor accés per a la seva recol·lecció (Zhang *et al.* 2013). En aquest context, destaca la potencialitat de les Salicàcies, com per exemple els pollancres, per a ésser utilitzades en fitoremediació (Laureysens *et al.* 2004; Meers *et al.* 2007, Massacci i Passatore 2014). Els pollancres mostren una bona tolerància a altes concentracions de metalls, una alta acumulació en les seves estructures i una alta variabilitat genotípica. Els pollancres es caracteritzen per una alta variabilitat i adaptabilitat a les restriccions ambientals, a més de disposar d'un important sistema d'arrels i un baix impacte en les cadenes tròfiques. Els pollancres també tenen un creixement relativament ràpid i són àmpliament utilitzats en tasques agroforestals degut a la seva alta producció de biomassa (dos Santos Utmazian *et al.* 2007; Pietrini *et al.* 2010a; Zacchini *et al.* 2009; Massacci i Passatore 2014).

5. ESPÈCIES VEGETALS ESTUDIADES

5.1. Bedoll comú (*Betula pendula* Roth.)

És un arbre caducifoli pertanyent a la família de les Betulàcies que pot superar lleugerament els 30 m d'alçada. Presenta un tronc amb escorça de color blanc amb taques romboïdals fosques. Les fulles són romboïdals, glabres, amb la base acabada en falca i el marge doblement serrat, inserides en branques penjants i flexibles. Els branquillons de l'any posseeixen glàndules resiníferes. Les flors masculines es troben en aments penjants de color marró i les flors femenines en aments cilíndrics erectes de color verd. Els fruits són en aqueni amb dues ales laterals.

Viu en terrenys silicis de l'estatge montà però ascendeix fins l'estatge subalpí, essent un arbre pioner i colonitzador, entre els 600 i 2.200 m d'altitud. Es troba preferentment en sòls humits, en zones poc atapeïdes, més aviat assolellades i properes a cursos d'aigua. S'estén per quasi tot Europa, Sibèria i nord del Marroc. Tenen un creixement molt ràpid, poden arribar a viure 100 anys i toleren freds intensos. Es caracteritzen per tenir un tronc prim de fins a 30 metres d'alçada, l'escorça blanquinosa i una copa més o menys bombada i irregular. La fusta del bedoll és groguenca i s'ha emprat en la fabricació de caixes i esclops; les fulles tenen propietats medicinals i tintòries. Sovint es cultiva com a arbre ornamental (Moro 2002; Bolòs *et al*. 2005).

5.2. Pi negre (*Pinus uncinata* Mill.)

Arbre perennifoli amb un tronc erecte, ramificat des de la base i que pot arribar als 25 metres d'alçada, encara que pot restar retorçat per efecte del vent o la neu. La copa és cònica o piramidal, i fosca com a conseqüència del dens fullatge. Les fulles són aciculars i es mantenen a les branques al voltant de 2 a 5 anys. Les fulles són aciculars distribuïdes de dos en dos o rarament en grups de tres, de 3-8 cm, rígides, de color verd fosc, i es mantenen en les branques al voltant de 2 a 5 anys. Els estròbils són ovoideo-cònics, de 5-7 x 2-3 cm, subsèssils, brillants, amb apòfisis molt prominents, recorbades cap el peduncle, amb centre excèntric. Les llavors mesuren entre 3 i 5 mm de llargària, negroses, amb ala de 10-14 mm. No presenta preferències edàfiques. Forma boscos a l'estatge subalpí, generalment entre els 1.000 i 2.700 m d'altitud. La seva àrea s'estén pel centre i oest dels Alps, Pirineus i Sistema Ibèric. Allà on entren amb contacte amb *P. sylvestris* s'hi poden trobar exemplars híbrids (*Pinus* x *rhaetica* Brügger). Resisteix molt bé els freds intensos i pateix amb les sequeres estivals, requerint així un sòl lleugerament humit. La fusta és bona per a fusteria i un excel·lent combustible. (Moro 2002; Bolòs *et al*. 2005).

5.3. Neret (*Rhododendron ferrugineum* L.)

Arbust perennifoli glabre, pertanyent a la família de les Ericàcies. Generalment molt ramificat, d'alçada entre 1 i 1,5 m, amb fulles coriàcies i de forma el·líptica, de 2-4 cm. de llargada i de color verd viu a la cara superior i to rovellat a la cara inferior. Les flors són lleugerament zigomorfes, disposades en raïms terminals a manera d'umbel·les, de coloració rosada molt intensa. Ovari súper. Fruit en càpsula. Viu en sòls àcids dels estatges subalpí i alpí, en boscos de coníferes i matollars, des de 1.300 a 2.600 m d'altitud als costers orientats al nord. El gènere *Rhododendron* inclou més de 700 espècies cultivades com a ornamentals i entre aquestes les del gènere Azalea, que es diferencia d'aquell perquè presenta les fulles caduques (Moro 2002; Bolòs *et al*. 2005; Larcher i Wagner 2004).

5.4. Pollancre (*Populus* spp.)

El pollancre (*Populus* spp.), pertany a un gènere que comprèn 29 espècies dins la família de les Salicàcies. Són arbres caducs o semi-caducs, de tronc senzill, de creixement ràpid (el més ràpid entre les espècies de clima temperat) i amb capacitat de rebrot. Les fulles joves presenten una forma relativament convergent en totes les espècies tret de *Populus alba*, que són lobulades; les fulles madures són més robustes que les joves. Són arbres dioics, excepte algunes espècies subtropicals, i la floració s'inicia a la primavera abans de la formació de les fulles (afavorint així la pol·linització), donant lloc a la formació d'inflorescències. Les càpsules i les seves llavors, amb morfologia adaptada per ser transportades pel vent (presenten pèls que formen el cotó), maduren junt amb l'aparició de les primeres fulles. Les fulles són simples, alternes, amb estípules, peciolades, i presenten els marges dentats (Eckenwalder 1996). Presenta un sistema d'arrels de diàmetre petit, fet que els confereix dinamisme en termes de creixement en el sòl (Pregitzer i Friend 1996).

Moltes espècies de pollancre són producte d'hibridacions naturals, i tenen un paper important en la generació de nou material genètic. La capacitat d'hibridació i de reproducció vegetativa dels pollancres ha contribuït a que es desenvolupi una oferta clonal molt extensa, amb genotips que reuneixen característiques desitjables per a diferents usos, fet que afavoreix la seva expansió i facilitat de cultiu (Sixto *et al*. 2007).

Els pollancres estan àmpliament distribuïts per l'hemisferi nord, formant part de boscs de clima temperat, montà o boreal, i poden viure en sòls inundats (Pregitzer i Friend 1996). Tenen una gran capacitat d'adaptar-se a diferents condicions mediambientals, que en part s'explica per la seva gran capacitat d'hibridació (Farmer 1996), així com de recolonitzar llocs on s'ha donat una alteració del medi. On es desenvolupen millor és en sòls frescs i profunds. Es consideren espècies altament productives, amb alts requeriments lumínics i hídrics (Sixto *et al*. 2007).

En aquesta tesi s'ha treballat amb plantes adultes de quatre espècies de pollancres de les quals es van seleccionar sis clons que actualment tenen un ús important en plantacions per la producció de biomassa.

Luisa Avanzo

Populus x canadensis Mönch. (pollancre del Canadà). Les plantes d'aquest clon són de creixement ràpid, tronc erecte i força ramificat Les fulles són serrades i poc ondulades. Des del punt de vista fitopatològic L. Avanzo és susceptible a la necrosi cortical parasitària. És molt resistent al virus del mosaic del tabac i resistent a la bronzatura. Aquest clon es pot trobar a Itàlia, França i Espanya (Picco *et al*. 2007).

58-861

Populus nigra L. (Pollancre). Forma poques branques en el primer any de creixement. Es pot trobar sobretot al nord d'Itàlia. Està adaptat a les condicions fresques i humides (Facciotto i Frison 1999).

Lux

Populus deltoides Batr. (Carolina). Des del punt de vista fitopatològic el clon Lux és molt resistent a la defoliació primaveral, a la necrosi cortical parasitària (mai presenta símptomes d'aquesta malaltia), i és sensible al virus del mosaic. El clon Lux es pot trobar a Itàlia, França i Espanya (Picco *et al.* 2007).

I-214

Populus x canadensis Mönch. (pollancre del Canadà). És el clon de pollancre que més es cultiva a Itàlia. Presenta fusta erecta o lleugerament sinuosa, mitjanament ramificat. Des del punt de vista fitopatològic el clon presenta una alta resistència a la defoliació primaveral, al virus del mosaic (del qual mai en presenta símptomes); resistència suficient a la necrosi cortical. El clon I-214 es pot trovar a Itàlia, Àustria, Bèlgica, Alemània, Espanya i França (Picco *et al.* 2007).

Adige

Populus x canadensis Mönch. (pollancre del Canadà). Presenta un avançament de la senescència foliar en el mes d'agost. El tronc és erecte amb ramificacions més o menys accentuades. Des del punt de vista fitopatològic el clon és sensible a la necrosi cortical parasitària. El clon es pot trovar a Itàlia (Picco *et al.* 2007).

Eridano

Populus deltoides x *maximowiczii.* Dels sis clons estudiats aquest és l'únic mascle. Les plantes d'aquest clon presenten un tronc erecte, verd i amb poques ramificacions. Les fulles són de color verd a la cara superior i blanques a la inferior. Des del punt de vista fitopatològic el clon és molt resistent al virus del mosaic i a la necrosi cortical parasitària. El clon Eridano es pot trobar a Itàlia (Picco *et al.* 2007).

6. OBJECTIUS

L'objectiu general de la tesi consisteix en la caracterització de les respostes ecofisiològiques relacionades amb el procés fotosintètic en espècies d'interès forestal davant de diverses condicions d'estrès ambiental, considerant que aquestes esdevindran més severes en un futur com a conseqüència de l'activitat humana i que poden arribar a limitar en major grau el seu desenvolupament.

Tenint en compte que les condicions ambientals tenen efectes diferents en les plantes en funció de l'espècie, de l'hàbitat que ocupen i del tipus d'estrès, entre altres factors, els objectius concrets de la tesi foren:

1. Realitzar la caracterització ecofisiològica de tres espècies representatives de l'estatge sub-alpí: *Betula pendula* Roth (bedoll), *Rhododendron ferrugineum* L. (neret) i *Pinus uncinata* Mill. (pi negre) davant condicions ambientals associades amb el canvi climàtic com són el dèficit hídric, l'augment dels nivells de CO_2 ambiental, irradiància i d'infeccions per patògens.
2. Estudiar les respostes fisiològiques de cinc clons de pollancre: *Populus deltoides* Batr. - clon Lux, *Populus nigra* L. - clon 58-861 i *Populus* × *canadensis* Mönch. - clons Luisa Avanzo i I-214 davant condicions ambientals limitants pròpies del clima mediterrani i a les infeccions per patògens, per determinar la seva tolerància en vistes a ésser aplicats en tasques de restauració ambiental.
3. Determinar la capacitat de dos clons de pollancre: *Populus* × *canadensis* Mönch. - clon I-214 i *Populus deltoides* × *maximowiczii* - clon Eridano exposats a concentracions elevades de Zn, per acumular el metall en les seves estructures i determinar la seva tolerància a la toxicitat mitjançant la determinació de les respostes de bescanvi de gasos, fotoprotectores i antioxidants, en vistes a la seva aplicació en tasques de fitorremediació.

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La Dra**. ISABEL FLECK BOU,** com a directora de la Tesi Doctoral titulada **“Respostes fotosintètiques de diverses espècies d’interès forestal en front al dèficit hídric, concentració elevada de CO_2 i contaminació per metalls pesants**” presentada per el doctorand **JORDI FERNÀNDEZ MARTÍNEZ** informa sobre el factor d’impacte i la participació del doctorand en els articles inclosos en la memòria d’aquesta Tesi Doctoral.

Article **“Effect of environmental stress factors on ecophysiological traits and susceptibility to pathogens of five *Populus* clones throughout the growing season”** publicat a la revista Tree Physiology, índex d’impacte (2013/14) de 3.405. En aquest article, que correspon al capítol 3 de la tesi doctoral, es descriuen les respostes ecofisiològiques de cinc clons de pollancre al llarg del seu creixement durant l’estiu en condicions naturals, a fi de determinar la seva resistència a factors d’estrès biòtic i abiòtic cara a la seva utilització en programes de fitoremediació. El doctorand va participar en tots el mostrejos i anàlisi ecofisiològics tant en camp com en laboratori amb excepció de la determinació de fenols totals que es realitzà als laboratoris del IBAF-CNR (Roma). En l’apartat fitopatològic hi va també participar G. Elena, estudiant de màster de la UB, i els resultats de pigments cloroplàstics van ser avaluats conjuntament amb B. Fernàndez-Marín de la Universitat del País Vasc. J. Fernàndez va realitzar les anàlisi estadístiques i va participar activament en la redacció de l’article, demostrant una gran capacitat d’anàlisi dels resultats, constant com a primer autor.

Article “**Photosynthetic and Growth Responses of *Populus* Clones I-214 and Eridano submitted to elevated Zn concentrations”** publicat a la revista Journal of Geochemical Exploration, índex d’impacte (2013/2014) de 2.432. En quest article que correspon al capítol 4 de la tesi doctoral es descriu l’efecte de concentracions elevades de Zn sobre el procés fotosintètic, la producció de biomassa i la tolerància a aquest metall pesant en fulles de diversa edat de plàntules dels clons de pollancre I-214 i

Eridano. El doctorand va participar en el disseny dels experiments i en la determinació dels processos de bescanvi de gasos, sistema que va dominar ràpidament. També es va familiaritzar en tècniques de determinació de contingut mineral en plantes. Realitzà el tractament estadístic i participà activament amb M. Zacchini en la discussió de l'acumulació i translocació de Zn en diverses estructures de les plantes. Va redactar l'article assessorat pels altres coautors, constant com a primer autor.

Article **"Gas-exchange , photo- and antioxidant protection, and metal accumulation in I-214 and Eridano *Populus* sp. clones subjected to elevated zinc concentrations"** publicat a la revista Environmental and Experimental Botany, índex d'impacte (2013/2014) de 3.003. En aquest article es descriu l'efecte de concentracions elevades de Zn sobre l'aparell fotosintètic i els mecanismes de fotoprotecció y sistemes antioxidants de dos clons de pollancre: I-214 i Eridano. El doctorand va participar en el disseny experimental i en la organització del creixement de les plantes sota els diferents tractaments. Va realitzar les mesures de bescanvi de gasos, fluorescència de les clorofil·les, discriminació isotòpica del C, determinació de contingut mineral, característiques estructurals. Les determinacions del contingut en pigments cloroplàstics es realitzaren a la Universitat del País Basc i les de fenols totals al CNR (Roma). Va realitzar el tractament estadístic i va redactar l'article amb la participació dels altres coautors, constant com a primer autor.

I per que consti als efectes oportuns,

Dra. Isabel Fleck Bou

Barcelona, 17 de març del 2015

RESULTATS

Els resultats obtinguts han permès obtenir un major coneixement sobre les respostes d'algunes espècies vegetals davant les condicions del medi que, degut a la influència directa o indirecta de l'activitat humana, han esdevingut o esdevindran cada vegada més limitants. En aquesta tesi, el fet d'haver dut a terme estudis en espècies que creixen en estatges altitudinals diferents han permès ampliar la informació sobre l'efecte d'algunes condicions en el desenvolupament de les plantes. Aquest és el cas dels estudis ecofisiològics sobre l'efecte del dèficit hídric estival en les plantes, on s'han estudiat les respostes d'espècies de l'estatge sub-alpí i una espècie desenvolupada en el clima mediterrani. La informació que se n'ha obtingut ha permès fer una aproximació sobre les respostes d'aquestes espècies en un futur on les sequeres seran més abundants al llarg del territori.

L'interès per estudiar les respostes de diverses espècies vegetals davant condicions associades al canvi climàtic i a la pol·lució resultant de l'activitat humana, han portat a estructurar els resultats de la tesi en dos apartats: **"Caracterització ecofisiològica de les respostes de diverses espècies representatives de l'estatge sub- alpí en front el canvi climàtic"** i **"Respostes fotosintètiques, de fotoprotecció i antioxidants en clons de *Populus* sp. en front de dos tipus de situacions d'estrès: el dèficit hídric estival i concentracions elevades de Zn"**.

En el primer apartat s'han descrit les respostes del bedoll, el neret i el pi negre davant d'episodis de dèficit hídric estival propis d'aquest estatge, de condicions atmosfèriques de CO_2 elevat a curt termini, de condicions d'alta irradiància i davant d'infeccions per patògens (**capítols 1 i 2**), tenint en compte que les condicions esmentades esdevindran més severes en el futur com a conseqüència del canvi climàtic. L'estudi es realitzà gràcies al projecte "Caracterización ecofisiológica de las respuestas de distintas especies representativas del piso subalpino al cambio climático" financiat pel Ministerio de Medio Ambiente y Medio Rural y Marino (066/2010).

En el segon apartat dels resultats de la tesis s'han tractat les respostes d'una espècie adaptada al clima Mediterrani, el pollancre, en front de les condicions de dèficit hídric pròpies de l'estiu (**capítol 3**). S'ha fet un estudi comparatiu entre 5 clons per saber quins d'ells presenten les millors característiques per desenvolupar-se en aquestes condicions i quins presenten una menor susceptibilitat als patògens foliars, mostrant així major tolerància als estressos abiòtics i biòtics per així poder emprar-los en tasques de restauració ambiental, com ara la fitoremediació. Per aprofundir en aquest darrer aspecte, sobretot partint de la necessitat d'obtenir un major coneixement sobre l'impacte de la pol·lució resultat de l'activitat humana en la vegetació, s'ha determinat l'efecte de les altes concentracions de Zn en el medi de creixement en unes altres dues varietats de pollancre que ja han mostrat una bona productivitat en condicions de camp, per obtenir informació sobre el seu desenvolupament, la seva capacitat de tolerar aquestes condicions i la seva aplicabilitat en tasques de fitoremediació (**capítols 4 i 5**). Aquests resultats formen part del projecte "Cuantificación de sistemas antioxidantes en Populus spp. mediante NIRS (Near-Infrared Reflectance Spectroscopy)" financiat pel Ministerio de Educación y Ciencia (AGL2008-0244/FOR).

CARACTERITZACIÓ ECOFISIOLÒGICA DE LES RESPOSTES DE DIVERSES ESPÈCIES REPRESENTATIVES DE L'ESTATGE SUB-ALPÍ EN FRONT EL CANVI CLIMÀTIC

Capítol 1: Respostes fotosintètiques d’espècies sub-alpines davant de condicions associades al canvi climàtic

PHOTOSYNTHETIC RESPONSES OF SEVERAL SUB-ALPINE SPECIES UNDER AMBIENT CONDITIONS ASSOCIATED WITH CLIMATE CHANGE

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CLIMATE CHANGE AND SUBALPINE SPECIES PHOTOSYNTHESIS

Abstract

Here, over two consecutive summers we studied the photosynthetic responses of three species representative of a subalpine forest of the Central Catalan Pyrenees: mountain pine (*Pinus uncinata* Mill.), birch (*Betula pendula* Roth) and rhododendron (*Rhododendron ferrugineum* L.) to circumstances associated with climate change such as lower water availability, higher exposure to elevated [CO_2] and higher solar radiation incident at Earth's surface. Information about the photosynthetic process and their limitants is essential in order to predict both the capacity of these species to adapt to conditions associated with climate change and the likely changes in plant communities. Short-term high [CO_2] conditions gave rise to increased photosynthesis rates (*A*) and water use efficiency (WUE), especially in birch and mountain pine, whereas stomatal conductance (g_s) was not altered in either of these species. Birch vulnerability to drought limited their photosynthesis by stomatal closure, which in turn induced photoinhibition and earlier foliar senescence. Rhododendron was especially affected by high irradiance levels, showing early photosynthesis saturation at low values, the highest chlorophyll content, lowest gas-exchange rates and the lowest levels of photoprotection. Mountain pine showed highest *A*, photosynthetic capacity (A_{max}) and light saturated rates of net CO_2 assimilation (A_{sat}) which were maintained under decreasing water availability. Furthermore, maximum quantum yield (F_v/F_m), thermal energy dissipation, PRI and SIPI radiometric index, and ascorbate content indicated improved photoprotection with respect to the other species. Values of the maximum velocity of carboxylation of RuBisco ($V_{c\ max}$) indicated an *A* limitation by N availability in this species.

***Keywords**:* elevated CO_2; drought; birch, rhododendron; mountain pine; photosynthesis.

1. Introduction

Climate change models predict that for forest ecosystems there will be a rise from the current atmospheric CO_2 ([CO_2]) concentration of approximately 390 ppm to between 730 and 1020 ppm by 2100 (Canadell *et al*. 2007), a decline in precipitation and higher exposure to high irradiance levels (Wild *et al*. 2005, Christensen *et al*. 2007, IPCC 2014). High-elevation and high-latitude ecosystems are expected to be particularly sensitive to ongoing changes in atmospheric composition and climate change (Kostiainen *et al*. 2004, Inauen *et al*. 2012, Körner 2012, Streit *et al*. 2014). In the Pyrenees, models have projected a 10 to15% decrease in annual precipitation where the maximum decrease will occur in the summer. Furthermore, the mean annual temperature will increase by 2.8 to 4ºC with the highest increases occurring in summer and with pronounced seasonal variations (López-Moreno *et al*. 2008). To date, changes in environmental patterns have already been observed in the Central-Pyrenees. Between 1953 and 2010 a 38% decrease in precipitation and a 1.6 ºC increase in temperature during the summer were registered in Estany de Sant Maurici close to the area that was studied (data provided by the Agencia Estatal de Meteorología (AEMET)). High-mountain species will probably be forced by temperature increase to even higher altitudes (where this is possible) (Hughes 2000, Wieser and Tausz 2007, Körner 2012). In the Central Pyrenees, advances in the upper limit of the forest of up to 80 cm per year over the last century were registered (Camarero 2009). Furthermore, a drastic reduction in the potential ranges of mountain conifer species has been predicted. The geographical area of one of the studied species, *Pinus uncinata* Mill. is likely to decrease by 75%-90% by the end of the century and the mean altitude of its habitat will rise from 2,340 to 2,472 m (Benito-Garzón *et al*. 2008, Pérez *et al*. 2011). It is also expected that the population of sub-alpine bushes such as *Rhododendron ferrugineum* L., also studied in the present work, will be particularly affected, undergoing a decrease of 80%-98% in its geographical area (Pérez *et al*. 2011). Furthermore, *R. ferrugineum* facilitates the development of *P. uncinata* seedlings and the growth of both species has been correlated (Grau *et al*. 2012). This would therefore indicate that changes in the *R. ferrugineum* population could determine the distribution of *P. uncinata* in the supraforestal threshold.

The effects of elevated [CO_2] on photosynthesis will play a decisive role since vegetation can act as a net sink by fixing between 1 and 3 GT of C per year (Melillo *et al*. 1993). Trees contribute to c. 50% of this net annual uptake (Bonan 2008). Forests cover approximately 43% of the Earth’s surface and are responsible for 70% of terrestrial primary production, therefore, the study of the impact of elevated [CO_2] and other environmental limitants of photosynthesis is of particular relevance (Körner *et al*. 2005, Ryan 2013). Moreover, adaptation of forest management to climate change requires an understanding of the effects of climate on forests (Keenan 2015).

Current studies on tree photosynthesis demonstrate a wide range of responses to elevated CO_2, however, on average a 30%−55% increase in photosynthesis is typically observed (Ainsworth and Rogers 2007). While C3 photosynthesis shows a well-defined immediate increase in response to elevated [CO_2], long-term responses are far more variable and 'down-regulation' of photosynthesis (loss of photosynthetic potential) was observed in numerous experiments (Spinnler *et al*. 2002, Watanabe *et al*. 2011). In contrast, in other studies no photosynthetic down-regulation occurred (Paoletti *et al*. 2007).

Future drier climate conditions may lead to a decrease in the carbon balance as reported some studies (Ogaya *et al*. 2014). Drought provokes stomatal closure, which in turn minimizes water loss but at the same time limits CO_2 influx into plants thereby notably affecting photosynthetic processes. Moreover, it also induces a series of changes in the biochemistry of photosynthesis which involve a decline in Rubisco activity and a reduction in Rubisco protein (Bota *et al*. 2004, Flexas *et al*. 2004), impairment of ATP synthesis and photophosphorylation (Tezara *et al*. 1999) and when drought is severe, these changes lead to disruptions in the photosynthetic apparatus (Oukarroum 2009) thereby affecting productivity.

One consequence of drought-induced limitations on photosynthesis is the exposure of plants to excess energy. This, if not safely dissipated may be harmful to photosystem PSII because of over-reduction of the reaction centers and increased production of reactive oxygen species (ROS) in the chloroplasts (Smirnoff 1993) which can in turn damage the photosynthetic apparatus. The xanthophyll-cycle (Demmig-Adams and Adams 1996) and a number of enzymatic and non-enzymatic antioxidants such as ascorbate are present in chloroplasts and act to prevent ROS formation or accumulation (Asada 1999).

The aim of the study was to ascertain the photosynthetic responses of three species representative of the vegetation of a subalpine zone: rhododendron *(Rhododendron ferrugineum* L.), mountain pine (*Pinus uncinata* Mill.) and birch (*Betula pendula* Roth) in front of predicted environmental change. Mountain pine and birch are respectively an evergreen and a deciduous tree (Felicísimo 2011, Sutinen *et al*. 2002) and rhododendron is an evergreen shrub that grows in sheltered habitats (Carrillo 2006). The following questions were addressed: a) how photosynthetic related parameters will respond with a decline in water availability b) how are these parameters affected by changes in the ambient CO_2 concentration and increasing irradiation? c) what will the main photosynthetic limitant be in the studied species? We hypothesized that birch due to their deciduous and rhododendron due to their shade-tolerant characteristics would be specially affected by a decrease in annual precipitation and increased irradiance being photosynthetic responses and productivity improved in mountain pine under predicted future environmental conditions.

Information regarding how different species may respond to these future conditions within their natural emplacement will help to predict the responses of characteristic sub-alpine species to global climate change and to establish likely variations in their populations.

2. Material and methods

2.1. Study site and plant material

The study was carried out in two areas of the Aigüestortes i Estany de Sant Maurici National Park in the Central Pyrenees, Catalonia, Spain. The first area (42º 32' 58.51'' N 0º 53' 19.29'' E) is close to Estany Llebreta at an altitude of 1,617 m and the second area (42º 34' 21.32'' N 0º 56' 32.61'' E) is close to Estany Llong at an altitude of 1,985 m. The soil of the first area is composed of granitic stones derived from rockslides and herbaceous cover is scarce. The second area is also characterized by granitic soil; however, it has a shallow layer of top soil with organic matter and herbaceous cover. The species that were studied were birch (*Betula pendula* Roth.) in the first area and rhododendron (*Rhododendron ferrugineum* L.) and mountain pine (*Pinus uncinata* Mill.) in the second. The average age of the trees was approximately 130 years for mountain pine and 16 years for birch. The climate varies between atlantic and continental, where winters are long and cold with sub-zero temperatures and snow cover lasting for the most part of the year. In contrast, summers are short and are characterized by their mild temperatures and chilly nights. The mean annual temperature stands around 5.2ºC and the annual thermal range is 16ºC. Mean annual precipitation lies between 1,200 and 1,300 mm and the average monthly precipitation is greater than 100 mm (Allué 1990). The average number of days of precipitation is 134, with 53 days of these in the form of snow. The climatological characteristics of the areas studied during the period of study are shown in Table 1.

2.2. Experimental design and sampling

Measurements and sampling in the forest were performed for three weeks over two campaigns (July and September) in 2011 and 2012. Measurements were carried out on fully expanded leaves (from the previous year in mountain pine and rhododendron) with a southerly orientation and from individuals with similar levels of irradiance exposure. Leaf samples for ascorbate content determination were collected: 2 leaves per birch individual

(5 individuals in total) and 10 leaves per mountain pine and rhododendron individual (5 individuals in total for each), frozen in liquid nitrogen on site and maintained at -80ºC in a laboratory freezer. Subsequently, leaf samples were milled and lyophilized (Virtis Lyophiliser, Freezemobile 6EL, Gardiner, N.Y., USA).

2.3. Gas-exchange and chlorophyll fluorescence measurements

During both the 2011 and 2012 measurements, net photosynthesis (*A*) and stomatal conductance (g_s) were measured at the forest sites with a LI-6400 gas exchange-system (Li-COR, Lincoln, Nebraska, USA) equipped with a Light source (LI-COR 6400-02B LED). Intrinsic water use efficiency (WUE) was calculated as A/g_s. Measurements were carried out around midday (11:30am-16:00pm) (local time) and at two atmospheric CO_2 concentrations (C_a): 370 or 750 ppm. Other ambient conditions were established from those determined *in situ* in July and September. In July, the following conditions in the measuring chamber were used: air flux: 500 $\mu mol \cdot s^{-1}$, temperature: 20 - 23ºC, Vapour Pressure Deficit (VPD) 1.5 ± 0.2 KPa in 2011 and 1.8 ± 0.3 kPa in 2012; saturating photosynthetic photon flux density (PPFD) : 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for birch and 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for rhododendron and mountain pine, water molar fraction: 3 ± 1 mmol $H_2O \cdot mol\ air^{-1}$ for birch and 1 ± 0.5 mmol $H_2O \cdot mol\ air^{-1}$ for rhododendron and mountain pine in 2011, and 2 ± 1 mmol $H_2O \cdot mol\ air^{-1}$ for birch and 1.5 ± 1 mmol $H_2O \cdot mol\ air^{-1}$ for rhododendron and mountain pine in 2012.. In September, the following conditions were established: air flux: 500 $\mu mol \cdot s^{-1}$, temperature: 15-18 ºC, VPD: 1.5 ± 0.3 kPa in 2011 and 1.00 ± 0.2 kPa in 2012; saturating PPFD: 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for birch and 1,200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for rhododendron and mountain pine, water molar fraction: 1.7 ± 0.7 mmol $H_2O \cdot mol\ air^{-1}$ for all species in 2011, and 1.5 ± 1 mmol $H_2O \cdot mol\ air^{-1}$ for birch and 1.0 ± 0.5 mmol $H_2O \cdot mol\ air^{-1}$ for rhododendron and mountain pine in 2012. Measurements were carried out on 3-4 leaves located between 2 to 5 m from the base (when trees) in 4-5 individuals per species, sampling campaign and year. These conditions were also established for gas exchange and chlorophyll fluorescence response curves that were carried out for each species in July and September 2011.

From these, curves for CO_2 assimilation in response to increasing intercellular CO_2 concentration (A/C_i) and to increasing irradiance (A/PPFD) were obtained. To calculate A/C_i curves, responses at different ambient [CO_2] (C_a) (50 to 1200 $\mu l\ l^{-1}$) were carried out. Analyses of A/C_i curves throughout the study allowed for the determination of the changes in net CO_2 assimilation at saturating C_i (A_{max}), maximum carboxylation velocity of

Rubisco ($V_{c,max}$), maximum potential rate of electron transport contributing to RuBP regeneration (J_{max}) and stomatal limitation (l) to light saturating *A* conditions for the different leaves. Estimations of $V_{c,max}$ and J_{max} were made by fitting a maximum likelihood regression below and above the inflexion of the A/C_i response using the method as described by McMurtrie and Wang (1993). Stomatal limitation (l) which is the decrease in light-saturated net CO_2 assimilation attributable to stomata was calculated as in Farquhar and Sharkey (1982).

To perform A/PPFD curves, C_a was set at 370 and/or 750 ppm and the range of PPFD was set from 0 to 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Analyses of A/PPFD curves allowed for the determination of the changes in apparent quantum yield of CO_2 fixation (Φ_{CO2}, initial slope and equivalent to the ratio of a given light-dependent product to the number of absorbed photons), the light-saturated rate of net CO_2 assimilation at ambient CO_2 (A_{sat}), the light compensation point (PC_{light}) and photosynthesis saturation irradiance ($PPFD_{sat}$). Curves (g_s/C_a) for stomatal conductance in response to increasing C_a levels (50 to 1200 ppm) were also determined. Curves were performed on 4-5 individuals per species and campaign.

Steady-state modulated chlorophyll fluorescence was determined simultaneously with gas exchange measurements using a leaf fluorescence chamber (LFC) coupled to the Li-Cor 6400 system at ambient (370 ppm) or elevated CO_2 (750 ppm) and at an *A* saturating PPFD of 1500 $\mu E \cdot m^{-2} \cdot s^{-1}$ for birch and 1200 $\mu E \cdot m^{-2} \cdot s^{-1}$ for mountain pine and rhododendron. Light-adapted components of chlorophyll fluorescence (steady-state yield (F), maximum fluorescence yield (F'_m) and quantum yield of PSII photochemistry (Φ_{PSII} equivalent to $(F'_m\text{-}F)/F'_m$)) (Genty *et al.* 1989) were measured. qP, photochemical quenching of chlorophyll fluorescence (equivalent to $(F'_m\text{-}F)/(F'_m\text{-}F'_o)$ and F'_v/F'_m intrinsic efficiency of open PSII centers during illumination (equivalent to $(F'_m\text{-}F'_o)/F'_m$)) were estimated according to Oxborough and Baker (1997). After dark adaptation of leaves (30 minutes for birch, 40 minutes for rhododendron and 45 minutes for mountain pine, where it was previously established that these timings provided sufficient adaptation time to allow for the complete oxidation of PSII reaction centers) with Dark leaf clips (DLC-8) (Walz, Effeltricht, Germany), F_o (minimum fluorescence yield), F_m (maximum fluorescence yield) and F_v/F_m (maximum quantum yield of PSII photochemistry (equivalent to $(F_m\text{-}F_o)/F_m$)) were obtained with a modulated fluorimeter Mini-PAM Walz (Walz, Effeltricht, Germany). Chlorophyll fluorescence was determined in 3 leaves per individual for 4-5 individuals per species, campaign and year.

Curves for chlorophyll fluorescence in response to increasing irradiance were performed at 370 and 750 ppm C_a with a PPFD of 0 to 1500 μmol·m^{-2}·s^{-1}. The analysis of these curves allowed for the determination of changes in Φ_{PSII}, qP, (1 - F'_v/F'_m) and ETR. (1 - F'_v/F'_m) is related to the efficiency of open reaction centers and excess excitation energy dissipation as heat involving the xanthophyll cycle (Demmig-Adams and Adams 1996). The electron transport rate (ETR) was determined according to Krall and Edwards (1992), where ETR = Φ_{PSII} X PPFD X 0.5 X 0.84, 0.5 is a factor that assumes equal distribution of energy between the two photosystems and 0.84 is considered to be the most common leaf absorbance for C3 plants (Björkman and Demmig 1987).

2.4. Total chlorophyll content

Total chlorophyll content was measured with a leaf chlorophyll meter (SPAD-502, Minolta, Osaka, Japan) on at least 5 leaves at two orientations (North and South) and on 5 individuals per species in July and September 2011.

2.5. Leaf reflectance measurements and index

Leaf reflectance was measured on 3 leaves per 5 individuals of each species with a portable spectral analysis system (USB4000, Oceanoptics) operated with Spectrasuite (Oceanoptics) software. Ten scans per sample were integrated (integration time 50 ms). The photochemical reflectance index (PRI) and the Structure-independent pigment index (SIPI) were derived from the spectra. PRI, a method to remotely assess photosynthetic efficiency using narrow-band reflectance was calculated as $[(R_{531}-R_{570})/(R_{531}+R_{570})]$, where R_n is the reflectance at n nm (Peñuelas *et al*. 1995). SIPI is connected to the Carotenes/Chlorophyll *a* relationship (Peñuelas and Filella 1998) and was calculated as $[(R_{800}-R_{445})/(R_{800}-R_{680})]$.

2.6. Ascorbate content

15 mg of lyophilized leaf samples were extracted with metaphosphoric acid (6% (w/v)) and centrifuged at 10,000g at 4ºC. Ascorbate (AscA) content was determined according to Foyer *et al*. (1983) and measured by the change in A_{265} using a spectrophotometer (Cecil Aquarius CE7400).

2.7. Statistical analysis

All statistical procedures were performed using SPSS for Windows (v. 15.0, SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to test the main effects on the measured parameters against appropriate error terms, species, campaign (July and September), CO_2 effect and year. A multiple comparison test of the means was carried out using the Duncan post-hoc test. Statistical significance was set at $p \leq 0.05$.

3. Results and Discussion

3.1. Gas exchange responses to increased CO_2 concentration and PPFD

Gas-exchange results showed that at elevated [CO_2] (750 ppm) there were higher net photosynthesis rates (*A*) in the three species studied in both of the summers of 2011 and 2012 when compared to ambient [CO_2] (370 ppm). This was especially notable in birch and mountain pine where rates were practically double (Fig. 1). This short-term effect of [CO_2] is attributed to an increase of CO_2 in the chloroplasts and to a reduction of photorespiration (Drake *et al*. 1997).

The summer of 2011 was characterized by scarce precipitations, mainly towards the end of the season, whereas in 2012 rainfall was more abundant (Table 1). The expected reduction in water availability associated to climate change would likely benefit differently the selected species. In fact, at ambient CO_2, a decrease in *A* was observed in birch in late summer (September 2011) (Fig.1) as a consequence of a decline in water availability, whereas mountain pine showed the highest *A* and maintained these rates throughout the season. In summer 2012, *A* did not decline in September in any species owing to a greater availability of water in comparison to the summer of 2011.

Birch presented lower *A* rates in late summer than in early summer (July) 2011, at elevated [CO_2], demonstrating that, despite higher CO_2 availability, photosynthesis was limited by water deficit conditions. In fact, stomatal closure occurred in birch in late summer 2011 as highlighted by the decline in g_s, while in summer 2012 no decrease in g_s occurred in any species (Fig. 1 and 2). Results indicate that there was a stomatal limitation of *A* in birch at both [CO_2].

Rhododendron showed gas-exchange rates in accordance with those reported (Larcher and Wagner 2004). *A* values were lower than for birch and mountain pine at both [CO_2], in part owing to its condition as a shade-adapted plant which has inherently low photosynthetic rates and lacks the ability to effectively increase light-saturated photosynthesis (Björkman 1981). Rhododendron also showed the lowest g_s in both years. The observed values around 50 $mmol \cdot m^{-2} \cdot s^{-1}$ are indicative of severe water stress (Flexas *et al*. 2002).

In our study, none of the species studied showed any variation of g_s with increasing [CO_2] (Fig.2). A meta-analysis of FACE studies reported that all functional groups respond to long-term exposure to elevated CO_2 by reducing g_s, but reductions were slightly lower for trees (Ainsworth and Rogers 2007). *Pinus taeda* guard cells have been shown to be unresponsive to elevated CO_2 in porometry studies (Ellsworth *et al*. 2011) and the same was reported for deciduous forest trees after 8 years of elevated [CO_2] (Bader *et al*. 2010, Streit *et al*. 2014). In field-grown trees, long-term exposure to elevated CO_2 in conifers resulted in small or no significant decreases in g_s, in contrast to woody deciduous and herbaceous plants even more marked under drought (Medlyn *et al*. 2001). In general, species with low g_s such as rhododendron are less affected by elevated CO_2 (Morison 1985). At elevated [CO_2], an increase in water use efficiency (WUE) was observed in all species in both years as a consequence of enhanced *A* and constant g_s (Fig.1). In general, the lowest WUE at elevated [CO_2] was observed for rhododendron.

Results of the A/C_i response curves obtained in summer 2011 showed a higher photosynthetic capacity (A_{max}) for birch and mountain pine with respect to rhododendron, especially in early summer when stomatal limitation of photosynthesis (l) (Fig. 3) was at its lowest because of higher water availability. Also, A_{max} declined in both birch and mountain pine in late summer. In birch, this was likely to be due to stomatal limitation as demonstrated by the decline in both l and g_s. In mountain pine, this was related to a decrease in the maximum velocity of carboxylation of Rubisco ($V_{c,max}$) (Fig. 3) indicative of a reduction in active Rubisco and therefore of nitrogen content (Jach and Ceulemans 2000) which was likely to have been translocated to younger leaves by the end of the summer. In rhododendron, lower A_{max} and $V_{c,max}$ is related to its adaptation to shaded habitats.

Results of the A/PPFD response curves (Fig.4) also showed the susceptibility of birch to water deficit during the drier summer of 2011 with a decline in Φ_{CO2} and A_{sat} at ambient and elevated [CO_2] whereas they did not decrease in either mountain pine or rhododendron. Mountain pine showed the highest Φ_{CO2} indicating improved efficiency of the utilization of photochemically-derived energy for CO_2 fixation. At elevated [CO_2], Φ_{CO2} did not increase in any species, although, increasing the atmospheric CO_2/O_2 ratio allowed for a greater proportion of absorbed light energy to be utilized for net carbon fixation. This could potentially lead to the alteration of the A/PPFD relationship (Long and Drake 1991). In birch, A_{sat} and $PPFD_{sat}$ increased in late summer at high [CO_2] with respect to ambient [CO_2] (Fig. 4) thus, this shows that high [CO_2] contributed to reduce the negative effect of drought on photosynthesis. Of the three species, rhododendron showed saturation of photosynthesis at the lowest PPFD and lowest PC light (Fig. 4), this being due to its adaptation to shade. Moreover, the observed increase in A_{sat} at high [CO_2] in early summer indicated that short-term elevated [CO_2] may facilitate the ability of these shade-tolerant plants to compete for light and improve their carbon balance (Pearcy and Björkman 1983).

Rhododendron had the highest total chlorophyll content (Fig. 5) in both campaigns, this being associated with its adaptation to low irradiances (Lichtenthaler *et al*. 2007). This species invests more nitrogen in light-capturing systems rather than in RuBisco (Evans and Poorter 2001) and as a consequence, it showed lower photosynthesis and light saturation irradiance as previously discussed.

3.2. Photochemical characteristics

The photosynthetic apparatus is particularly susceptible to suboptimal growth conditions such as low water availability (Zhou *et al*. 2007, Tezara *et al*. 2008, Peña-Rojas *et al*. 2004), low nitrogen status (Hymus *et al*. 2001), or greater than saturating light doses (Albert *et al*. 2011). Photochemistry was especially affected in birch under reduced water availability in late summer at ambient [CO_2] (Table 2), a decrease in F_v/F_m occurred while mountain pine showed F_v/F_m values of around 0.83 reflecting non-stressful conditions (Björkman and Demmig, 1987) and highest Φ_{PSII} and qP in the drier summer of 2011 (Table 2). Photoinhibition was observed in rhododendron in 2012 as a result of the scarce precipitations over 20 consecutive days in July (0.5mm) inducing foliar senescence (Warren *et al*. 2014).

Thermal dissipation was highest in mountain pine and rhododendron (Table 2) under decreased water availability in late summer 2011 as pointed the values of F'_v/F'_m (open PSII reaction centers efficiency). They are inversely related to excess energy dissipation in the form of heat by means of the xanthophyll cycle and contribute to the photoprotection of the photosynthetic apparatus (Demmig-Adams and Adams 1996).

The responses of chlorophyll fluorescence parameters to increasing irradiance (Fig. 6) were determined in summer 2011 and provided information on the light intensities that gave rise to limitations in photochemistry at ambient and elevated [CO_2]. Mountain pine did not show any variation in any fluorescence parameter with increasing PPFD. Birch showed a statistically significant decrease at ambient [CO_2] in late summer in Φ_{PSII} and ETR at high light intensities (above 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD) whereas in rhododendron it occurred at lower PPFD (around 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Results also support the notable limitation of photochemistry in rhododendron due to excess light exacerbated by lower water availability in September. The possible future loss of snow cover in winter due to increases in temperatures (Larcher and Siegwolf 1985) would expose rhododendron to high light intensities that could be damaging and give rise to photoinhibition (Adams *et al*. 1995).

Short term high [CO_2] did not generate negative effects on photochemistry, showing all species similar trends in the fluorescence parameters with increasing PPFD to those at ambient [CO_2] (Fig. 6). Variable effects on the efficiency of photochemistry under long-term exposure to elevated [CO_2] have been reported (Taub *et al*. 2000, Hymus *et al*. 2001, Martínez-Carrasco *et al*. 2005, Gutiérrez *et al*. 2009).

3.3. Photoprotective responses

The observed differences in photoprotection were related to the CO_2 assimilation capacity of the studied species. Rhododendron, with low photosynthetic activity, showed the lowest photochemical reflectance index (PRI) index values in July 2012 with respect to the other species, especially at midday (Fig. 7), therefore indicating a high degree of participation of the xanthophyll cycle in the dissipation of excess energy not used in photosynthesis (Peñuelas *et al*. 1995) in accordance with results for F'_v/F'_m as previously mentioned.

Among the hydrophilic antioxidants, ascorbate (AscA) is quantitatively the most abundant of these and is localized in the main cell compartments of plant tissues (Gill and Tuteja 2010). It is one of the most important intracellular defenses against ROS-induced oxidative damage interacting with H_2O_2 in particular. AscA was found entirely in its reduced form during the summers of both years (Fig. 8). In both years, its levels were highest in mountain pine and lowest in birch. In mountain pine, high AscA levels, and low F'_v/F'_m values indicate a significant contribution of the different photoprotective mechanisms to maintaining a suitable F_v/F_m throughout summer drought. SIPI index values reflect the carotene and xanthophyll content with respect to chlorophyll in tissues. The highest index, which was observed in mountain pine, indicated that it had a higher level of those pigments that contribute to energy dissipation as heat and therefore a higher photoprotective capacity (Fig. 7). In contrast, birch had the lowest values of AscA and SIPI. This was connected to its deciduous physiology which invests less in photoprotection in non-permanent structures. The decrease in AscA and SIPI observed in rhododendron in late summer 2011 marked the onset of oxidative stress due to excess irradiance and drought.

4. Conclusions

Short-term exposure to high [CO_2] improved the photosynthetic rates, maintained stomatal conductance and increased WUE of the three species, especially that of *P. uncinata* followed by *B. pendula*, a fact that could compensate for episodes of low water availability.

P. uncinata showed the highest photosynthetic rates and photoprotective capacity, where Rubisco and therefore N availability are in all probability the main photosynthesis limitants at the end of the summer. Current summer water availability in their natural emplacement did not alter CO_2 assimilation, photochemistry and water relations in this species.

B. pendula might be especially affected by scarce precipitations. Stomatal closure decreased CO_2 assimilation and electron transport which caused the efficiency of the photosynthetic apparatus to be low and increased susceptibility to photoinhibition, thus leading to earlier foliar senescence.

R. ferrugineum photosynthesis can be especially limited by high light intensities, where it becomes saturated at low PPFD and shows decreases in the efficiency of PSII reaction centers. As a consequence, this leads to increased susceptibility to photoinhibition. Furthermore, oxidative damage can be exacerbated by stomatal closure during summer drought.

As hypothesized, it was seen that mountain pine would adapt best to changes brought about by a future climate change scenario, provided that it were able to avail of an adequate supply of nitrogen. To the contrary, this would act as a limiting factor in terms of adaptability.

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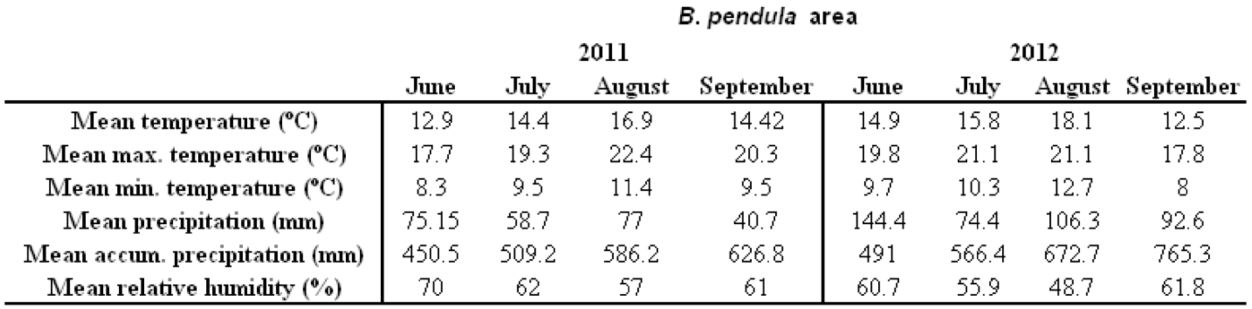
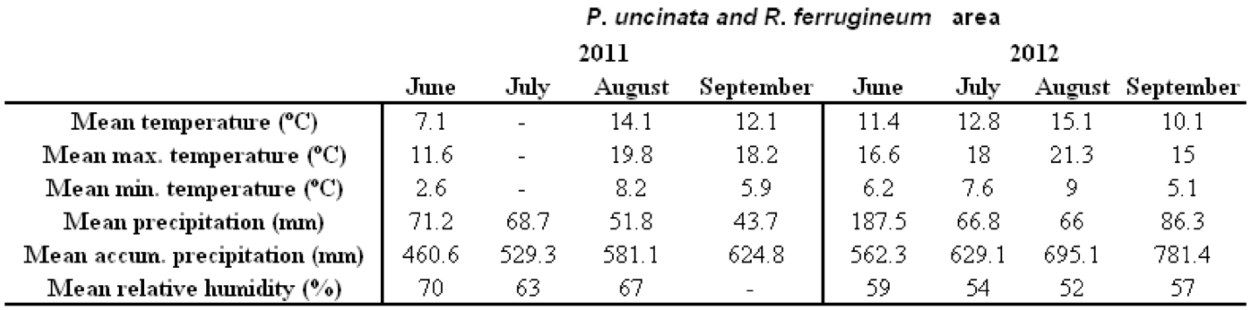
Suplemental material

Table 1. Climatic characteristics during the studied period.



B. pendula area

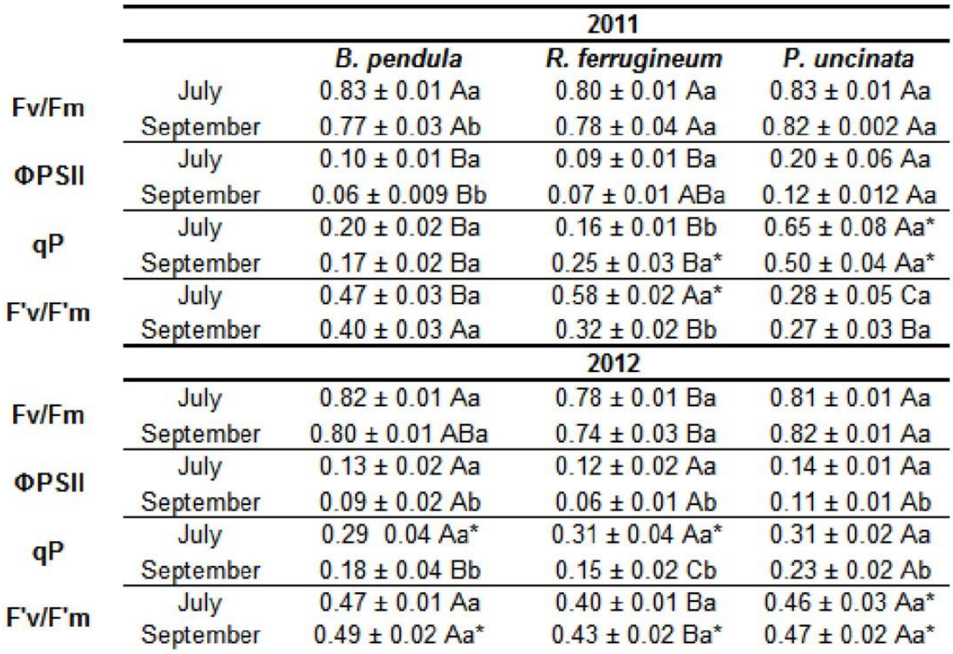
	2011				2012			
	June	July	August	September	June	July	August	September
Mean temperature (ºC)	12.9	14.4	16.9	14.42	14.9	15.8	18.1	12.5
Mean max. temperature (ºC)	17.7	19.3	22.4	20.3	19.8	21.1	21.1	17.8
Mean min. temperature (ºC)	8.3	9.5	11.4	9.5	9.7	10.3	12.7	8
Mean precipitation (mm)	75.15	58.7	77	40.7	144.4	74.4	106.3	92.6
Mean accum. precipitation (mm)	450.5	509.2	586.2	626.8	491	566.4	672.7	765.3
Mean relative humidity (%)	70	62	57	61	60.7	55.9	48.7	61.8



P. uncinata and R. ferrugineum area

	2011				2012			
	June	July	August	September	June	July	August	September
Mean temperature (ºC)	7.1	-	14.1	12.1	11.4	12.8	15.1	10.1
Mean max. temperature (ºC)	11.6	-	19.8	18.2	16.6	18	21.3	15
Mean min. temperature (ºC)	2.6	-	8.2	5.9	6.2	7.6	9	5.1
Mean precipitation (mm)	71.2	68.7	51.8	43.7	187.5	66.8	66	86.3
Mean accum. precipitation (mm)	460.6	529.3	581.1	624.8	562.3	629.1	695.1	781.4
Mean relative humidity (%)	70	63	67	-	59	54	52	57

Table 2. Chlorophyll fluorescence parameters at 370 ppm [CO_2]: maximum quantum yield (F_v/F_m), PSII quantum yield (Φ_{PSII}), photochemical quenching (qP) and efficiency of the PSII open reaction centers (F'_v/F'_m) of birch, rhododendron and mountain pine leaves. Significant differences ($p \leq 0.05$) are expressed as A, B, C (differences between species); a, b, c (between July and September) or with an asterisk (between years).



		2011		
		B. pendula	***R. ferrugineum***	***P. uncinata***
Fv/Fm	July	0.83 ± 0.01 Aa	0.80 ± 0.01 Aa	0.83 ± 0.01 Aa
	September	0.77 ± 0.03 Ab	0.78 ± 0.04 Aa	0.82 ± 0.002 Aa
ΦPSII	July	0.10 ± 0.01 Ba	0.09 ± 0.01 Ba	0.20 ± 0.06 Aa
	September	0.06 ± 0.009 Bb	0.07 ± 0.01 ABa	0.12 ± 0.012 Aa
qP	July	0.20 ± 0.02 Ba	0.16 ± 0.01 Bb	0.65 ± 0.08 Aa*
	September	0.17 ± 0.02 Ba	0.25 ± 0.03 Ba*	0.50 ± 0.04 Aa*
F'v/F'm	July	0.47 ± 0.03 Ba	0.58 ± 0.02 Aa*	0.28 ± 0.05 Ca
	September	0.40 ± 0.03 Aa	0.32 ± 0.02 Bb	0.27 ± 0.03 Ba
		2012		
Fv/Fm	July	0.82 ± 0.01 Aa	0.78 ± 0.01 Ba	0.81 ± 0.01 Aa
	September	0.80 ± 0.01 ABa	0.74 ± 0.03 Ba	0.82 ± 0.01 Aa
ΦPSII	July	0.13 ± 0.02 Aa	0.12 ± 0.02 Aa	0.14 ± 0.01 Aa
	September	0.09 ± 0.02 Ab	0.06 ± 0.01 Ab	0.11 ± 0.01 Ab
qP	July	0.29 0.04 Aa*	0.31 ± 0.04 Aa*	0.31 ± 0.02 Aa
	September	0.18 ± 0.04 Bb	0.15 ± 0.02 Cb	0.23 ± 0.02 Ab
F'v/F'm	July	0.47 ± 0.01 Aa	0.40 ± 0.01 Ba	0.46 ± 0.03 Aa*
	September	0.49 ± 0.02 Aa*	0.43 ± 0.02 Ba*	0.47 ± 0.02 Aa*

Figure 1. Net Photosynthesis (*A*), stomatal conductance (g_s) and water use efficiency (WUE) of birch, rhododendron and mountain pine leaves. Significant differences ($p \leq 0.05$) are expressed as A, B, C (differences between species); a, b, c (between July and September); α, β (between CO_2 concentration); or with an asterisk (between years).

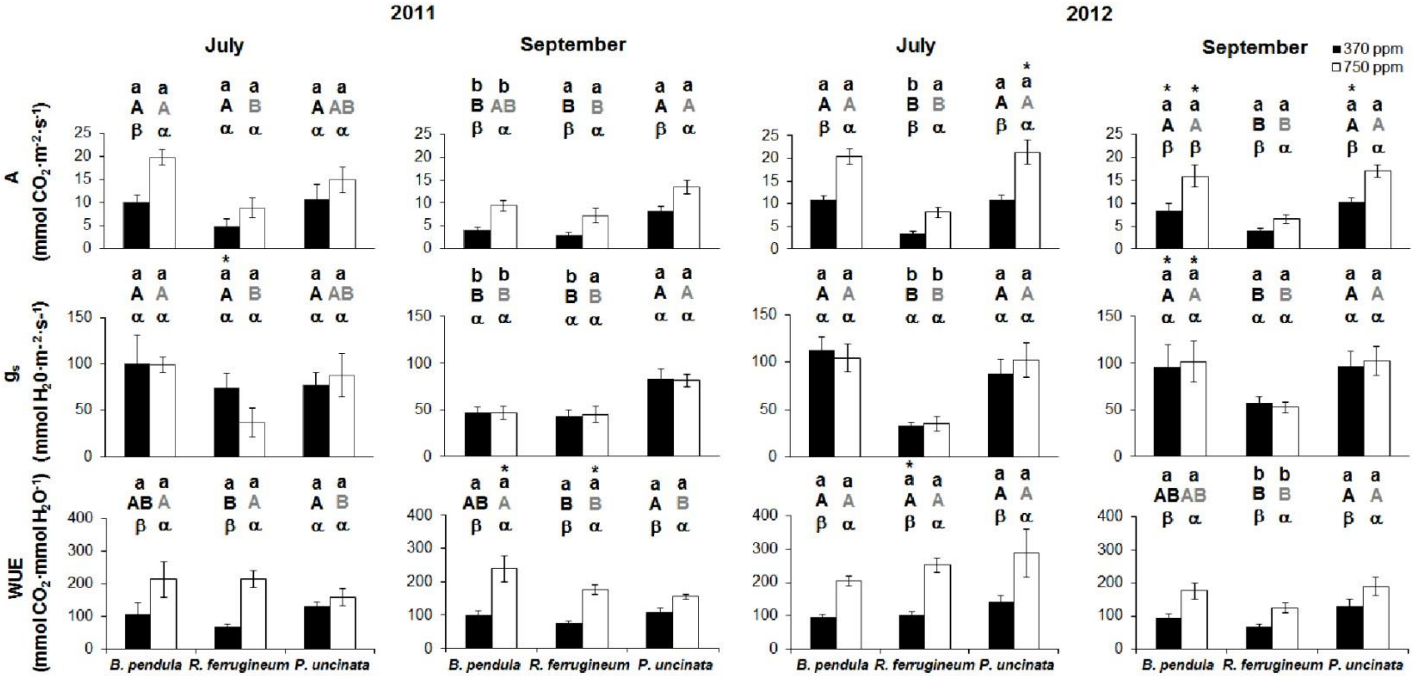


Figure 2. Stomatal conductance (g_s) responses to increased ambient CO_2 of birch, rhododendron and mountain pine leaves measured in 2011.

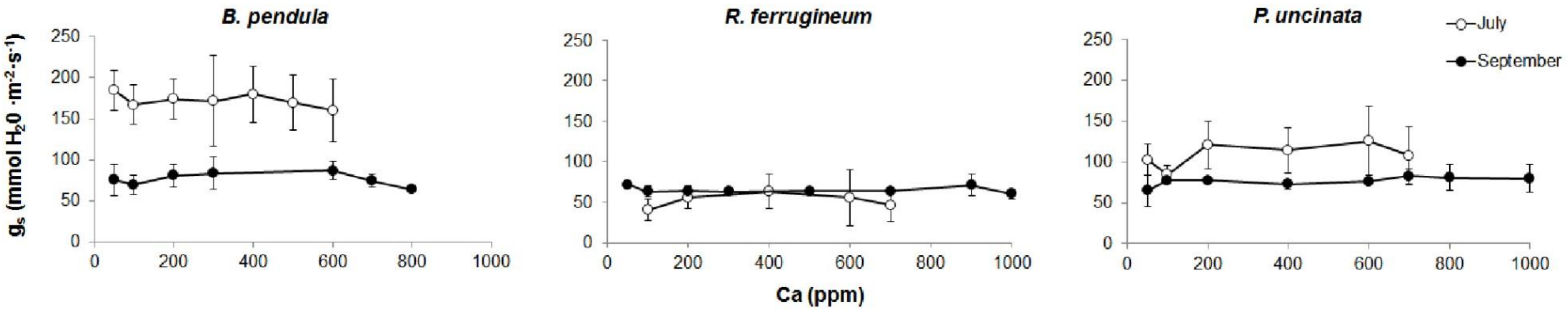


Figure 3. Maximum photosynthetic rate (A_{max}), maximum velocity of RuBP carboxylation by Rubisco ($V_{c,max}$), stomatal limitation (l) and maximum capacity of RuBP regeneration (J_{max}) of birch, rhododendron and mountain pine leaves measured in 2011. Significant differences ($p \leq 0.05$) are expressed as A, B, C (differences between species) and a, b, c (between July and September).

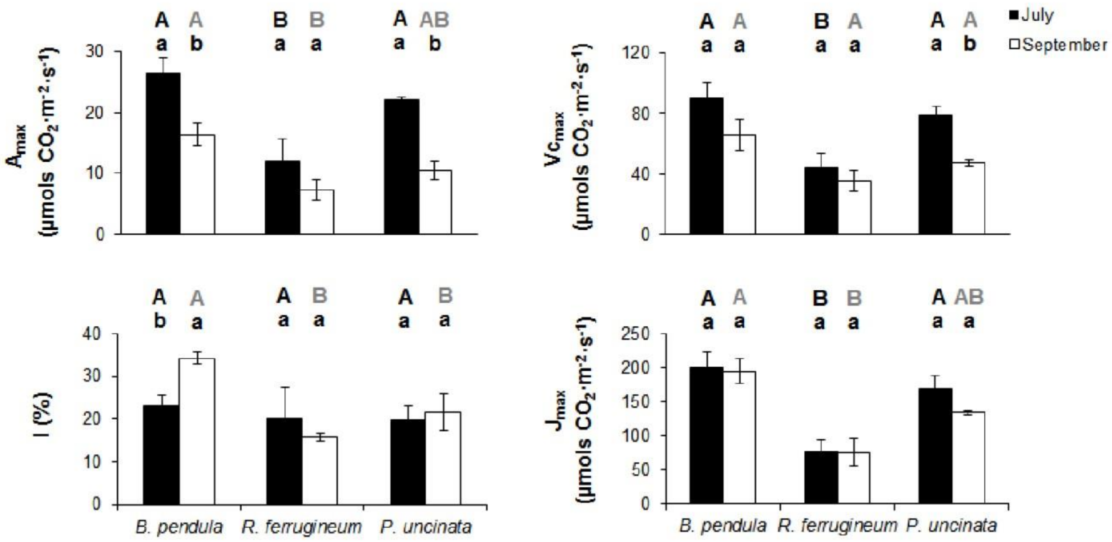


Figure 4. Apparent quantum yield of CO_2 (Φ_{CO2}), light compensation point (PClight), photosynthetic rate at light saturation (A_{sat}) and saturating photosynthetic photon flux density ($PPFD_{sat}$) of birch, rhododendron and mountain pine leaves measured in 2011. Significant differences ($p \leq 0.05$) are expressed as A, B, C (differences between species); a, b, c (between July and September); and α, β (between CO_2 concentration).

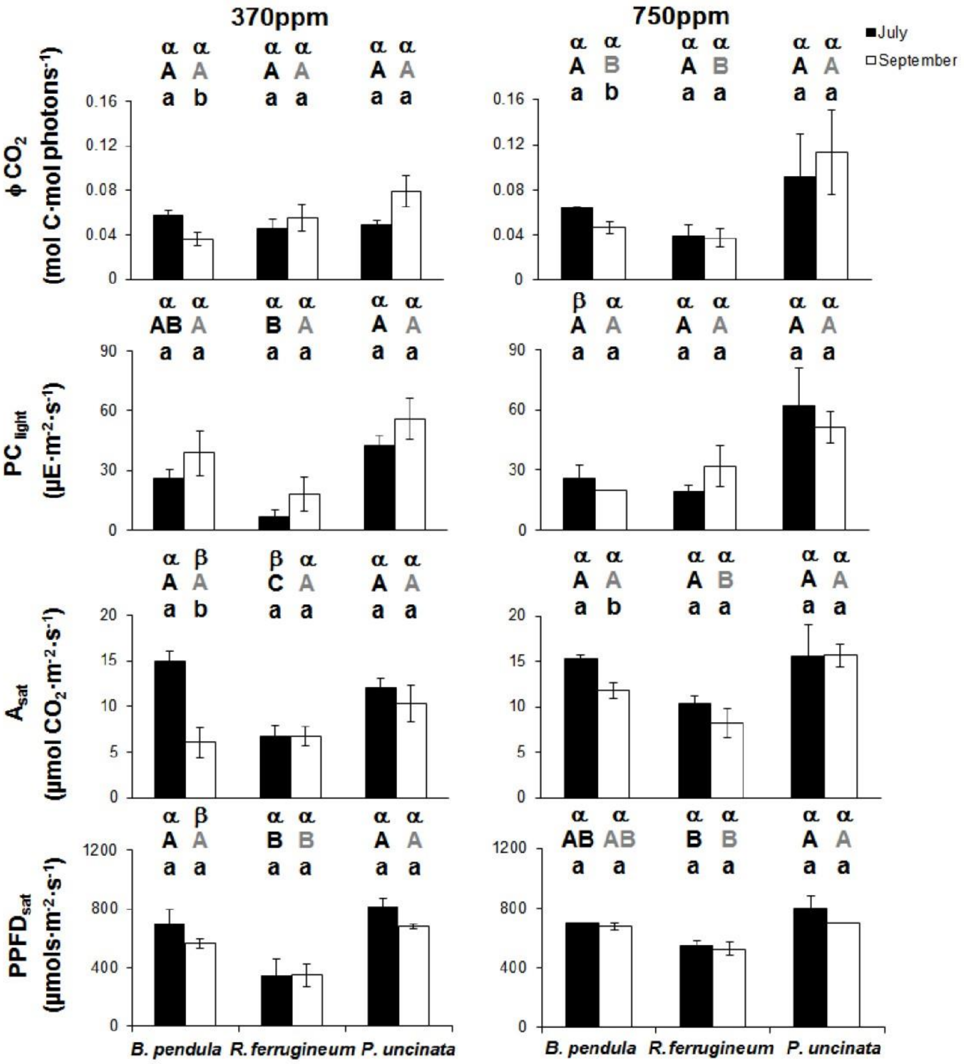


Figure 5. Total chlorophyll content of birch, rhododendron and mountain pine leaves measured in 2011. Significant differences ($p \leq 0.05$) are expressed as A, B, C (differences between species) and a, b, c (between July and September).

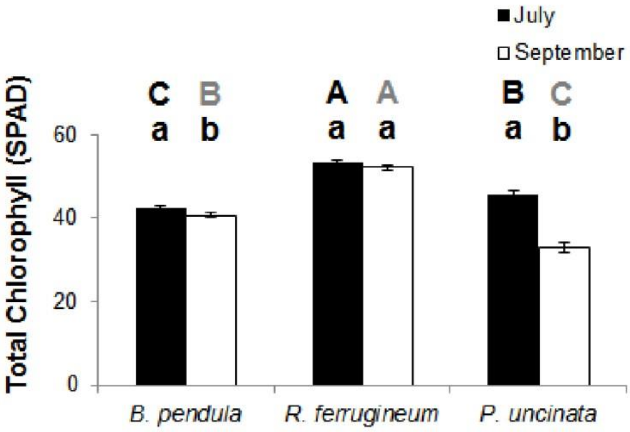


Figure 6. Responses of PSII quantum yield (Φ_{PSII}), photochemical quenching (qP), efficiency of PSII open reaction centers related to thermal dissipation (1 - F'_v/F'_m) and electron transport rate (ETR) to photosynthetic photon flux density (PPFD) in birch, rhododendron and mountain pine leaves measured in 2011.

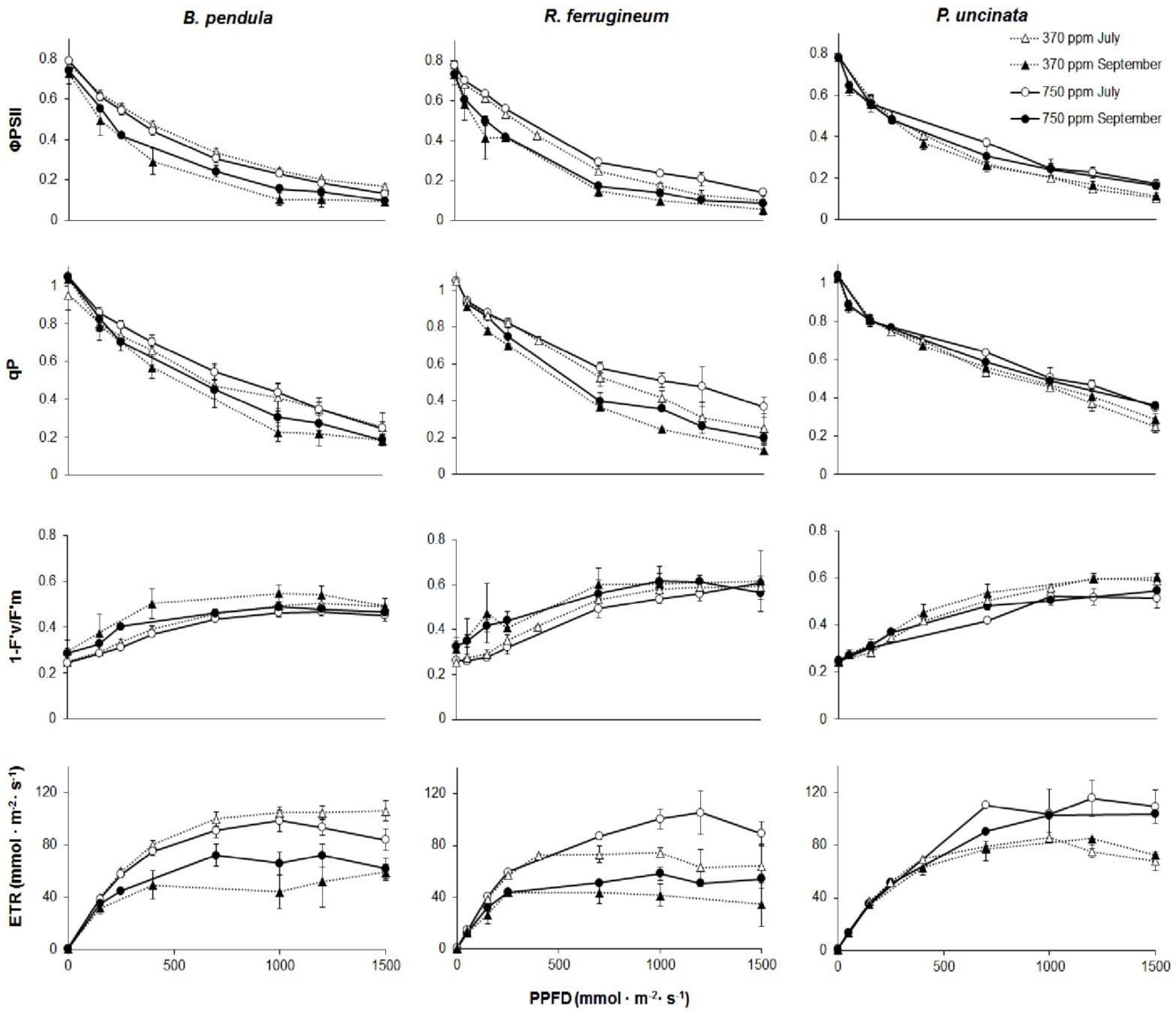


Figure 7. PRI and SIPI radiometric index values for birch, rhododendron and mountain pine leaves measured in 2012 (only in July for PRI). Significant differences ($p \leq 0.05$) are expressed as A, B, C (differences between species) and a, b, c (between times of day for PRI; between July and September for SIPI).

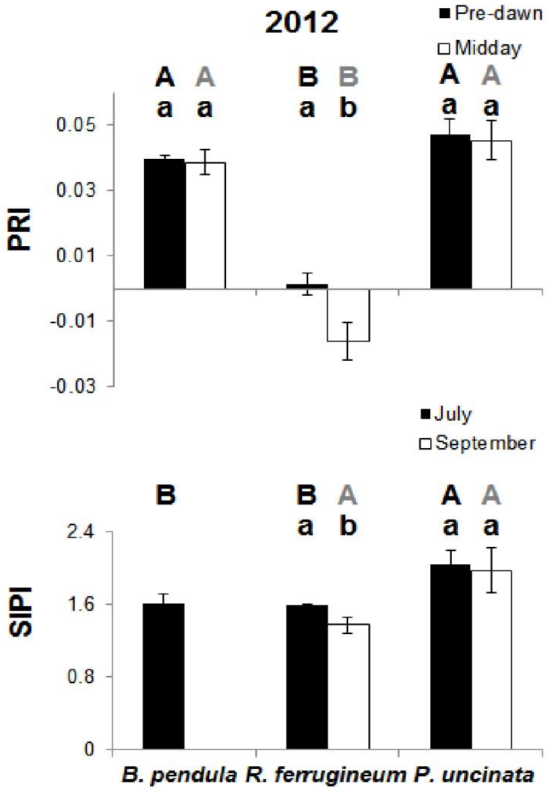
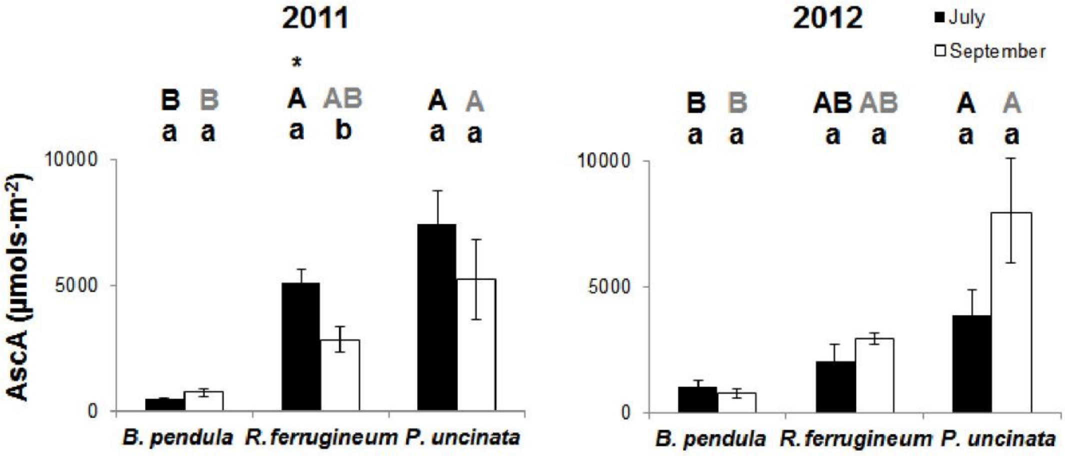


Figure 8. Reduced ascorbate (AscA) content of birch, rhododendron and mountain pine leaves. Significant differences ($p \leq 0.05$) are expressed as A, B, C (differences between species), a, b, c (between July and September) or with an asterisk (between years).



Capítol 2: Déficit hídric estacional en el bosc sub-alpí: efectes en el bescanvi de gasos, composició isotòpica del carboni, contingut mineral i susceptibilitat a la infecció per patògens en diferents espècies

SEASONAL WATER DEFICIT IN A SUB-ALPINE FOREST: EFFECTS ON GAS EXCHANGE, CARBON ISOTOPE COMPOSITION, MINERAL CONTENT AND SUSCEPTIBILITY TO PATHOGEN INFECTION OF SEVERAL SPECIES

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WATER DEFICIT RESPONSES OF SUBALPINE SPECIES

Abstract

According to predictions for global climate change, annual rainfall distribution in Western Europe will undergo alterations and there will be increased number of episodes of drought where high mountain systems will be particularly vulnerable. To ascertain how high mountain forests will cope with a future decline in water availability and the associated increase in the rate of infections, a study was conducted on three species which are representative of a subalpine forest: birch (*Betula pendula* Roth.), rhododendron (*Rhododendron ferrugineum* L.) and mountain pine (*Pinus uncinata* Mill.) over the course of two consecutive summers. Birch was especially susceptible to long-term low water availability showing a notable decline in net photosynthesis (*A*) and stomatal conductance (g_s), which lead to photoinhibition and to early leaf senescence as was demonstrated in values obtained for photosynthetic nitrogen use efficiency (PNUE), C/N ratio and foliar N. Stomatal limitation of photosynthesis was confirmed from results obtained from gas-exchange and ^{13}C isotope discrimination (Δ^{13}C). Birch was the most susceptible to rust infection but the effect on photochemistry was more notable in rhododendron as was indicated by maximum quantum yield (F_v/F_m) and chlorophyll values. Rhododendron showed signs of sensitivity to both long-term drought and in particular, to short periods of low water availability (days to weeks) that negatively affected gas-exchange and photochemistry. The observed deficit of P, N and Fe contributed to the photosynthetic impairment. Mountain pine was the most tolerant species since alterations in gas exchange, water relations and photoinhibition were not observed during periods of low water availability. In part, this can be attributed to the obtained leaf structural characteristics. Mountain pine showed the highest investment of N in soluble protein (Rubisco) and the highest K, Fe and Mg content, which in turn contributed to the highest *A* rates.

Key words: birch, drought, nutrients, rust, pine, rhododendron, structural parameters.

Introduction

The Earth’s climate has been altered by greenhouse emmissions and this will continue into the forseeable future. Forest ecosystems will be affected as a result of changes expected to take place in climate variables and there is a greater risk of extreme weather events such as prolonged drought, floods and heat waves. Forests are not only essential for the maintenance of terrestrial biodiversity, but also act as net carbon sinks mitigating the impact of the forecast increases in atmospheric CO_2 concentration (IPCC 2014). As highlighted by Lindner *et al*. (2010), the long life span of trees makes European forests especially vulnerable and in particular those at high mountain ranges (see Theurillat *et al*. 2001). A large variability in structural and functional traits and responses to seasonal water deficit has been observed, and this may be crucial for the coexistence of species and biodiversity (Ackerly 2004).

Furthermore, research into plant responses to water stress is important as most climate-change scenarios suggest an increase in aridity in many areas of the globe (Petit *et al*. 1999). In addition to the expected changes in precipitation patterns with rains of greater intensity but reduced frequency, snow will become less present in many areas and earlier runoff dates will affect soil moisture (see review by Anderegg *et al*. 2013). In a future warming environment, tree water loss will also increase with soil warming (Wieser *et al*. 2015).To add to this, future climate models indicate that for alpine forests the total precipitation for the April–September period could fall on average by as much as 20 to 30% by the end of the 21st century (Houghton *et al*. 2001). This would come on top of a substantial increase in the likelihood of heat waves (Beniston 2004), eventually leading to an overall intensification of droughts. It has been reported that Pyrenees populations of conifers would profit from the reduction of water availability since deciduous forests such as those made up predominantly of *Betula pendula* Roth have a higher demand for precipitations and therefore, lower rainfall would lead to them being displaced by conifer populations (Herranz *et al*. 2009).

Indeed, adaptations to the predicted drought conditions will take place and these will include stomatal closure and the inhibition of numerous photosynthetic enzymes. The latter will lead to a decrease in photosynthetic capacity and, when drought is severe, to disruptions in the photosynthetic apparatus (Chaves *et al*. 2009). Under mild water stress conditions, stomatal closure has been described as being the main factor limiting photosynthesis; while with increasing drought, non-stomatal limitation has also been reported (Peña-Rojas *et al*. 2004). Water availability also plays a central role in modifying leaf structure (Peña-Rojas *et al*. 2005). Changes in the leaf mass per area ratio (LMA) and its components, leaf density (D) and thickness (T) (Dijkstra 1989) will occur, thereby contributing to changes in photosynthetic capacity (Niinemets 2001). D affects CO_2 diffusion to the site of carboxylation, which can be particularly significant in sclerophylls (Terashima *et al*. 2001). Leaf size is also sensitive to climate: site-mean leaf size typically scales with water availability and, to a lesser degree, with temperature

(Wilf *et al*. 1998).

Carbon isotope discrimination ($\Delta^{13}C$) is negatively correlated with time-integrated water use efficiency (WUE) in C3 plants (Knight *et al*. 1994) which is due to both the differential diffusivity across the stomatal pathway and to Rubisco (which discriminates against ^{13}C during RuBP carboxylation). As described above, morphological characteristics can affect internal resistances; thus, leaf thickness and leaf density can be an important source of variation in $\Delta^{13}C$.

In terms of growth in forest ecosystems, this is often limited by nutrient supply, (Sigurdson 2013). Drought stress contributes negatively to nutrient availability since nutrient uptake by roots is reduced (Pinkerton and Simpson 1986) and nutrient transport from the roots to the shoots is also restricted by reduced transpiration rates (Kramer and Boyer 1995).

In plants, nitrogen is the nutrient that is required in the largest quantities and therefore can be considered as an indicator of productivity. Indeed, more than 50% of N is used in the photosynthetic apparatus (Hikosaka and Terashima 1996) and consequently, leaf N content strongly correlates with photosynthetic capacity (Field and Mooney 1986). A decrease in N concentration can reduce the rates of the enzymatic and electron-transport functions of photosynthesis (Crous *et al.* 2008). Overinvestment in Rubisco by many plants is an adaptation to temporary variations in N supply with Rubisco serving as an N store (Millard 2007). Environmental stress increases the rate of leaf senescence, which involves the degradation of proteins such as Rubisco (Lim *et al.* 2007). Furthermore, Phosphorous (P), Magnesium (Mg), Iron (Fe) and Potassium (K) are key plant nutrients that play important roles in the photosynthetic metabolism. Phosphate is a component of cellular membranes, nucleic acids and nucleoproteins and is essential for energy storage and transfer, carbohydrate metabolism, photosynthesis and respiration (Vance *et al*. 2003). Phosphate deficiency negatively affects plant photosynthesis, growth and yield. Mg is a component of the chlorophyll molecule and enzyme activator (Rubisco formation and utilization of ATP). Fe is a constituent of enzymes involved in photosynthesis and is necessary for chlorophyll biosynthesis. In addition, K has a particular importance in photosynthesis, osmoregulation, enzyme activation, protein synthesis, ion homeostasis, maintenance of the anion-cation balance and stomatal aperture (Marschner 1995).

The direct effects of climate change on individual plants and plant communities may occur even in the absence of pathogens, but it might also bring about changes in plants that will affect their interactions with them (Niinemets 2001). A shift in climate conditions may play an important role as biotic diseases are expected to increase in prevalence and severity (Juroszek and von Tiedemann 2011).

Changes in plant architecture may affect microclimates and thus change the risk of infection (Burdon 1987). Pathogens that typically affect water-stressed hosts are likely to have an increased impact on forests where there is a reduction in precipitation (see review by Sturrock *et al*. 2011) and the distribution of hosts and diseases will shift to areas which have more suitable growing conditions. The proliferation of diseases such as rust and other fungi pathogens that infect the leaves of plants such as *Chrysomyxa rhododendri* whose host is *Rhododendron* spp. (Ganthaler *et al* 2013) will likely increase with increasing temperatures and drought conditions (Desprez-Loustau *et al*. 2007).

In order to predict the effects of future environmental changes, specifically the decrease in precipitation on photosynthetic performance, productivity and survival capacity of sub-alpine forests, we first need to know how species will cope with a decline in water availability. Of particular interest is the study of the responses of species representative of subalpine communities in order to determine their adaptation capacity to water deficit conditions and to obtain more information about the effect of future reductions of precipitation on those ecosystems.

In summary, therefore, the objective of the study was to characterize water relations and gas-exchange responses of mountain pine (*Pinus uncinata* Mill), birch (*Betula pendula* Roth) and rhododendron (*Rhododendron ferrugineum* L.) over the course of different precipitation periods for two consecutive summers, with particular emphasis on responses to rainfall limitation. All three species exhibit different functional characteristics: rhododendron is an understory bush with evergreen leaves whereas mountain pine and birch are evergreen and deciduous trees respectively.

Another key objective was to characterize the presence and susceptibility of the studied species to pathogen infections in their natural placement during a period of low water availability in order to ascertain their resistance capacity. We also aimed to characterize the effect of the disease on the photosynthetic process in both the early and advanced stages of the infection.

The following questions were addressed: a) how will water relations of the different species being studied be altered by decreased water availability both in the short and long-term? b) since a limited supply of nutrients can be expected under drought conditions, is the current nutrient status of the studied species adequate? c) will the susceptibility to pathogen infections increase under these circumstances?

Knowledge of the effects of abiotic and biotic stress factors on plants will be key in understanding climate change effects on natural vegetation and in the implementation of improved management practices.

Material and methods

Study site and plant material

The study was carried out in two areas at the Aigüestortes i Estany de Sant Maurici National Park, in the Central Pyrenees, Catalonia, Spain. The first area (42º 32' 58.51" N 0º 53' 19.29" E) is close to Estany Llebreta at an altitude of 1,617 m and the second area (42º 34' 21.32" N 0º 56' 32.61" E) is close to Estany Llong at an altitude of 1,985 m. The climate varies between atlantic and continental, where winters are long and cold with sub-zero temperatures and snow cover lasting for the most part of the year. In contrast, summers are short and are characterized by their mild temperatures and chilly nights. The mean annual temperature stands around 5.2ºC and the annual thermal range is 16ºC. Mean precipitation lies between 1,200-1,300 mm and the monthly precipitation is greater than 100 mm. The climatological characteristics during the period of study are shown in Fig. 1. The studied species were birch (*Betula pendula* Roth.) in the first area and rhododendron (*Rhododendron ferrugineum* L.) and mountain pine (*Pinus uncinata* Mill.) in the second. The average age of the trees was approximately 130 years for mountain pine and 16 years for birch. Measurements and sampling in the forest were performed for three weeks over two campaigns (July and September) in 2011 and 2012. Measurements were carried out on fully expanded leaves (from the previous year in mountain pine and rhododendron), with a southerly orientation and from individuals with similar levels of irradiance exposure.

Gas-exchange and chlorophyll fluorescence measurements

Net photosynthesis (*A*), stomatal conductance (g_s) and transpiration rate (*E*) were measured with a gas exchange-system LI-6400 (Li-COR, Lincoln, Nebraska, USA), equipped with a Light source (LI-COR 6400-02B LED). The instantaneous water use efficiency (WUE) was calculated as *A*/*E*. Measurements were carried out around midday (11:30 am-16:00 pm) (local time) at 370 µl l^{-1} ambient CO_2 concentration (C_a). Other ambient conditions were established from those determined *in situ* in July and

September. In July, the following conditions in the measuring chamber were used: air flux: 500 µmol·s^{-1}, temperature: 20 - 23ºC, Vapour Pressure Deficit (VPD) 1.5 ± 0.2 kPa in 2011 and 1.8 ± 0.3 kPa in 2012; saturating photosynthetic photon flux density (PPFD) : 1500 µmol·m^{-2}·s^{-1} for birch and 1200 µmol·m^{-2}·s^{-1} for rhododendron and mountain pine, water molar fraction: 3 ± 1 mmol H_2O·mol air^{-1} for birch and 1 ± 0.5 mmol H_2O·mol air^{-1} for rhododendron and mountain pine in 2011, and 2 ± 1 mmol H_2O·mol air^{-1} for birch and 1.5 ± 1 mmol H_2O·mol air^{-1} for rhododendron and mountain pine in 2012. In September, the following conditions were established: air flux: 500 µmol·s^{-1}, temperature: 15-18 ºC, VPD: 1.5 ± 0.3 kPa in 2011 and 1.00 ± 0.2 kPa in 2012; saturating PPFD: 1500 µmol·m^{-2}·s^{-1} for birch and 1,200 µmol·m^{-2}·s^{-1} for rhododendron and mountain pine, water molar fraction: 1.7 ± 0.7 mmol H_2O·mol air^{-1} for all species in 2011, and 1.5 ± 1 mmol H_2O·mol air^{-1} for birch and 1.0 ± 0.5 mmol H_2O·mol air^{-1} for rhododendron and mountain pine in 2012. Measurements were carried out on 3-4 leaves located between 2 to 5 m from the base (when trees) in 4-5 individuals per species, sampling campaign and year.

After dark adaptation of leaves (30 minutes for birch, 40 minutes for rhododendron and 45 minutes for mountain pine, timings previously established which provided sufficient adaptation time to allow for the complete oxidation of PSII reaction centres) F_o (minimum fluorescence yield), F_m (maximum fluorescence yield) and F_v/F_m (maximum quantum yield of PSII photochemistry (equivalent to $(F_m\text{-}F_o)/F_m$)), were obtained with the modulated fluorimeter Mini-PAM Walz (Walz, Effeltricht, Germany).

Hydration and relative water content of leaves

Hydration (H) was calculated as $((M_f - M_d) / M_d)$, where M_f is plant fresh mass and M_d is plant dry mass (after oven-drying samples at 65ºC until a constant weight was reached). The relative water content (RWC) was determined in the laboratory from three replicates of 2 leaves per individual for birch and 10 leaves per individual for rhododendron and pine, taken at midday in July and September 2012. RWC was calculated as $[(M_f\text{-}M_d)/(M_{fs}\text{-}M_d)\cdot 100]$ where M_{fs} is plant fresh saturated mass (after rehydrating samples for 24h in the dark at 4ºC).

Leaf structural parameters

Thirty south-oriented leaves per species and campaign in 2011 and 2012 were selected. Sampling leaf area (LA) was determined with an imaging software (ImageJ version

1.45s). Leaf mass per area (LMA) was calculated as M_d/LA, and the components Thickness (T) and Density (D) were calculated as M_f/LA and $[(M_d/M_f)\cdot 100]$ respectively (Dijkstra, 1989, Niinemets, 1999).

Leaf carbon isotope composition and mineral content

Leaf samples were collected (2 leaves per birch individual (5 individuals) and 10 leaves per pine and rhododendron individual (5 individuals)), frozen in liquid nitrogen on site and stored at -80ºC until analyses were carried out. After lyophilization of the samples (Alpha 1-4 LD Plus lyophilizer, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), the carbon isotopic composition ($\delta^{13}C$) and total Carbon and Nitrogen (N) content were determined from approximately 0.8 mg of ground lyophilized material using an elemental analyzer (EA1108, Series 1, Carlo Erba Instrumentazione, Milan, Italy) coupled to a mass spectrometer (Delta C, Finnigan MAT, Bremen, Germany) at the Serveis Científico-Tècnics (UB). Carbon isotopic composition of soluble extracts ($\delta^{13}C$) was determined from approximately 50 mg of ground samples according to Nogués *et al*. (2004). $\delta^{13}C$ values were determined using a standard reference which was calibrated against Pee Dee Belemnite (PDB) carbonate and used to calculate the carbon isotope discrimination ($\Delta^{13}C$) according to the following: $\Delta^{13}C = 1000\cdot(\delta_a-\delta_p)/(1+\delta_p)$, where δ_a and δ_p are values for the air ($\delta^{13}C = -10.37$ determined at the forest sites) and the plant respectively (Farquhar *et al.* 1989).

The mineral content of Phosphorus (P), Potassium (K), Iron (Fe) and Magnesium (Mg) was determined according to Munter *et al*. (1984) from lyophilized leaves (one sample per individual, 5 individuals per species in July and September of 2010 and 2011) and analysed with a inductively coupled plasma mass spectrometry (Elan-6000 ICP-MS, PerkinElmer, Massachusetts, USA) at the Serveis Científico-Tècnics (UB).

Incidence and severity of rust infection

We evaluated the incidence of rust infection firstly based on the percentage of individuals (10) of each species that showed visual symptoms of the disease. Then the incidence in ten infected individuals was evaluated in 20 randomly selected leaves and from this, the percentage of infected leaves (presence of pustules) was calculated. 20 leaves were selected at random in an individual and in total 5 individuals per clone were evaluated. Severity was considered as the degree of the infection in any given individual. Six degrees of severity were established from the percentage of leaf area covered by rust, as shown in Table 1. This parameter was determined in 5 plants per species. For each individual, we examined 5 leaves and in the case of birch we also considered leaves from branches at two heights: low branches (0-2 m height) and high branches (> 2 m).

Chlorophyll fluorescence and chlorophyll content of infected leaves

Before measurements were taken, leaves that were representative of each infection severity degree were selected: in birch, leaves showed degrees of severity of up to 5, while in rhododendron the maximum degree of severity was 3. In birch and rhododendron, after at least 45 minutes dark-adaptation, the maximum quantum yield of PSII photochemistry (F_v/F_m) was determined with a Mini-PAM Walz (Walz, Effeltricht, Germany) modulated fluorimeter. This was carried out on a minimum of 3 leaves per severity degree and per individual, however, orientation and branch height were not taken into consideration. In total, measurements were taken on 5 birch trees and 7 rhododendron bushes in July and on the same individuals in September. Chlorophyll fluorescence imaging was carried out with a pulse-amplitude-modulated chlorophyll fluorimeter Imaging-PAM (MICRO-version (Walz, Effeltrich, Germany)) operated with the Imaging Win v.2.21d (Heinz Walz) software. Chlorophyll parameters were obtained for one squared image area of 26 x 34 mm^2 per leaf. F_v/F_m measurements were performed on pustules and on non-affected areas of infected leaves.

At the end of the period of study (September 2011), the total chlorophyll content was determined *in situ* in 5 individuals per species on at least 3 leaves with the same degree of infection by means of a leaf chlorophyll meter (SPAD-502, Minolta, Osaka, Japan).

Statistical analysis

All statistical procedures were performed using 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, species, campaign (July, September) and year in which the parameters were measured. A multiple comparison test of the means was carried out using the Duncan post-hoc test. Statistical significance was set at $p \leq 0.05$.

Results and Discussion

Gas exchange and water relations under reduced water availability affected each of the studied species differently

The different precipitation regimes of 2011 and 2012 produced long term effects (in 2011) and short term effects (in 2012) on the water relations and photosynthetic responses of the studied species. In 2011, in the period from January to September, mean accumulated precipitation was low when compared with the same period of 2012 (626.8 mm and 765.3 mm respectively). This was caused by a 46% decrease in rainfall from June onwards when compared to the same period of 2012 (Fig. 1). This decrease gave rise to low water availability for plants over a long period of time. In 2012, no evidence of long term effects of low water availability were observed in the studied parameters. However, in 2012, while rainfall was scarce (0.5 mm) for 20 consecutive days in July, it was abundant throughout September. This decrease in precipitations in July 2012 gave rise to low water availability for plants over a short period of time.

The mean anual VPD up until September was slightly lower in 2011 than for the same period of 2012 (0.5 kPa and 0.58 kPa respectively). However, in the period from June to September greater differences in VPD between both years were observed: in 2011 mean summer VPD was 0.67 kPa, whereas for the same period of 2012 it was 0.82 kPa. This was due to higher temperatures in the summer of 2012 (monthly mean values from June to September 2011 were as follows: 13 ºC, 14.4 ºC, 16.9 ºC, 14.4 ºC; and in 2012: 14.9 ºC, 15.7 ºC, 18.1 ºC, 12.5 ºC). We detected small differences in VPD between July and September 2011 (0.64 kPa and 0.67 kPa respectively) and a higher VPD in July of 2012 than in September (0.82 kPa and 0.58 kPa respectively).

In late summer 2011 birch and rhododendron showed a reduction in stomatal conductance (g_s) (54% and 43% respectively) reaching values of under 50 $mmol \cdot m^{-2} \cdot s^{-1}$ (Table 1), this being indicative of severe water stress (Flexas *et al*. 2002). In birch, transpiration (*E*) and CO_2 assimilation (*A*) rates in September were 54% and 59% lower than in July respectively (Table 1) indicating that there was a stomatal limitation of photosynthesis. Moreover, the decline in F_v/F_m revealed that photoinhibition had been induced by seasonal water deficit.

Stomatal closure can limit CO_2 assimilation while at the same time maintaining operational RWC and H of the leaf. However, in birch, a signficant decline in H was also observed which would reflect both decreases in soil water reserves (Table 1) and an advanced stage of foliar senescence (Valjakka *et al.* 1999, Warren *et al*. 2014). As no differences in VPD were observed between July and September, the decline in g_s in birch and rhododendron in late summer is likely to have been linked to a lower soil water availability.

Gas-exchange did not decline in either species throughout the milder summer of 2012 In birch and mountain pine, *A* and g_s were similar in July 2011 and 2012 Thus, it can be deduced that the decline in *A* and g_s in birch in late summer 2011 was related to prolonged low water availability.

Rhododendron exhibited severe water stress in the period of low rainfall in early summer 2012 which was observed in a decrease in g_s of 56% when compared to values for early summer 2011. This was also reflected in the lower F_v/F_m values obtained and in a drop in RWC levels to below 80% (Table 1), considered to be inadequate, which are usually associated with high water deficit (González and González-Vilar 2001). In late summer 2012, g_s increased in rhododendron owing to rainfall in that period. These responses demonstrated the sensitivity of this species to recent, short-term changes in water availability and also possibly to VPD. This is probably due to the fact that this shrub has only access to water in the surface soil layers which are more susceptible to large oscillations in water potential (Dawson 1996) whereas in contrast, the deeper root systems of birch and mountain pine would give them access to deeper water reserves, therefore reducing their sensitivity to brusque changes in precipitation patterns.

Mountain pine was the species least affected by changes in water availability: g_s values always remained between 50–100 $mmol \cdot m^{-2} \cdot s^{-1}$ indicative of moderate water stress. Moreover, no summer variations of *A*, g_s, *E*, H and RWC were observed. In general, the highest *A* rates and instantaneous water use efficiency (WUE_{inst}) were seen in this species (double that in rhododendron). Conversely, it had the lowest H value which was related to higher leaf structural investment (Table 1). Values obtained for F_v/F_m remained around 0.83 throughout both summers (Table 1), indicating that mountain pine did not experience stressful conditions (Björkman and Demmig 1987).

Leaf structural characteristics and Carbon isotope discrimination reflected differences in water relations

At the beginning of summer 2011, leaf area (LA) was highest in birch but had decreased by late summer. This was then followed by the onset of leaf senescence and abscission. Coriaceous leaves in rhododendron and mountain pine needles have drought-resistant morphologies (Gratani and Bombelli 2000) and this allowed for the maintenance of leaf structure over the course of both summers. LMA, indicative of leaf hardiness and rigidity (sclerophylly) (Salleo *et al.* 1997) was highest in mountain pine followed by rhododendron, while in birch, low LMA suggested that there was a loss in leaf dry weight with respect to leaf expansion in order to increase the CO_2 assimilation surface (Bala Subramanian and Maheswari 1992). D was highest in mountain pine followed by rhododendron as a result of greater cell wall thickness and smaller and more tightly packed cells (Niinemets 2001). Birch increased D late on in both summers; in 2011, changes were mainly related to low water availability whereas in the milder 2012 it was as a result of leaf senescence. In both years, high T values were recorded for mountain pine **(**2-fold higher than rhododendron and 3-fold higher than birch). This was associated with an increase in photosynthetic protein per area and furthermore, explains the highest *A* that was measured (Hanba *et al*. 2002).

$\Delta^{13}C$ of leaf dry mass provides information about the photosynthetic characteristics throughout the course of leaf development and is inversely related to long-term WUE in C3 plants (Brugnoli and Farquhar 2000). $\Delta^{13}C$ in summer 2011 showed that WUE was lowest for birch and highest for mountain pine, while in summer 2012 no differences between species were observed (Fig. 3). In birch, $\Delta^{13}C$ was 1.9 per mil lower than during the same period of 2011 which accounts for an approximately 10% increase in WUE. In mountain pine, low $\Delta^{13}C$ and high LMA values suggest a dense mesophyll structure with strong internal CO_2 diffusion (Flexas *et al*. 2008, Fleck *et al*. 2010).

Furthermore, the $\Delta^{13}C$ in the soluble fraction tells us about photosynthetic characteristics in the short-term (days, hours). $\Delta^{13}C$ varies in accordance with the relative contribution of enzymatic (Rubisco) and diffusional fractionation processes where this variation is a linear function of intercellular versus ambient CO_2 concentration (C_i/C_a) (Farquhar and Richards 1984).

In birch, under the low water availability conditions of late summer 2011, the decline in $\Delta^{13}C$ and C_i of the soluble fraction (Fig. 3) was related to the decrease in g_s (Table 1). As discussed earlier, this fact highlights that there is stomatal limitation of photosynthesis in this species. In contrast, in late summer 2011 in the case of rhododendron, maintenance of values for *A*, increases in $\Delta^{13}C$ (dry mass and soluble fraction) and decreases in C_i and g_s, all pointed to non-stomatal photosynthesis limitation.

Birch showed a significant negative relationship between $\Delta^{13}C$ and LMA (Fig.4) making it the species that was most susceptible to undergo modifications in leaf structure under reduced water availability as was seen to occur in late summer 2011. Variations of $\Delta^{13}C$ in rhododendron and mountain pine could not be correlated with any changes in LMA. This is indicative of a dense mesophyll structure in both species. Furthermore, the observed independence of $\Delta^{13}C$ (and consequently WUE) from biomass parameters would indicate that there exists the potential to improve WUE without necessarily producing knock-on effects on leaf traits, thus avoiding a reduction in overall productivity (Monclus *et al*. 2005).

Nutritional characteristics of the studied species

N content per area in terms of both organic matter and the soluble fraction was at least two-fold higher in mountain pine than in the other species (Fig. 5). This was directly related to the highest *A* rate and T values recorded in this species. In both years, in birch approximately 10 % of total N was in the form of soluble N (Fig. 5) whereas in rhododendron this proportion was 15% and in mountain pine 20%, indicating that the highest investment in RuBisco occurred in the latter. In mountain pine, the decline in soluble N in late summer of both years suggested that nitrogen from RuBisco in old leaves had been translocated to young leaves (Camm 1993, Millard *et al.* 2001) which matches the reported decrease in $V_{c,max}$ in this species (Fleck *et al*. 2015). In early summer, PNUE in mountain pine and rhododendron was in general lower than in birch which would point to a greater investment of N in non-photosynthetic components (Takashima *et al*. 2004), this being characteristic of schlerophyllous leaves. The decrease in PNUE in birch in late summer accounted for lower N usage in photosynthesis and a greater investment in leaf structure, a fact related to leaf senescence processes and adaptation to water deficit.

With respect to the C/N ratio (Fig. 5), this was highest in mountain pine and lowest in birch in both years and is linked to a higher C allocation for structural purposes, which is characteristic of high LMA species (Reich *et al*. 1999). Increases in C/N in birch and rhododendron in late summer can be linked to early senescence processes such as protein degradation and N resorption (Wingler *et al*. 2006).

P content in birch and mountain pine remained stable or even increased in late summer in both years (Fig. 6). This is likely to have contributed to the maintenance of photosynthesis levels when N was remobilized, since P is positively related to the allocation of N to Rubisco (Jacob and Lawlor 1992). In addition, low N/P in both species is indicative of larger root systems (Poorter and Nagel 2000). P content was highest in mountain pine and lowest in rhododendron. P was more limiting than N in the latter, which was revealed by its higher N/P ratio (Fig.6). The decrease in P in rhododendron in late summer 2012 can be linked to its remobilization as was shown by the increase in C/P and had a direct impact on PSII efficiency (Conroy 1986) in accordance with the results for F_v/F_m.

Regarding the mineral concentration of K, Fe and Mg, which are expressed in terms of leaf area (Fig. 7), this was lowest for birch and highest for mountain pine. This would explain why the *A* rates of the latter were the highest (Richardson *et al.* 2011). Mineral concentration which was calculated in terms of leaf dry weight (mg g $^{-1}$DW) (Fig. 8) provided information about the deficiency thresholds of all three species. These were in accordance with reference values reported for N (Marschner 1995), P (Kuang *et al.* 2007), K (Zöttl and Hüttl 1989), Fe (Kabata-Pendias and Pendias 2001) and Mg (Kuang *et al.* 2007). It was seen that N, P, K and Mg were higher in birch than in the other species, which most likely were N and P limited. However, after long-term water deficit (late summer 2011) there was a deficit of N in birch. Furthermore, Fe deficit was observed in birch and rhododendron during low water availability periods. This deficit of both elements would contribute to photosynthesis limitation and early leaf senescence.

Incidence of pathogens during drought

The most significant infections were observed in birch and rhododendron and were caused by rust (Basidiomycota, Uredinales): *Melampsoridium betulinum* (Fries) Kleb. in birch and *Chrysomyxa rhododendri* (DC.) De Bary in rhododendron. Rust infection reduces the volume of photosynthetic tissue by damaging the chlorophylls (Kuprevich and Transhel, 1957) and causes premature leaf fall (Pohjonen, 1991) and may enhance herbivore performance (Lappalainen *et al.* 1995). Birch rust is a common pathogen in Europe, Asia and America (Kuprevich and Transhel 1957). *Chrysomyxa rhododendri* is hosted by the *Rhododendron* and *Picea* species and is endemic to alpine and boreal areas of Europe (Bennell 1985).

By means of the analysis of rust incidence and severity, it was possible to observe the propagation of the disease. In both July and especially September, birch had the largest number of individuals that were infected with rust (Table 2). This was linked to summer water deficit, which favoured rust infection and its development on the leaf surface (Grimmer *et al.* 2012). In July, in both species, the highest degree of severity in leaves that was observed was 3 (moderate), whereas in September degrees 4 and 5 were observed, especially in birch. This indicated that a severe or very severe infection level affected more than 50% of leaf area (Table 4). The effects of foliar pathogens include the reduction of CO_2 assimilation (Hajji *et al.* 2009) by directly affecting g_s (Pinkard and Mohammed 2006) and the photosynthetic apparatus (Fernàndez–Martínez *et al.* 2013).

Moreover, the effect of rust on photochemistry by the end of the summer was verified by the decline in F_v/F_m in infected leaves (Fig. 9). Although rhododendron was less infected than birch, F_v/F_m values indicated that photoinhibition was more severe. For example, in September F_v/F_m values of less than 0.75 were measured in leaves of rhododendron starting at the relatively low degree of severity of 1.5, whereas in birch, PSII efficiency was only severely affected from degrees of severity of 3-4 and upwards. Rhododendron is a shade-adapted species and its susceptibility to photoinhibition under high irradiation conditions during summer would likely contribute to a greater decline of F_v/F_m values in infected individuals. In addition, total chlorophyll content in infected leaves declined over course of the summer as infection progressed (Elena *et al*. 2014). In rhododendron a notable decline in Chl in leaves with only traces of infection (degree of severity 1) was observed, w hereas in birch there was only a decline in Chl in severely infected leaves (degree 4 and up) (Fig. 10).

Through chlorophyll fluorescence imaging it was possible to demonstrate the heterogeneity of the effect of rust i.e. a decrease in F_v/F_m only in zones where the fungus developed fructification (pustules) (Fig. 11). Images also showed that there was a higher incidence of rust in birch with higher pustule density. It was also noted that in late summer, although birch was more infected, F_v/F_m values for rhododendron pustules were much lower. This may indicate that under future climate change conditions the photochemistry of rhododendron might be affected more by rust infections than that of birch.

Concluding remarks

Current low water availability conditions in summer, which are likely to be exacerbated in the future, affected the species being studied in different ways. Birch and rhododendron were especially affected by a decrease in precipitation whereas mountain pine was shown to maintain its physiological characteristics. Birch was the most vulnerable species and was negatively affected by periods of long-term water deficit. Photosynthesis was limited by g_s which in turn provoked alterations in the photosynthetic apparatus. Rhododendron was susceptible to both long and short-term low water availability, which resulted in episodes of severe water stress. In birch, nutrient status was affected after long-term water deficit. Moreover, a limitation of N and Fe was observed which contributed to early leaf senescence. Also in rhododendron a decline in foliar N and P as well as results for C/N and C/P indicated that advanced nutrient resorption and leaf senescence took place in late summer. The observed deficit in N, P, and Fe would in turn have contributed to photosynthesis limitation. Mountain pine was N and P limited but the high N investment in soluble N (e.g. Rubisco protein) contributed to maintaining steady photosynthetic rates. Birch and rhododendron were susceptible to rust infection, which increased during summer water deficit. In the case of the latter, a shade–adapted species, the photochemistry was particularly affected.

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Conflict of interest

None declared.

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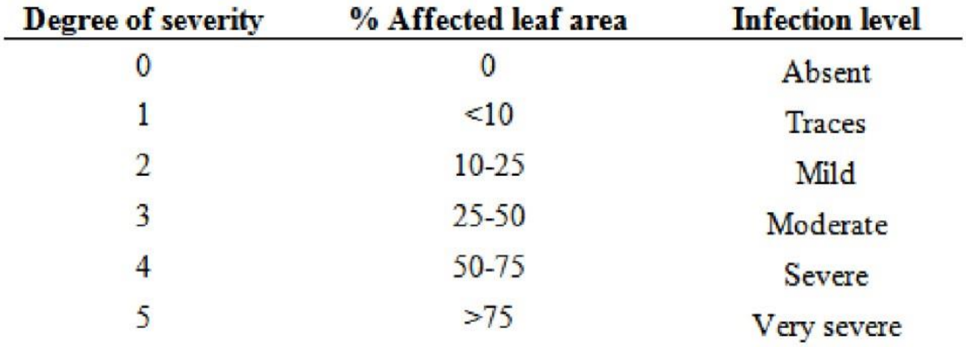
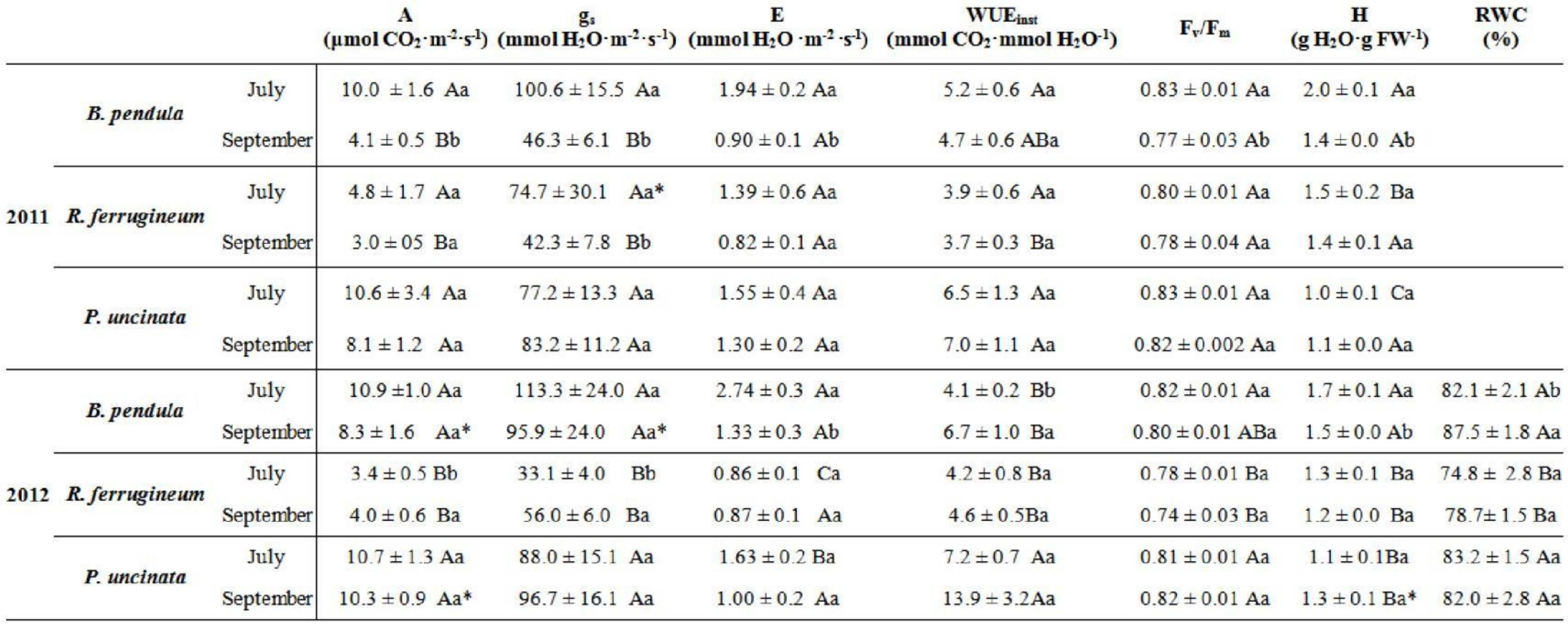
SUPLEMENTAL MATERIAL

Table 1. Different degrees of severity of the foliar infection caused by rust. Each percentage of affected area corresponds to a degree of severity.



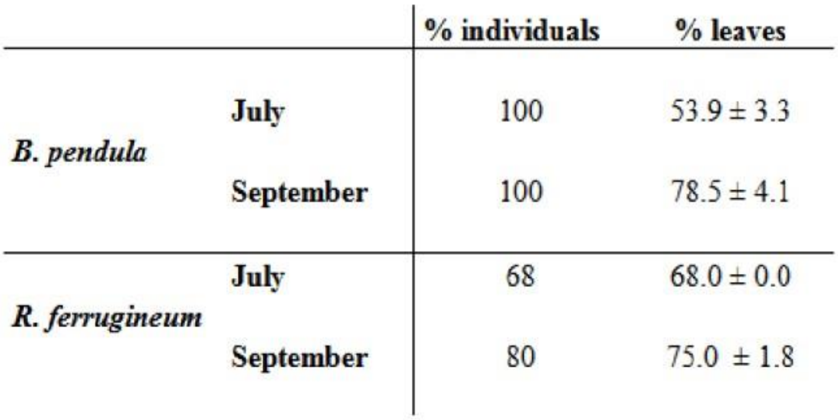
Degree of severity	% Affected leaf area	Infection level
0	0	Absent
1	<10	Traces
2	10-25	Mild
3	25-50	Moderate
4	50-75	Severe
5	>75	Very severe

Table 2. Net Photosynthesis (*A*), stomatal conductance (g_s), transpiration (*E*), instantaneous water use efficiency (WUE_{inst}), maximum quantum yield of PSII (F_v/F_m), hydration (H) and relative water content (RWC) of *Betula pendula*, *Rhododendron ferrugineum* and *Pinus uncinata*. Significant differences ($p \leq 0.05$) are expressed as A,B,C (between species); a,b,c (between July and September); or with an asterisk (between years).



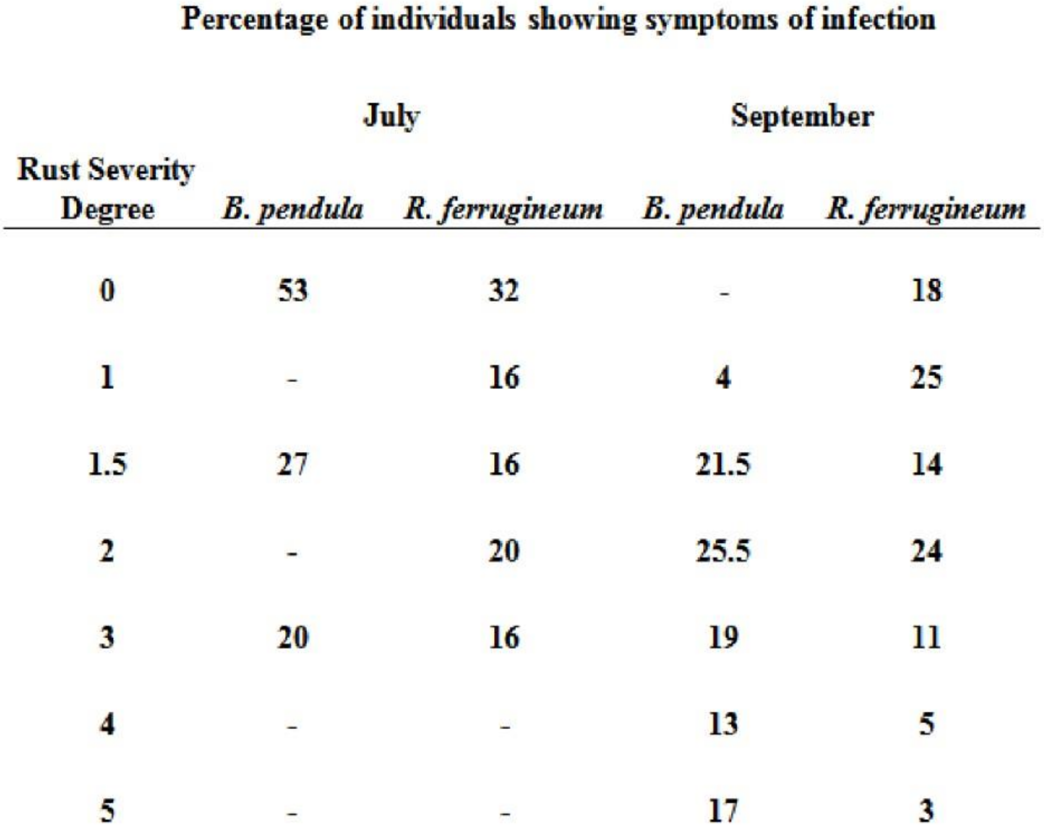
			A (μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$)	g_s (mmol $H_2O \cdot m^{-2} \cdot s^{-1}$)	E (mmol $H_2O \cdot m^{-2} \cdot s^{-1}$)	WUE_{inst} (mmol $CO_2 \cdot$ mmol H_2O^{-1})	F_v/F_m	H (g $H_2O \cdot$ g FW^{-1})	RWC (%)
2011	***B. pendula***	July	10.0 ± 1.6 Aa	100.6 ± 15.5 Aa	1.94 ± 0.2 Aa	5.2 ± 0.6 Aa	0.83 ± 0.01 Aa	2.0 ± 0.1 Aa	
		September	4.1 ± 0.5 Bb	46.3 ± 6.1 Bb	0.90 ± 0.1 Ab	4.7 ± 0.6 ABa	0.77 ± 0.03 Ab	1.4 ± 0.0 Ab	
	R. ferrugineum	July	4.8 ± 1.7 Aa	74.7 ± 30.1 Aa*	1.39 ± 0.6 Aa	3.9 ± 0.6 Aa	0.80 ± 0.01 Aa	1.5 ± 0.2 Ba	
		September	3.0 ± 05 Ba	42.3 ± 7.8 Bb	0.82 ± 0.1 Aa	3.7 ± 0.3 Ba	0.78 ± 0.04 Aa	1.4 ± 0.1 Aa	
	P. uncinata	July	10.6 ± 3.4 Aa	77.2 ± 13.3 Aa	1.55 ± 0.4 Aa	6.5 ± 1.3 Aa	0.83 ± 0.01 Aa	1.0 ± 0.1 Ca	
		September	8.1 ± 1.2 Aa	83.2 ± 11.2 Aa	1.30 ± 0.2 Aa	7.0 ± 1.1 Aa	0.82 ± 0.002 Aa	1.1 ± 0.0 Aa	
2012	***B. pendula***	July	10.9 ±1.0 Aa	113.3 ± 24.0 Aa	2.74 ± 0.3 Aa	4.1 ± 0.2 Bb	0.82 ± 0.01 Aa	1.7 ± 0.1 Aa	82.1 ± 2.1 Ab
		September	8.3 ± 1.6 Aa*	95.9 ± 24.0 Aa*	1.33 ± 0.3 Ab	6.7 ± 1.0 Ba	0.80 ± 0.01 ABa	1.5 ± 0.0 Ab	87.5 ± 1.8 Aa
	R. ferrugineum	July	3.4 ± 0.5 Bb	33.1 ± 4.0 Bb	0.86 ± 0.1 Ca	4.2 ± 0.8 Ba	0.78 ± 0.01 Ba	1.3 ± 0.1 Ba	74.8 ± 2.8 Ba
		September	4.0 ± 0.6 Ba	56.0 ± 6.0 Ba	0.87 ± 0.1 Aa	4.6 ± 0.5Ba	0.74 ± 0.03 Ba	1.2 ± 0.0 Ba	78.7± 1.5 Ba
	P. uncinata	July	10.7 ± 1.3 Aa	88.0 ± 15.1 Aa	1.63 ± 0.2 Ba	7.2 ± 0.7 Aa	0.81 ± 0.01 Aa	1.1 ± 0.1Ba	83.2 ± 1.5 Aa
		September	10.3 ± 0.9 Aa*	96.7 ± 16.1 Aa	1.00 ± 0.2 Aa	13.9 ± 3.2Aa	0.82 ± 0.01 Aa	1.3 ± 0.1 Ba*	82.0 ± 2.8 Aa

Table 3. Incidence of rust infection in July and September expressed as a percentage of infected individuals and as a percentage of the number of infected leaves of 20 randomly selected leaves of 5 infected individuals expressed as mean ± SE.



		% individuals	% leaves
B. pendula	**July**	100	53.9 ± 3.3
	September	100	78.5 ± 4.1
R. ferrugineum	**July**	68	68.0 ± 0.0
	September	80	75.0 ± 1.8

Table 4. Distribution of the different severity degrees observed in *Betula pendula* and *Rhododendron ferrugineum* in July and September as percentage of individuals showing symptoms of infection.



Percentage of individuals showing symptoms of infection

	July		**September**	
Rust Severity Degree	***B. pendula***	***R. ferrugineum***	***B. pendula***	***R. ferrugineum***
0	**53**	**32**	-	**18**
1	-	**16**	**4**	**25**
1.5	**27**	**16**	**21.5**	**14**
2	-	**20**	**25.5**	**24**
3	**20**	**16**	**19**	**11**
4	-	-	**13**	**5**
5	-	-	**17**	**3**

Figure 1. Vapour pressure deficit (VPD), Mean precipitation (mm) and Mean accumulated precipitation (mm) during 2011 and 2012 at the site of study. Grey columns indicate the periods when measurements and samplings were performed.

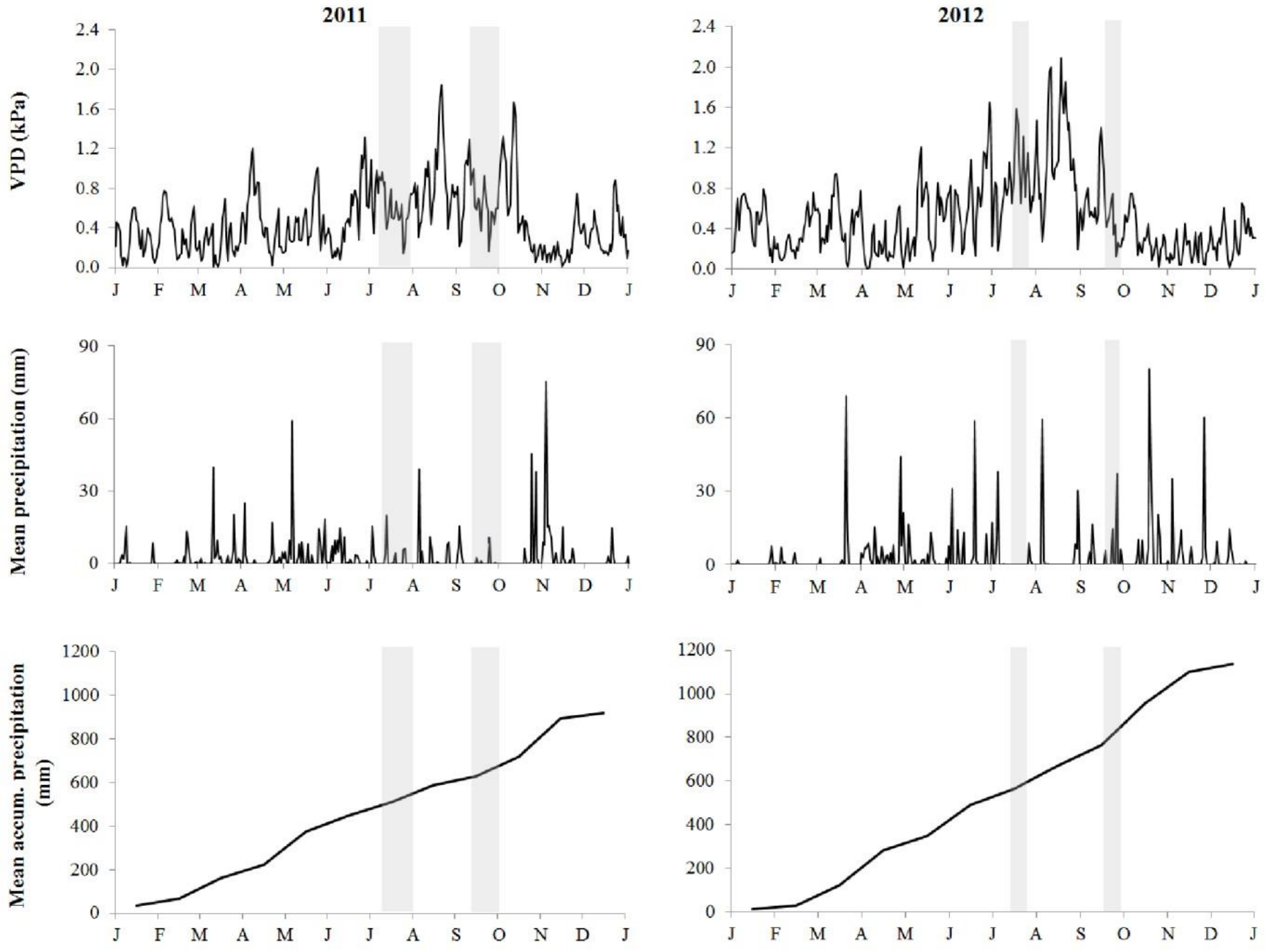


Figure 2. Leaf area (LA), Leaf mass area (LMA), Density (D) and Thickness (T), of *Betula pendula*, *Rhododendron ferrugineum* and *Pinus uncinata* leaves. Significant differences (p≤ 0.05) are expressed as A, B, C (differences between species), a, b (differences between July and September) and asterisk (differences between years).

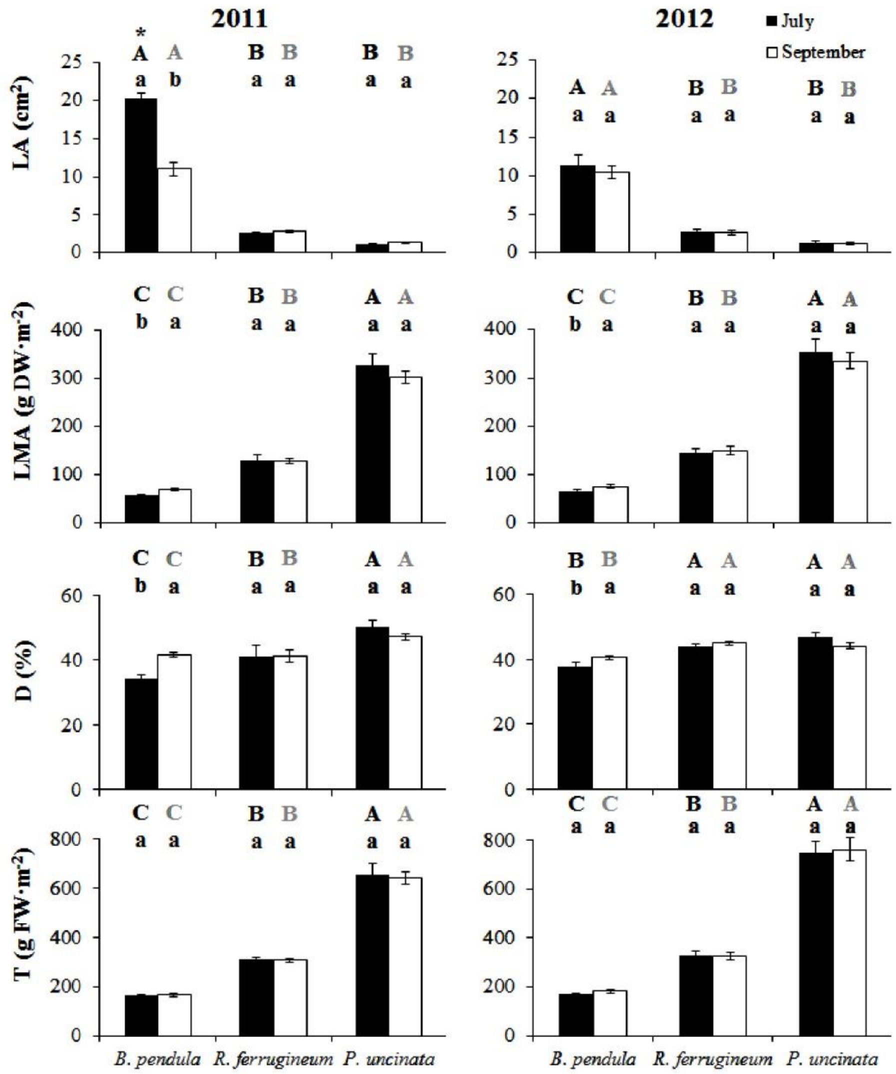


Figure 3. ^{13}C isotope discrimination (Δ^{13}C) in the dry mass and in the soluble fraction of *Betula pendula*, *Rhododendron ferrugineum* and *Pinus uncinata* leaves. Significant differences ($p \leq 0.05$) are expressed as A,B,C (differences between species), a, b (differences between July and September) and asterisk (differences between years).

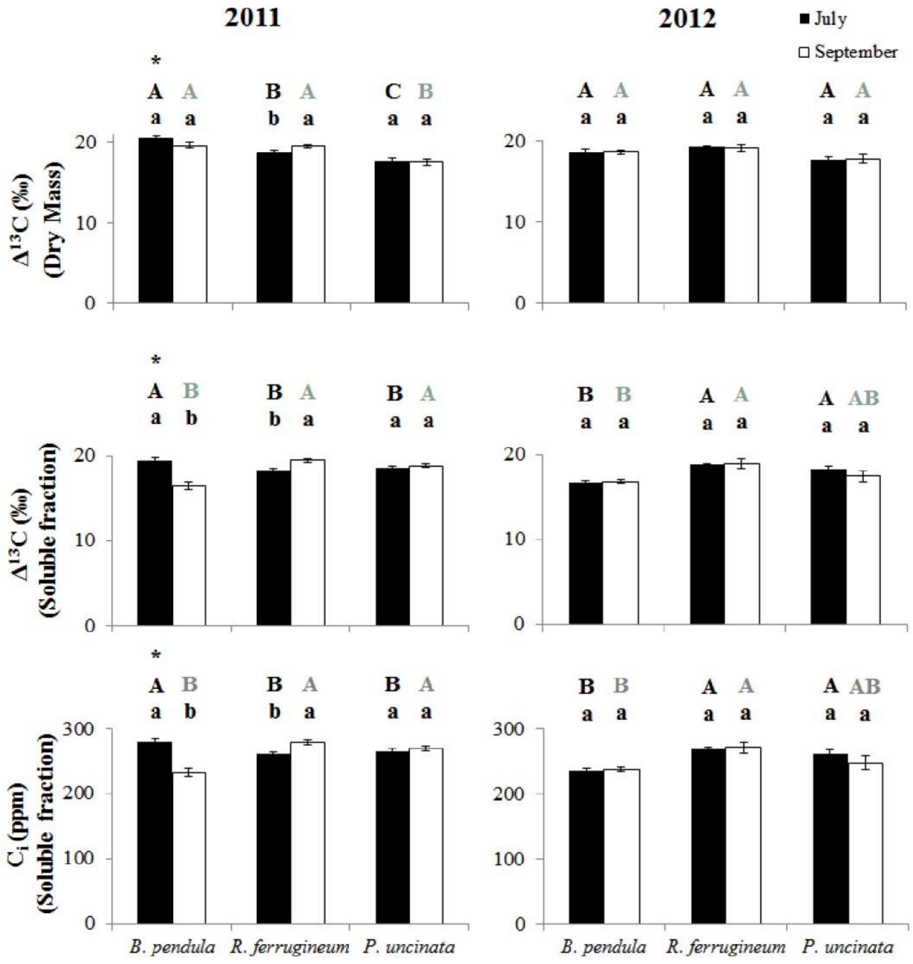


Fig 4. Relationship between Leaf mass area (LMA) and dry mass ^{13}C isotope discrimination (Δ^{13}C) in *Betula pendula*, *Rhododendron ferrugineum* and *Pinus uncinata* leaves.

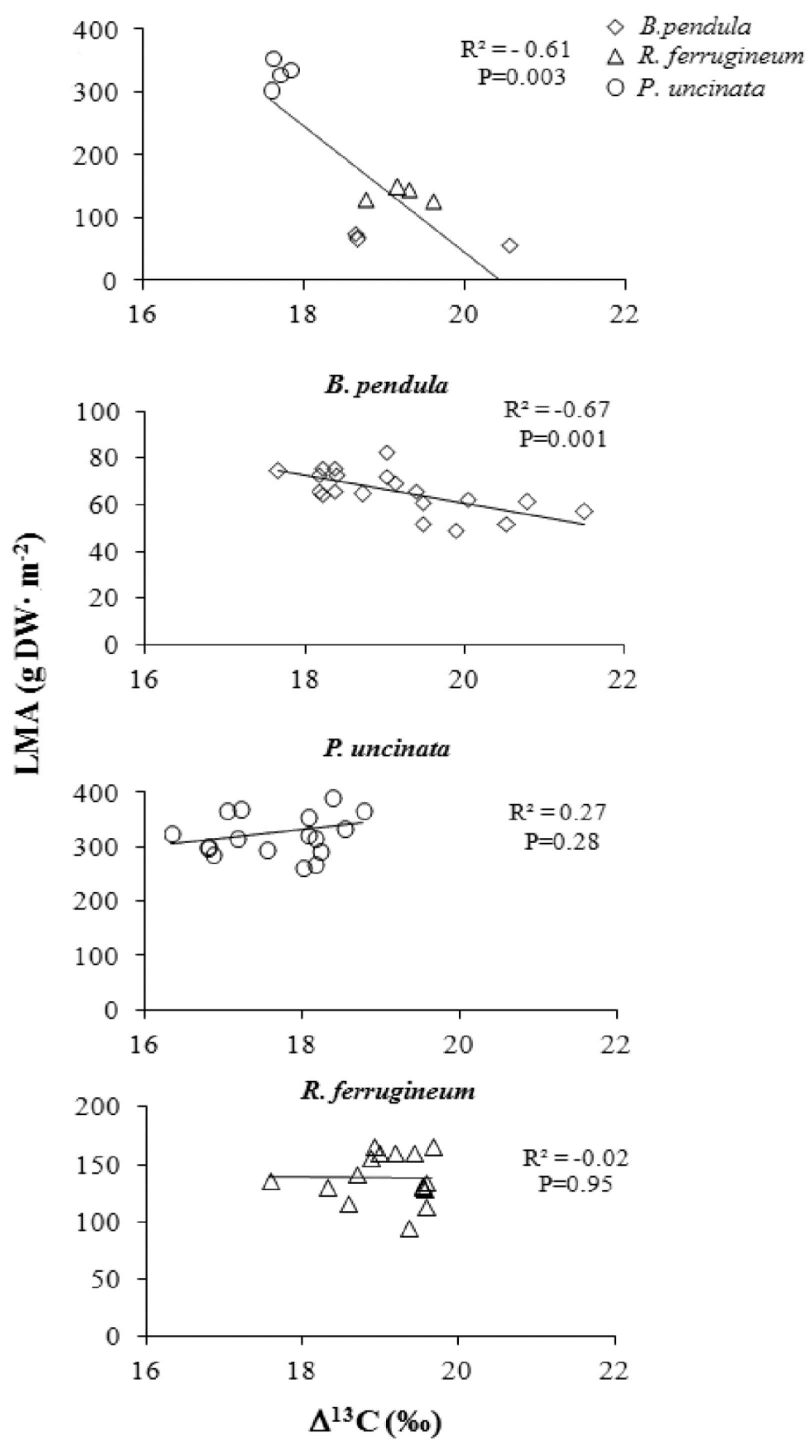


Figure 5. Total Nitrogen and soluble Nitrogen content calculated in terms of leaf area, photosynthetic nitrogen-use efficiency (PNUE) and C/N ratio of *Betula pendula*, *Rhododendron ferrugineum* and *Pinus uncinata* leaves. Significant differences ($p \leq 0.05$) are expressed as A, B, C (differences between species), a, b (differences between July and September) and asterisk (differences between years).

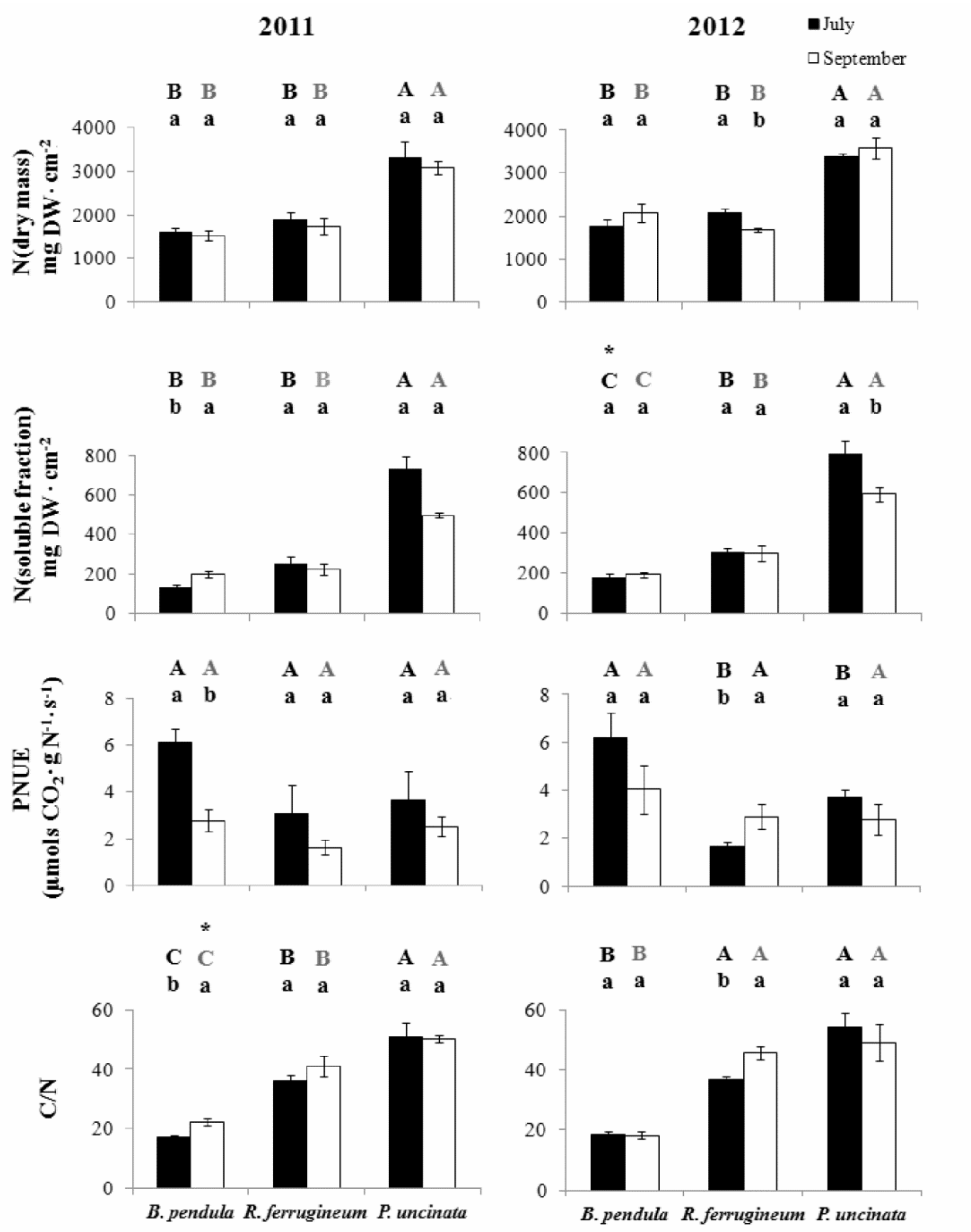


Figure 6. Phosphorus (P) content calculated in terms of leaf area, C/P ratio and N/P ratio of *Betula pendula*, *Rhododendron ferrugineum* and *Pinus uncinata* leaves. Significant differences (p≤ 0.05) are expressed as A, B, C (differences between species), a, b (differences between July and September) and asterisk (differences between years).

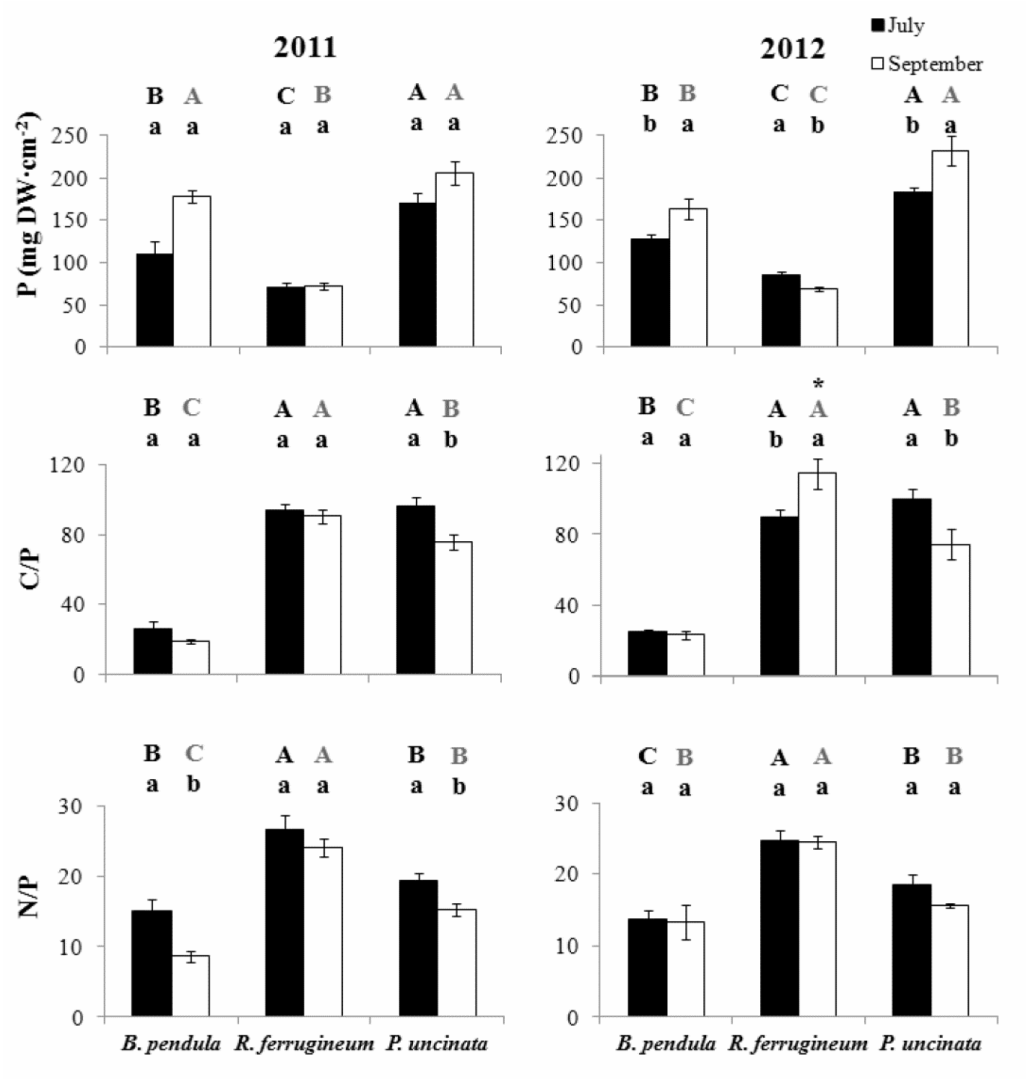


Figure 7. Potassium (K), Iron (Fe) and Magnesium (Mg) content of *Betula pendula*, *Rhododendron ferrugineum* and *Pinus uncinata* leaves calculated in terms of leaf area. Significant differences ($p \leq 0.05$) are expressed as A, B, C (differences between species), a, b (differences between July and September) and asterisk (differences between years).

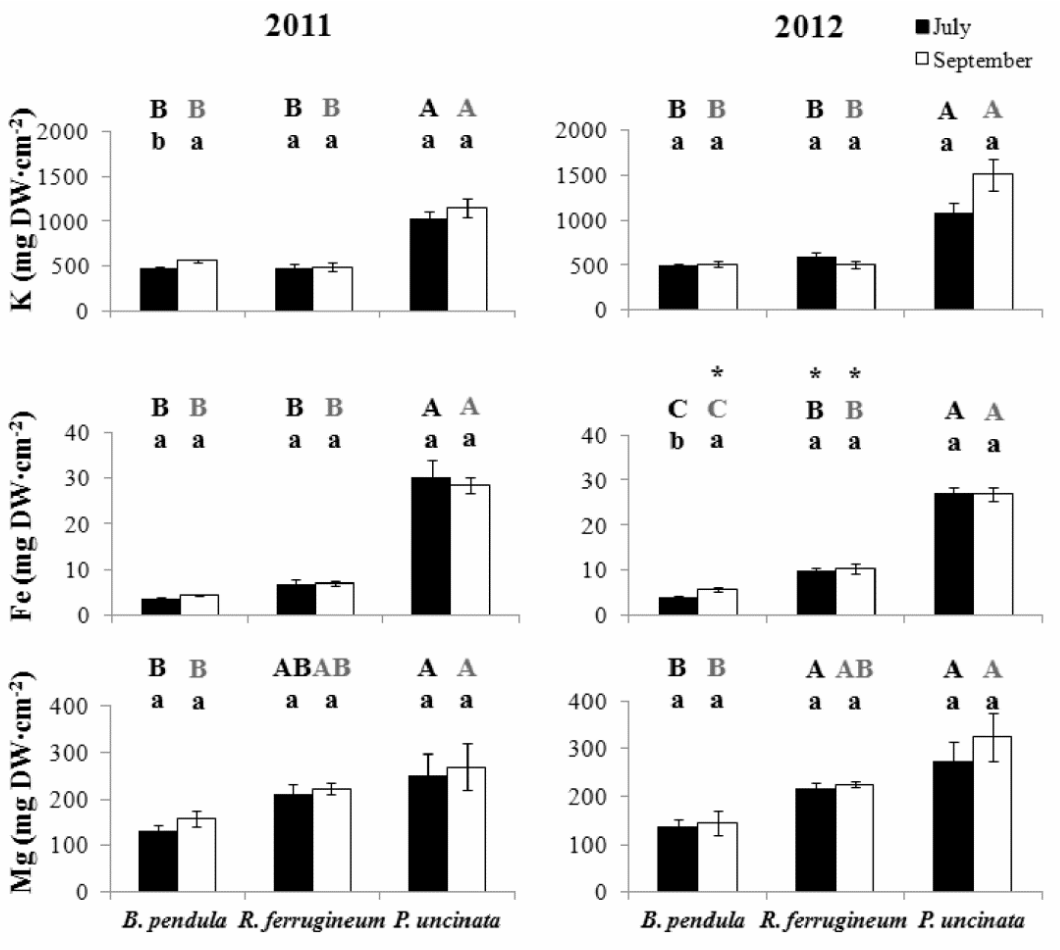


Figure 8. Nitrogen (N), Phosphorus (P) Potassium (K), Iron (Fe) and Magnesium (Mg) content of *Betula pendula*, *Rhododendron ferrugineum* and *Pinus uncinata* leaves calculated in terms of dry weight. Significant differences ($p \leq 0.05$) are expressed as A, B, C (differences between species); a, b (differences between July and September) and asterisk (differences between years).

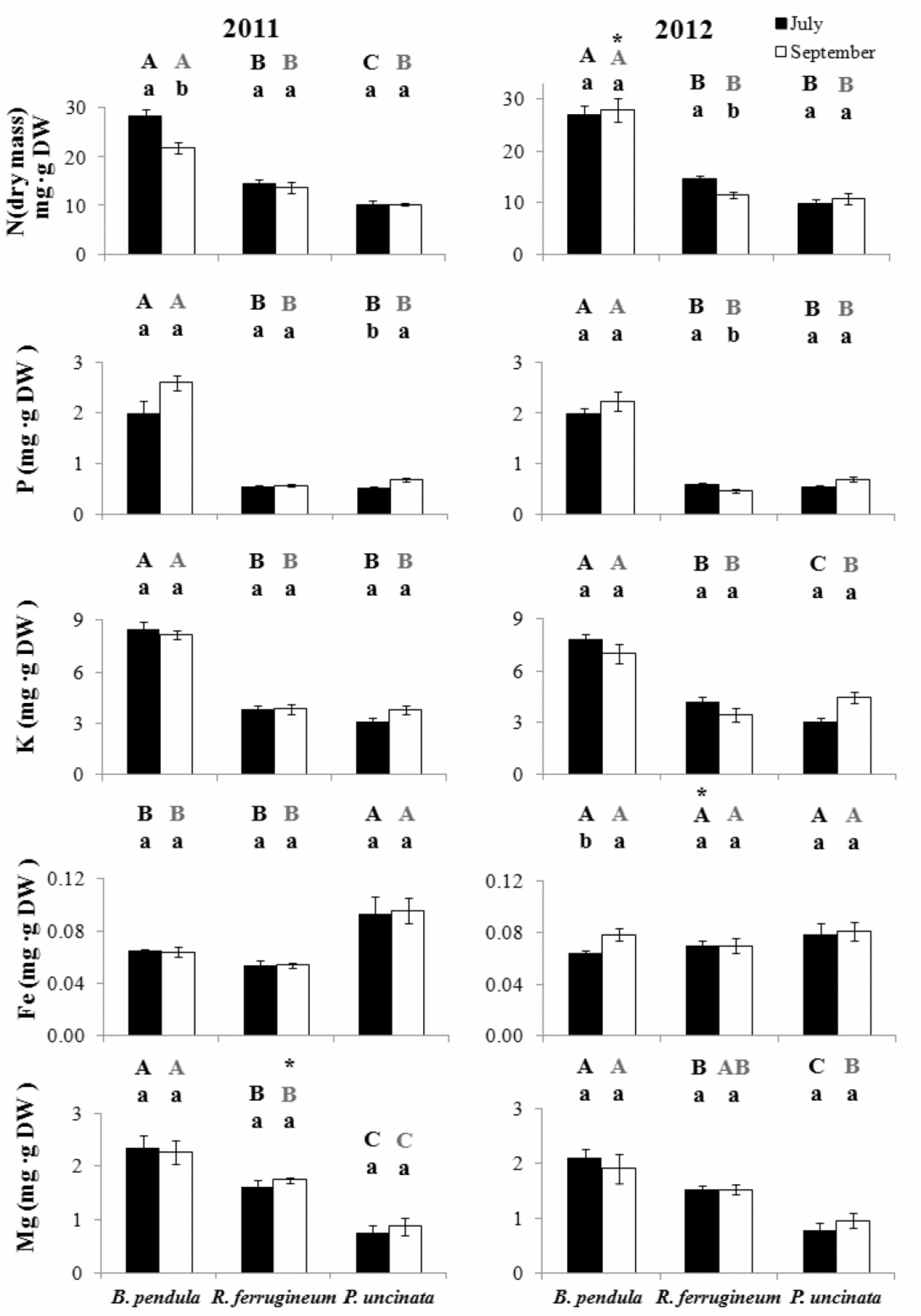


Figure 9. Maximum PSII quantum yield (F_v/F_m) at different degrees of severity of rust in leaves of *Betula pendula* and *Rhododendron ferrugineum* in July and September 2011. Significant differences ($p \leq 0.05$) are expressed as a, b (differences between degrees of severity) and A, B (differences between July and September).

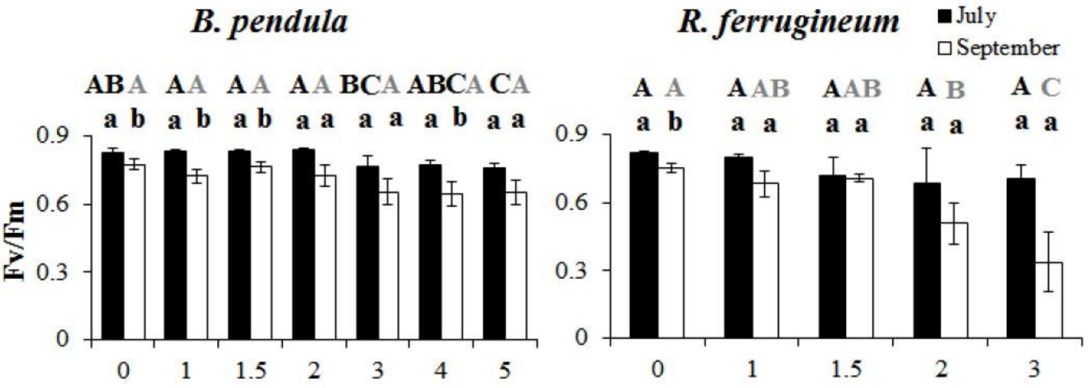


Figure 10. Total chlorophyll content in leaves of *Betula pendula* and *Rhododendron ferrugineum* with different degrees of severity of infection at the end of September 2011. Significant differences ($p \leq 0.05$) are expressed as A, B, C (differences between species), a, b (differences between degrees of severity).

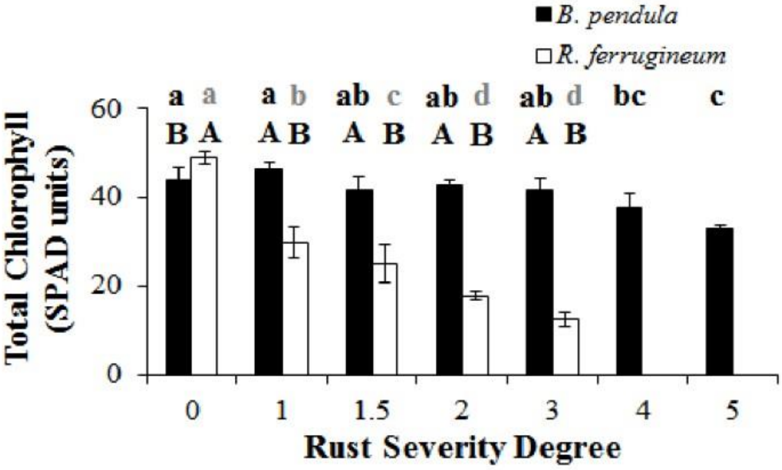
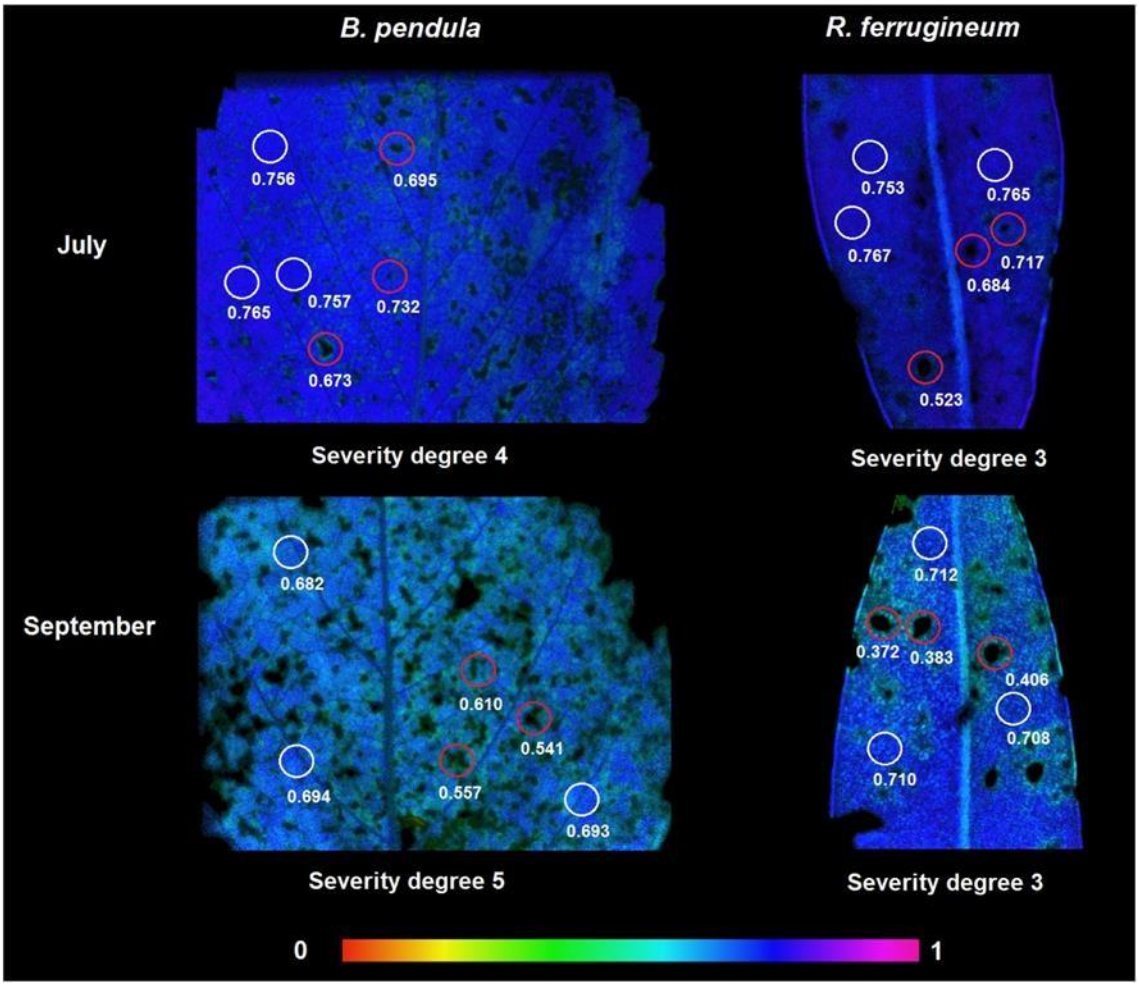


Figure 11. Chlorophyll fluorescence image showing the maximum PSII quantum yield (F_v/F_m) of infected pustules in red and control areas in white. Leaves were analysed in birch (severity degree 4) and in rhododendron (severity degree 3) in July, and in birch (severity degree 5) and in rhododendron (severity degree 3) in September. Images were selected taking into consideration the highest severity degrees observed in July and in September in each species.



RESPOSTES FOTOSINTÈTIQUES, DE FOTOPROTECCIÓ I ANTIOXIDANTS EN CLONS DE *Populus* EN FRONT DE DOS TIPUS DE SITUACIONS D'ESTRÈS: EL DÈFICIT HÍDRIC ESTIVAL I CONCENTRACIONS ELEVADES DE Zn

Capítol 3: Efecte de factors d'estrès ambiental en les característiques ecofisiològiques i susceptibilitat per patògens en cinc clons de *Populus* al llarg de l'estació de creixement

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Research paper

Effect of environmental stress factors on ecophysiological traits and susceptibility to pathogens of five *Populus* clones throughout the growing season

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The variability of ecophysiological traits associated with productivity (e.g., water relations, leaf structure, photosynthesis and nitrogen (N) content) and susceptibility to fungal and insect infection were investigated in five poplar clones (*Populus deltoides* Batr.—Lux clone; *Populus nigra* L.—58-861 clone and *Populus* × *canadensis* Mönch.—Luisa Avanzo, I-214 and Adige clones) during their growing season. The objective of the study was to determine their physiological responses under summer constraints (characteristic of the Mediterranean climate) and to propose clone candidates for environmental restoration activities such as phytoremediation. Relative water content, the radiometric water index and ^{13}C isotope discrimination ($\Delta^{13}C$) results reflected improved water relations in Adige and Lux during summer drought. Leaf structural parameters such as leaf area, leaf mass per area, density (D) and thickness (T) indicated poorer structural adaptations to summer drought in clone 58-861. Nitrogen content and $\Delta^{13}C$ results pointed to a stomatal component as the main limitant of photosynthesis in all clones. Adige and Lux showed enhanced photoprotection as indicated by the size and the de-epoxidation index of the xanthophyll-cycle pool, and also improved antioxidant defence displayed by higher ascorbate, reduced glutathione, total phenolics and α-tocopherol levels. Photoprotective and antioxidative responses allowed all clones to maintain a high maximum quantum yield of PSII (F_v/F_m) with the exception of Luisa Avanzo and 58-861 which experienced slight photoinhibition in late spring. The study of susceptibility to rust (*Melampsora* sp.) and lace bug (*Monosteira unicostata* Muls. and Rey) infections showed Adige and Lux to be the most tolerant. Overall, these two clones presented high adaptability to summer conditions and improved resistance to abiotic and biotic stress, thereby making them highly commendable clones for use in environmental remediation programmes.

Keywords: biotic stress, chloroplast pigments, photochemistry, poplar, water stress, water use efficiency.

Introduction

Poplars (*Populus* spp.) are fast-growing trees, traditionally used as a source of fuel, fibre, lumber and plywood. They are widely cultivated due to their high adaptability to different climate conditions and their large biomass yield. Poplars can be cultivated under short rotation coppicing, a management regime whereby plants are repeatedly cut back to a low stump (every 2–6 years) with the subsequent production of new shoots from the stump and/or roots (Blake 1983), thus allowing one to obtain 8–10 kg of dry biomass per plant per year (Ceulemans et al. 1992, Scarascia-Mugnozza et al. 1997, Kauter et al. 2003).

Furthermore, poplar is a model tree for forest plant genomics (Jansson and Douglas 2007). The *Populus trichocarpa* (Torr. and Gray) genome was completely sequenced (Tuskan et al. 2006) and a highly informative genetic mapping of *Populus nigra* L. species was carried out (Gaudet et al. 2008), thus representing a valuable tool in the identification of the genetic determinants of biomass production and environmental adaptation. Moreover, several studies have focused on the poplar genome-based analysis of qualitative and quantitative trait loci to evaluate the genetic control of some characteristics such as resistance to rust (Newcombe et al. 1996, Jorge et al. 2005), biomass yield and its distribution throughout the tree (Wullschleger et al. 2005), adaptation to elevated CO_2 (Rae et al. 2007), response to drought (Street et al. 2006) and cadmium tolerance (Induri et al. 2012). In recent years, the value of poplars in terms of ecological services has been highlighted (Tognetti et al. 2013). Among these, the ability of poplar species to mitigate the environmental impacts of human activities has received particular attention. In fact, the fast growth rate which characterizes these plants is of particular interest for the purposes of the sequestration of atmospheric CO_2 (Calfapietra et al. 2010) or the reduction of metal pollution in soils and water (Laureysens et al. 2004, Fernàndez et al. 2012). Adaptability to growth under pedo-climatic unfavourable conditions, i.e. low water and nutrient availability, is an important pre-requisite of candidate plants for phytoremediation. Therefore, the evaluation of a plant's ability to overcome growth factor limitations other than soil pollution is a very important step in order to assess its real potential in terms of phytoremediation in open-field applications. The importance of exploring the large variability which characterizes the *Populus* genus in order to evaluate genotypes with superior potential for phytoremediation has been raised (Dos Santos et al. 2007, Pietrini et al. 2010).

With regard to biotic stress factors in poplars, leaf rust caused by species of the genus *Melampsora* (Basidiomycota, Uredinales) is considered to be one of the most widespread and devastating diseases (Pinon and Frey 2005). *Melampsora* spp. depend on living host tissues for their development and reproduction. Their infection induces the depletion of sugars and nutrients, which affect growth, produce premature defoliation and predispose trees to other diseases and pests (Feau et al. 2007). Moreover, leaf rust disease produces a decrease in plant photosynthesis leading to lower biomass production (Agrios 2005). Poplars are also commonly affected by small insects such as lace bugs (*Monosteira unicostata* Muls. and Rey (Het: Tingidae; Miller 2004)). Lace bugs are usually host specific and can be very destructive to plants, feeding on the undersides of leaves by piercing the epidermis and extracting the sap which can result in premature leaf abscission. In poplars, pest management is carried out by using resistant clones or certified plant material, and through the use of good culture practices; for this reason, the determination of clone susceptibility is indispensable.

The aim of the work was to study the adaptability of selected poplar clones to environmental constraints typical of the Mediterranean climate, and their resistance to fungus (genus *Melampsora*) and insect (genus *Monosteira*) infections by tracking physiological characteristics and responses during growth. The poplar clones *Populus deltoides* Batr.—Lux clone; *Populus nigra* L.—58-861 clone and *Populus* × *canadensis* Mönch. Luisa Avanzo and I-214 clones were chosen for their outstanding performances in phytoremediation (Zacchini et al. 2009, Pietrini et al. 2010) while *Populus* × *canadensis* Mönch. Adige clone was chosen for its remarkable survival rate and resistance to mosaic virus (Facciotto and Frison 1999). Physiological characterization included parameters related to water relations and productivity, ^{13}C isotopic discrimination ($\Delta^{13}C$) and photochemistry. Chloroplast pigments, antioxidants (ascorbate (AscA), reduced glutathione (GSH) and total phenolics (TPhe)) and chlorophyll fluorescence measurements provided information on electron transport characteristics, excess energy dissipation processes and reactive oxygen species (ROS) scavenging and detoxification activities. The evaluation of different responses against abiotic and biotic stress of the selected clones will provide information for the selection of poplar clones that display a high growth capacity under adverse conditions such as those experienced in sites in which environmental restoration is required.

Materials and methods

Experimental site and plant material

Adult plants of five female poplar clones (*Populus deltoides* Batr. (Cottonwood)—Lux clone; *Populus nigra* L. (black poplar)—58-861 clone and *Populus* × *canadensis* Mönch. (hybrid black poplar)—Luisa Avanzo, I-214 and Adige clones) grown since 2001 in the IBAF-Institute experimental field near Rome in the Tevere valley were used for this experiment performed in 2009. Each clone was represented by 10–30 individuals and localized in multiple parcels characterized by a single-row design with inter-row distances of 220 cm and a spacing of 60 cm between plants within the row. Three randomly selected plants per clone were used for measurements and sampling. The soil is a typical alluvium and the climate is Mediterranean, with a mean maximum temperature registered in 2009 of 27.6 °C, a mean minimum temperature of 3.63 °C and an annual rainfall of 927.6 mm. Climatic data during the study were recorded at the Monterotondo (Rome) meteorological station (Italian Agrometeorological Network, CMA-EnteCRA, Italy) (Table 1).

Sampling and measurements

Sampling was performed in spring (18–25 May), early summer (6–13 July) and late summer (14–21 September) at midday (13.30–16.30 h) local time. Samples were obtained by

Table 1. Climatological data at Monterotondo (Rome) obtained from the Italian Agrometeorological Network (CMA-EnteCRA, Italy) for 1 month before the periods that samples and measures were taken.

	Spring 18.IV–18.V	Early summer 06.VI–06.VII	Late summer 14.VIII–14.IX
Total daily precipitation (mm)	75.60	142.40	38.20
Mean daily minimum temperature (°C)	8.78	14.67	17.04
Mean daily maximum temperature (°C)	23.49	29.97	33.69
Mean daily solar radiation (kJ/m^2)	21,068.45	27,193.77	22,984.39

selecting fully developed leaves from the entire flush of growth which were exposed to sun irradiance, omitting leaves formed with bud break and the youngest leaves of the growth flush. Two samples of 12–21 south-facing and fully developed leaves were randomly selected between 2 and 4 m height from three plants of each *Populus* clone (six samples per clone). Samples were immediately frozen in liquid nitrogen, stored in the laboratory at −80 °C, lyophilized (Virtis Lyophiliser, Freezemobile 6EL, Gardiner, NY, USA) and milled in a Cyclotec 1093/Foss Sample Mill (Tecator, Höganäs, Sweden) until analyses were carried out. Measurements of the relative water content (RWC), leaf biomass parameters, chlorophyll fluorescence and leaf reflectance were performed during the same sampling weeks at midday on three south-facing leaves of three trees per clone.

Relative water content and leaf biomass parameters

Relative water content was determined as $[(M_f - M_d)/(M_{fs} - M_d)] \times 100$, with M_f being plant fresh mass; M_{fs}, plant fresh saturated mass (after rehydrating samples for 24 h in darkness at 4 °C); and M_d plant dry mass (after oven-drying at 60 °C until a constant weight was achieved). Leaf area (LA) was determined with a Laser Leaf Area Meter (CI-203) (CID, Inc., Camas, WA, USA). Leaf mass per area (LMA) was determined as M_d/LA, and its components leaf thickness (T) and leaf density (D) were calculated as (M_f/LA) and $[(M_d/M_f) \times 100]$, respectively (Niinemets 1999).

Leaf reflectance measurements

The analysis of foliar reflectance (Peñuelas and Filella 1998) allowed for non-destructive evaluation of hydric responses by means of the calculation of the water index (WI). Leaf reflectance was measured with a portable spectral analysis system with artificial light (USB4000, Oceanoptics), operated with Spectrasuite (Oceanoptics) software. Ten scans per sample were integrated (integration time 50 ms). The WI was derived from the spectra and calculated as R_{900}/R_{970} where reflectance at 970 nm is associated with water absorption and 900 nm is a reference wavelength (Peñuelas et al. 1997). Methodological problems did not allow us to obtain WI values in May.

Leaf carbon isotope composition and nitrogen content

Carbon isotopic composition ($\delta^{13}C$) and total nitrogen (N) content were determined with ~1 mg of lyophilized material with an elemental analyser (EA1108, Series 1, Carlo Erba Instrumentazione, Milan, Italy) coupled to a mass spectrometer (Delta C, Finnigan MAT, Bremen, Germany). $\delta^{13}C$ values were determined using a standard reference calibrated against Pee Dee Belemnite carbonate and used to estimate $\Delta^{13}C$ as: $\Delta^{13}C = 1000 \times (\delta_a - \delta_p)/(1 + \delta_p)$, where δ_a and δ_p are values for air ($\delta^{13}C = -10.5\%$) and the plant, respectively (Farquhar et al. 1989).

Chlorophyll fluorescence

Minimum fluorescence yield (F_o), maximum fluorescence yield (F_m) and maximum quantum yield of PSII photochemistry (F_v/F_m) (equivalent to $(F_m - F_o)/F_m$) were determined in dark-adapted leaves (at least 40 min) with a Mini-Pam Photosynthesis Yield Analyser (Walz, Effeltrich, Germany).

Chloroplast pigments

Light-exposed leaf samples were collected for chloroplast pigment analysis from the same flush where chlorophyll fluorescence measurements were made. Chloroplast pigments and α-tocopherol (α-Toc) were extracted from 50 mg of lyophilized material with pure acetone buffered with $CaCO_3$ and centrifuged twice at 12,000 *g*. The supernatants were filtered through 0.2-µm PTFE filters (Teknokroma, Barcelona, Spain). The pigments were separated by high-performance liquid chromatography on a reversed-phase C18 column (Waters Spherisorb ODS1, 4.6 × 250 mm, Milford, MA, USA) and detected with a photodiode array detector, according to the method by García-Plazaola and Becerril (1999, 2001). Tocopherol detection and quantification were performed with a Scanning Fluorescence Detector (Waters 474) that was operated in series with the photodiode array detector according to García-Plazaola and Becerril (1999, 2001). The relative de-epoxidation state of the xanthophyll-cycle pigments was estimated by the ratio (A + Z)/(V + A + Z), abbreviated AZ/VAZ.

Ascorbate and glutathione

Fifteen mg of lyophilized leaf samples were extracted with metaphosphoric acid (6% (w/v)) and centrifuged at 10,000 *g* at 4 °C. Ascorbate content was determined according to Foyer et al. (1983) and measured by the change in A_{265} using a spectrophotometer (Cecil Aquarius, CE7400, Cecil Instruments, Cambridge, UK). Glutathione content was obtained according to the Noctor and Foyer protocol (1998). Reduced glutathione was determined and measured by the change in A_{412}.

Total phenolics

Lyophilized leaves were homogenized with 20 volumes of an 80% methanol solution, extracted twice and centrifuged at 14,000 *g* for 10 min. Total phenolics content was determined according to Singleton and Rossi (1965) and measured by A_{735} (with a spectrophotometer; Perkin Elmer, Norwalk, CT, USA). Total phenolics were expressed as gallic acid equivalents.

Incidence and severity of rust infection and incidence of lace bugs

The incidence of *Melampsora* sp. and *M. unicostata* was evaluated in each clone on the basis of the percentage of individuals showing visual symptoms (presence of rust pustules/lace bug faeces). The incidence in each infected individual was evaluated by the percentage of infected leaves from 15 randomly selected leaves per individual and in three individuals per clone. The degree of severity was established from the percentage of LA covered by *Melampsora* pustules (uredinia) as shown in Table 2. The study of both pathogens was performed in September when rust infection typically reaches a more advanced stage.

Statistical analysis

Statistical procedures were performed using SPSS for Windows vs. 15.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance was used to test the main effects against appropriate error terms, of clone and season (spring, early summer, late summer) on the measured parameters. A multiple comparison test of the means using the Duncan post hoc test was applied.

Results and discussion

Water relations, Δ¹³C and structural attributes of leaves

The Mediterranean climate is characterized by summer droughts with significant consequences for plant growth (Mitrakos 1980, Camarero et al. 2010). In May, for 3 weeks before the start of our study, rainfall was scarce (~4.6 mm) and climatological data indicated that the lowest precipitation and the highest temperatures occurred in late summer (Table 1). The decline in water availability during the summer drought was observed by the results obtained for hydric and leaf structural parameters. In fast-growing species, such as poplar, productivity is highly dependent on water availability (Tschaplinski et al. 1994) and leaf structure (Ceulemans 1990). Improved water relations were observed in Adige and Lux during growth. These two clones showed the highest RWC at the end of the summer (September) (Figure 1a). A decrease in RWC from May to September was observed in all clones except in Lux, which showed constant values. Relative water content in May (above 90% in all clones) might be related to stomatal closure and to a reduction in cellular expansion. In September, RWC was <80% in L. Avanzo, I-214 and especially in 58-861, which was reflected in a leaf water potential equal to or lower than –1.5 MPa and pointed to a high water deficit in plants and consequent metabolic changes such as photosynthesis disruption, an increase in respiration, and proline and abscisic acid accumulation (González and González-Vilar 2001). Since the radiometric index WI is related to stomatal conductance (g_s) (Serrano et al. 2010), WI results showed

Table 2. Degrees of severity of the foliar infection produced by *Melampsora* sp. Each percentage of affected LA corresponds to a degree of rust severity.

Severity degree	% Affected LA	Infection level
1	0	Absent
1.5	<1	Traces
2	1–5	Mild
3	6–25	Moderate
4	26–50	Severe
5	>50	Very severe

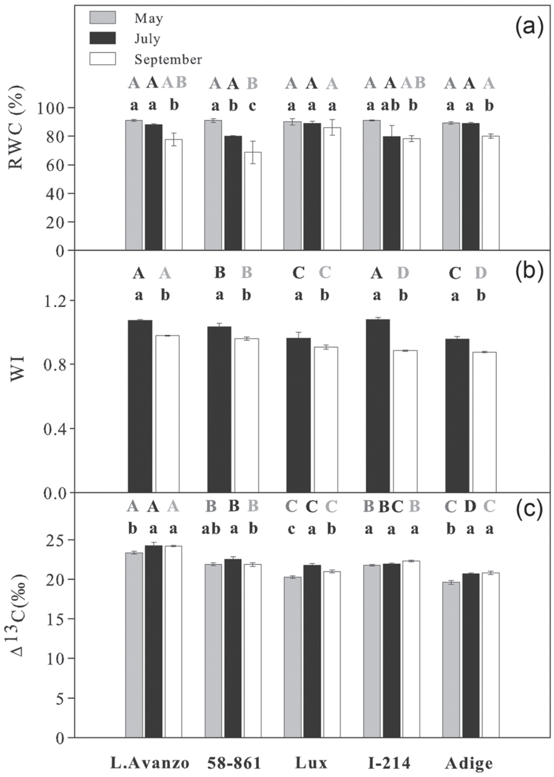

Figure 1. (a) % Relative water content, (b) water index and (c) Δ¹³C of the different clones throughout the growing season (May, July, September). Different letters correspond to significant differences at $P < 0.05$ between clones (A–C) or between sampling periods (a–c). Values are the mean ± SE of three replicates on three plants per clone. Methodological problems did not permit to obtain WI values in May.

lower g_s in Adige and Lux in July and September. Water index values also indicated that there was a decline in leaf water content in September in all clones (Figure 1b).

^{13}C isotope discrimination is negatively correlated with time-integrated water-use efficiency (WUE) in C3 plants (Knight et al. 1994), defined as the ratio of biomass accumulation to transpiration. The lower $\Delta^{13}C$ in Adige and Lux and higher $\Delta^{13}C$ in L. Avanzo (Figure 1c) reflected their differences in WUE, as was also demonstrated in RWC and WI results, previously discussed. In Adige and Lux, a $\Delta^{13}C$ of ~2 per mil lower than that of other clones accounted for the increase in WUE of ~30% (Ehleringer et al. 1992). ^{13}C isotope discrimination varies with the relative contribution of enzymatic and diffusional fractionation processes (Farquhar and Richards 1984). Results confirm that Adige and Lux displayed the lowest g_s as expected from WI data. High WUE in May (except in I-214) and in September in 58-861 and Lux would indicate changes in g_s that could contribute to maintaining a high RWC.

The seasonal reduction in water availability was also reflected in leaf structure (Peña-Rojas et al. 2005). Lux, Adige and I-214 displayed improved leaf structural adaptations during their growth period in order to maintain high water content as RWC, WI and $\Delta^{13}C$ results demonstrated, particularly in the two first clones. The highest LA was observed in Lux and the lowest in 58-861 (Figure 2a). High-yielding clones display a larger total LA and individual LA in a range of *P. deltoides* × *P. nigra* clones (Marron et al. 2005, Monclus et al. 2005). In L. Avanzo, Lux and Adige, a notable increase in LA occurred from May to July; whereas in L. Avanzo and Lux, the trend showed a decline from July to September. A decline in LA reflects the limitations due to water loss through transpiration during summer. On the other hand, Adige and Lux showed the highest LMA during the study (Figure 2b), where the increase in LMA during ontogeny is related to an increase of leaf dry mass accumulation after expansion ceases, cuticle thickening and secondary cell wall development (Marron et al. 2008). Leaf mass per area increased in September in all clones except in I-214. The two components of LMA, thickness (*T*) and density (*D*), are not necessarily interdependent and may be controlled by different environmental variables (Witkowski and Lamont 1991). The increase in leaf *T* in 58-861, Lux and Adige during growth (Figure 2d) explains the additional mesophyll layers which imply a higher photosynthetic capacity (Hanba et al. 2002). From May to July, clones showed a decrease in *D* whereas after July it increased (Figure 2c). An increase in *D* is associated with thicker cell walls and with smaller and more tightly packed cells (Niinemets 2001). Increases in LMA are due to an increased *D* as reported for greenhouse-grown cuttings during drought (Marron et al. 2003).

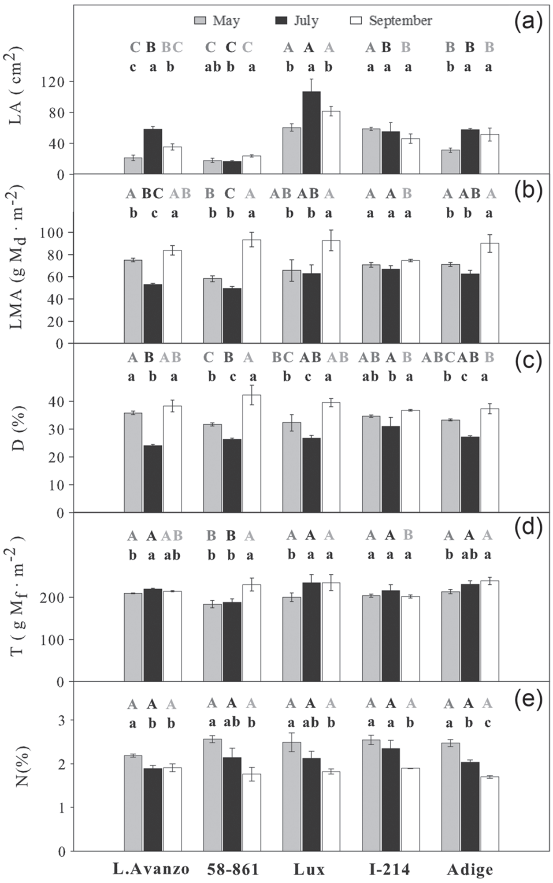

Figure 2. (a) Leaf area, (b) leaf mass per area, (c) leaf density (*D*), (d) leaf thickness (*T*) and (e) N content of the different clones throughout the growing season (May, July, September). Different letters correspond to significant differences at $P < 0.05$ between clones (A–C) or between sampling periods (a–c). Values are the mean ± SE of three replicates on three plants per clone.

Nitrogen content, considered an indicator of productivity (Ceulemans 1990), declined in all clones (Figure 2e), reflecting its remobilization during plant ontogeny. We observed a negative correlation between N and LMA values in Lux ($r^2 = -0.75$, data not shown), whereas no relationship was obtained between $\Delta^{13}C$ and LMA values in any clone. The observed independence of $\Delta^{13}C$ (and therefore of WUE) from biomass parameters would indicate that there is a potential to improve WUE in poplar without necessarily reducing the overall productivity (Monclus et al. 2005, Chamaillard et al. 2011).

Photoprotection and antioxidant response

The maximum quantum yield of PSII (F_v/F_m) has been used as an indicator of the functional state of the photosynthetic apparatus. Most of our *Populus* clones showed values within the typical range for non-severely stressed plants (0.75–0.85, Björkman and Demmig 1987) and maximum values were reached in September (Figure 3a). Throughout the period of study, Adige showed the highest F_v/F_m values followed by I-214 and Lux. The slightly but significantly lower values of F_v/F_m for

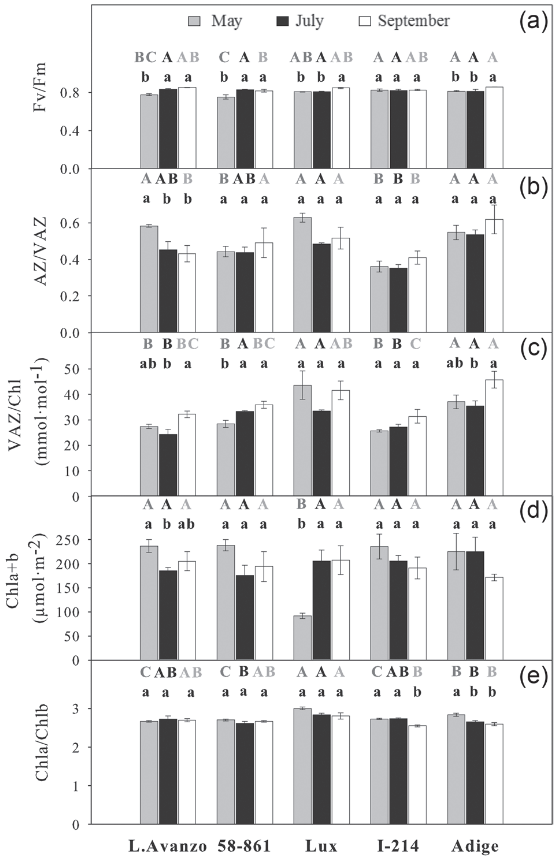

Figure 3. (a) Maximum quantum yield of PSII (F_v/F_m), (b) de-epoxidation degree of xanthophyll cycle pigments (AZ/VAZ), (c) total content of xanthophyll cycle pigments (V + A + Z/Chl), (d) total chlorophyll content (Chlorophyll *a* + *b*) and (e) Chl*a*/*b* ratio of the different *Populus* clones throughout the growing season (May, July, September). Different letters correspond to significant differences at $P < 0.05$ between clones (A–C) or between sampling periods (a–c). Values are the mean ± SE of six replicates (12–21 leaves each one) per clone for chlorophyll pigments, and three replicates on three plants per clone for F_v/F_m measures.

L. Avanzo and 58-861 clones in May indicated a slight photoinhibition which was probably related to the water deficit in this period (Flexas et al. 2004).

Summer water stress normally induces an increase in leaf stomatal closure and a consequent low CO_2 assimilation (Lawlor and Cornic 2002). This fact can lead to a dangerous excess of excitation energy in the thylakoids. To counteract it, plants are able to dissipate part of the excessive absorbed light energy as heat through a process mediated by the de-epoxidation of xanthophyll-cycle pigments (Demmig-Adams and Adams 2006). The values of the de-epoxidation index (AZ/VAZ) observed in the *Populus* clones varied within a range of 0.36–0.62, indicating different levels of photoprotection (Figure 3b). Lux and Adige showed the highest capacity for excess energy dissipation of heat during the whole growing season, while I-214 showed the lowest AZ/VAZ values. September was shown to be the month with the highest energy dissipation in clones except for L. Avanzo and Lux. Concomitantly, the highest VAZ/Chl ratio was reached in September for most of the clones, with Lux and Adige showing the highest levels of all the clones throughout the growing season (Figure 3c). Lux presented markedly lower photosynthetic pigment content in May (e.g., Chla + Chlb, Figure 3d) probably due to an immature state of the leaves. At this stage, the high VAZ/Chl and AZ/VAZ observed might indicate that zeaxanthin (Z) offers protection against excessive light during leaf development (Schindler et al. 1994).

Total chlorophyll content declined over the course of the growing season in all clones (Figure 3d). This fact occurred in parallel with the remobilization of N and also with an increase of leaf *D*. Lux showed the highest Chla/Chlb ratio (Figure 3e) during growth, indicating an improved adaptation to high light intensities.

In addition to its role in excess energy dissipation, Z together with other lipophilic molecules of the chloroplast, such as β-carotene (β-Car) and α-Toc, plays a fundamental role in the plant antioxidant response. Zeaxanthin and β-Car are efficient quenchers of triplet Chl and $^1O_2$ (Young and Britton 1990, Havaux et al. 2007, Gill and Tuteja 2010, Dall'Osto et al. 2012), while α-Toc scavenges lipid peroxyl radicals in photosynthetic membranes (Trebst et al. 2002). Furthermore, the three antioxidants can interact synergistically as radical scavengers (Böhm et al. 1997, Havaux and Niyogi 1999). In our study, no remarkable differences were noticed in β-Car content among clones and the months of study, but considerable differences were noticed in α-Toc content (Figure 4a and b). A marked increase occurred in September in all clones especially in Adige and 58-861 probably as a result of both an enhanced antioxidant response and ontogenic accumulation of α-Toc during leaf ageing (García-Plazaola et al. 2003).

Among the hydrophilic antioxidants, ascorbate and glutathione are considered to be the most important intracellular defences against ROS-induced oxidative damage and are localized in the main cell compartments (Gill and Tuteja 2010, Gest et al. 2013). On the other hand, phenolics, mainly localized in vacuoles and the cell wall, are series of diverse secondary metabolites (flavonoids, tannins, hydroxycinnamate esters and lignin) that are abundant in plant tissues (see the review by Grace and Logan 2000), and which play a double role as antioxidants and on plant–herbivore interactions (Reichardt et al. 1991). Ascorbate is quantitatively the most abundant antioxidant in plant tissues (Noctor and Foyer 1998) and interacts with ROS, particularly with H_2O_2, and is required for the regeneration of α-Toc (Beyer 1994) and the formation of Z (Müller-Moulé et al. 2002). Moreover, effective resistance to prolonged stress requires increased AscA regeneration, a process partially dependent on GSH in higher plants (Foyer and Halliwell

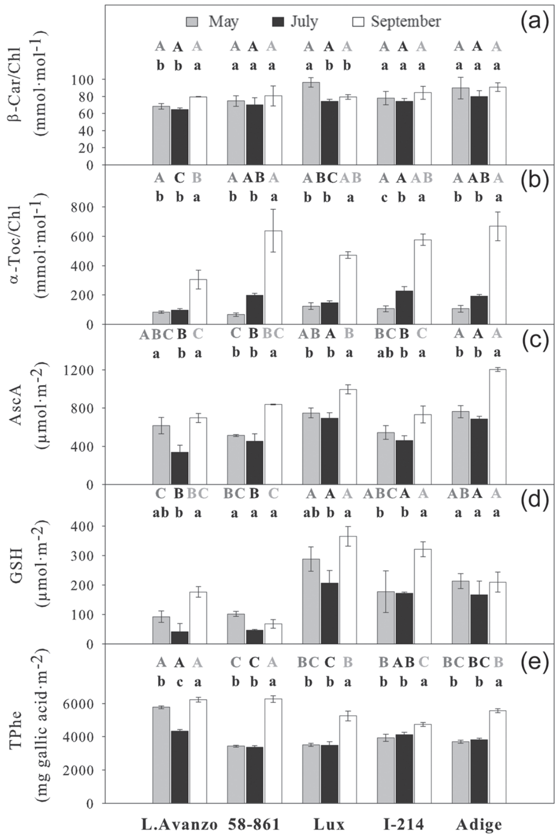

Figure 4. (a) β-carotene content, (b) α-tocopherol content, (c) total ascorbate content, (d) reduced glutathione content and (e) total phenolics content of the different *Populus* clones throughout the growing season (May, July, September). Different letters correspond to significant differences at $P < 0.05$ between clones (A–C) or between sampling periods (a–c). Values are the mean ± SE of six replicates (12–21 leaves each one) per clone.

1976). This was further correlated in Adige and Lux clones (with high values of AZ/VAZ and α-Toc), which displayed the highest AscA content during the growth period and (however only in the case of Lux) also the highest GSH content (Figure 4c and d). On the other hand, 58-861 displayed low GSH values in September, whereas total glutathione increased (data not shown), indicating that most glutathione was in an oxidized state and that this clone had more difficulties than others in maintaining the glutathione pool in a reduced state under long-term stress conditions.

Following a similar trend to that of AscA and GSH content, high TPhe levels were observed in all clones in September and also in L. Avanzo in May (Figure 4e). This clone showed the highest TPhe levels of all clones throughout the growing season. The hydrophilic antioxidant levels and the previously mentioned increase in the photoprotective mechanisms in L. Avanzo in May point to a greater requirement in this clone for protection mechanisms in this period.

Overall, *Populus* clones showed higher antioxidative responses in September probably due to the water deficit conditions and the advanced ontogenic state of the plants. High F_v/F_m (>0.8) in this period might indicate that antioxidant protection mechanisms were working efficiently in all clones. High AscA and GSH levels in September could be related to α-Toc and Z synthesis.

Susceptibility to infection

All clones showed a remarkable presence of rust infection except for Adige in which only 20% of individuals were affected and ~14% of their leaves showed signs of infection (Table 3). Luisa Avanzo displayed signs of severe rust infection (levels 4–5) in ~86% of leaves, whereas Adige was the most resistant clone revealing the lowest degree of infection only presenting traces of the fungus (level 1.5) in 11% of leaves. Lux

Table 3. Lace bugs and rust incidence and rust severity degree in *Populus* clones in September. The incidence of both pathogens was evaluated on the basis of the percentage of individuals of each clone showing visual symptoms of the disease. The incidence in each individual was evaluated by the percentage of the number of infected leaves from 15 randomly selected leaves in 3 infected individuals per clone. Rust severity was considered as the degree of the infection in an individual. This parameter was determined in three plants per clone. For each individual, we examined 24 leaves (3 leaves per position, facing north, south, west and east, and at two heights: at the base (2 m from the soil surface) and top of the plants). The degree of severity was established from the percentage of LA covered by *Melampsora* rust.

	L. Avanzo	58-861	Lux	I-214	Adige
% Lace bug incidence					
% Individuals	0	100	0	100	0
% Leaves	0	100	0	93.3	0
% Rust incidence					
% Individuals	100	100	100	100	20
% Leaves	100	86.6	73.3	93.3	14.1
Degree of rust severity					
1	0.0	25.0	23.6	1.4	88.9
1.5	0.0	40.3	44.4	20.8	11.1
2	0.0	19.4	15.3	27.8	0.0
3	19.4	13.9	13.9	30.6	0.0
4	45.8	1.4	2.8	19.4	0.0
5	40.3	0.0	0.0	0.0	0.0

showed a low degree of severity of the infection and 58-861 and I-214 were affected mildly. *Melampsora* rust has been reported to affect chlorophyll, water relations and photochemical processes in sensitive clones (G. Elena, J. Fernàndez-Martínez, M. Zacchini, A. Moret and I. Fleck, unpublished data). With respect to lace bugs (*M. unicostata*), we observed a marked presence of the insect in 58-861 and I-214 leaves (Table 3), whereas in L. Avanzo, Lux and Adige the faeces of the insect were not found. The susceptibility of L. Avanzo and 58-861 clones to infection by these pathogens could be related to increased ROS formation in September (Tiedemann 1997) and/or to their lower pool of glutathione that can play an important role in biochemical reactions against the development of the disease (Kuzniak and Sklodowska 1999).

Concluding remarks

We conclude that changes in leaf structure in Adige and Lux contributed to maintain highest RWC during summer drought. This fact, in addition to their improved photoprotective and antioxidant responses, contributed to the preservation of PSII functionality under periods of water deficit. Furthermore, these two clones showed very low susceptibility to *Melampsora* sp. and *M. unicostata* infections. The evaluation of the adaptation to environmental constraints represents a primary step for the selection of poplar clones for different uses of this multifunctional wood species. This study highlighted the suitability of Lux and Adige for cultivation in unfavourable abiotic and biotic conditions, opening up interesting prospects for their use in environmental restoration in the Mediterranean region.

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Conflict of interest

None declared.

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Capítol 4: Respostes fotosintètiques i de creixement dels clons de *Populus* I-214 i Eridano sotmesos a concentracions elevades de Zn

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Photosynthetic and growth responses of *Populus* clones Eridano and I-214 submitted to elevated Zn concentrations

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ABSTRACT

The effect of two toxic levels of Zn were analysed in two poplar clones (*Populus deltoides* x *maximowiczii* — clone Eridano, and *Populus* x *canadiensis euramericana* Mönch. — clone I-214), both of which are commonly used in plantations in Italy. We examined their capacity to accumulate and tolerate toxic levels of Zn and the effect of this accumulation on plant growth and development, leaf morphology, gas-exchange and nutrient composition. Rooted cuttings were treated for three weeks in hydroponic culture with 0 (control), 1 mM and 5 mM zinc chloride ($ZnCl_2$). Both clones showed Zn toxicity effects in their physiological responses, especially at 5 mM [Zn], but Eridano displayed high tolerance at 1 mM [Zn]. Growth and biomass production declined mainly in I-214, with a severe reduction of root growth. Biomass parameters and tolerance index (Ti) indicated a higher capacity of Eridano to tolerate toxic levels of Zn. Leaf morphological changes were observed, and appeared earlier in I-214 at 1 mM [Zn], with a decline in leaf area and thickness, and an increase in leaf density. Gas-exchange parameters pointed to a drastic decrease in net photosynthesis (A), stomatal conductance (g_s) and transpiration rate (E) at 5 mM [Zn], although Eridano showed good tolerance at 1 mM [Zn]. With increasing Zn in the nutrient solution, Zn concentration increased in all plant structures, accumulating mainly in roots, especially in Eridano. The heavy metal accumulation was similar at 1 mM and 5 mM [Zn] for each clone, and the capacity of both clones to take up Zn from the medium was reduced. The concentration of Zn accumulated in young leaves accounted for the decline observed in biomass, photosynthesis, Fe and chlorophyll content. In hydroponic culture with highly toxic Zn levels, Eridano showed a higher capacity of accumulation, translocation to aerial parts, and tolerance than I-214, thus appearing as a very promising clone for the phytoremediation of Zn-polluted substrates.

1. Introduction

Zinc (Zn) is an essential micronutrient and a component of thousands of proteins involved in plant metabolism (nitrogen metabolism, photosynthesis and auxin synthesis) (Marschner, 1995, and review by Broadley et al., 2007). At appropriate concentrations zinc increases plant productivity, growth and development of fruits. However, at supraoptimal concentrations it becomes toxic, with negative effects on ion transport and metabolic processes, such as transpiration, photosynthesis and enzymatic activities related to nitrogen metabolism, and it inhibits growth (Cakmak, 2000; Vaillant et al., 2005).

Increasing pollution due to heavy metals has become a serious environmental concern (Alkorta et al., 2004), and zinc concentration in water and soil has risen as a result of human activities such as mining or production of wastewater. Excess Zn can affect the uptake of other nutrient elements such as Cu, Mn and Fe, and the deficiency of these elements may cause oxidative stress (del Río et al., 1991). High levels of Zn may also contribute to the formation of reactive oxygen species (ROS) that can directly affect plant growth, photosynthetic activity, water relations and metabolism (Cuypers et al., 2001). Since plants are at the bottom of trophic chains, high concentrations of Zn in plants also generate secondary problems. Zn excess displaces other metals from active sites in proteins and also induces chlorosis in young leaves due to the fact that Zn has a similar ion radius to Fe or Mg (Marschner, 1995).

In recent years, the capacity of plants to accumulate metals in their organs has been proposed as a suitable tool for the remediation of metal-polluted substrates. This natural technique, called phytoremediation, can allow the reduction or complete removal of mild and diffuse metal contamination of soils and waters. It also avoids the heavy impacts on ecosystems and the high costs associated with traditional techniques (Cunningham and Ow, 1996). In this context, the potentiality of Salicaceae, such as poplars and willows, for the remediation of soil and water from metal pollution has been highlighted (Kuzovkina and Quigley, 2005; Laureysens et al., 2004; Meers et al., 2007). In particular, it has been reported (Dos Santos Utmazian et al., 2007; Pietrini et al., 2010; Zacchini et al., 2009) that metal-treated poplars

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showed good tolerance, a high bioconcentration factor and suitable genotype variability for the traits that are useful for phytoremediation. In fact, poplars are characterised by high variability and adaptability to environmental constraints, in addition to having an extended root system and a low impact on trophic chains. Poplars also grow relatively fast, and they are widely used in agroforestry because of their high biomass production, which can be used to generate heat or electric power. The possibility of obtaining both ecological benefits (*i.e.* remediation and energy production) through a particular plant cultivation management technique, called short rotation coppicing (SRC), has recently been proposed for Salicaceae (Witters et al., 2009). SRC relies on the intensive cultivation of fast-growing trees at close spacing, in short productive cycles with recurrent plant cutting at the base of the trunk and the harvest of the above-ground biomass.

All these growth and cultivation characteristics indicate that poplars are highly suitable candidates for phytoremediation. Thus, studies examining the physiological performance of poplars exposed to metals are of particular interest. Their aim is to identify the biological processes that enable poplars to tolerate and accumulate metals in their tissues, and thus evaluate their potential for phytoremediation. For this purpose, hydroponics studies are commonly performed to evaluate the effects of metal exposure on poplars (Borghi et al., 2007; Dos Santos Utmazian et al., 2007; Gaudet et al., 2011; Pietrini et al., 2010) at physiological, biochemical and molecular levels. These results are reported to be highly comparable with those obtained by growing plants on a large in upper scale systems (Watson et al., 1999; Zacchini et al., 2009). In this context, an *in vitro* culture approach has also been proposed as a suitable method to investigate the effects of metal treatment on poplars, and to ascertain the phytoremediation potentiality of these plants without the limitation imposed by metal bioavailability (Castiglione et al., 2007; Di Lonardo et al., 2011; Iori et al., 2012). It is also worth mentioning that the poplar has recently emerged as a model tree for forest science in studies addressing molecular, morpho-physiological and biochemical mechanisms (Jansson and Douglas, 2007; Wullschleger et al., 2002;) and that the genome of *Populus trichocarpa* has been completely sequenced (Tuskan et al., 2006).

The aim of this study was to evaluate the capacity of rooted cuttings of poplar clones Eridano (*Populus deltoides* x *maximowiczii*) and I-214 (*Populus* x *canadiensis euramericana* Mönch.), exposed to two toxic levels of Zn to tolerate and concentrate the metal in plant parts. Zinc concentrations were chosen according to Di Baccio et al. (2005), which evaluated the toxicity of this metal on poplar plants.

Trees of these clones, are commonly used in north-Italian plantations for biomass production. When grown on soil amended with industrial wastes, they exhibit different metal accumulation potential (Sebastiani et al., 2004). Moreover, the clones showed differing antioxidant capacity in the response to ozone stress (Di Baccio et al., 2008). In our study, Zn concentration was measured in roots, shoots, and leaves that had fully expanded before the Zn treatment, and in leaves that fully expanded after the treatment, in order to evaluate Zn distribution throughout plant development. The effects of the metal on plant growth, the morpho-anatomical characteristics, the gas-exchange parameters and the absorption of other metals and nutrients were also measured to assess the potentiality of these clones for phytoremediation purposes.

2. Materials and methods

2.1. Plant material and treatments

Rooted cuttings (20 cm long) of poplar clones, Eridano (*P. deltoides* x *maximowiczii*) and I-214 (*P.* x *canadiensis euramericana* Mönch.) were grown in greenhouse at 65%–75% relative humidity, 26 °C, and maximum photosynthetic photon flux density (PPFD) of around $1000\ \mu mol \cdot m^{-2} \cdot s^{-1}$ at midday. Thirty six plants were grown in hydroponic culture for three weeks. Six plants per treatment were taken at random and were supplied twice a week with 6 L of nutrient solution (Moore, 1974) with 0 (control), 1 mM and 5 mM zinc chloride ($ZnCl_2$) at pH 6.5. Leaves already present and fully developed before the treatments were designated as old leaves, whereas leaves fully developed after the beginning of the treatments were designated as young leaves. After measurement of gas-exchange and chlorophyll content on attached leaves, plant parts were separated into young leaves, old leaves, stem, woody cuttings and roots for the determination of biomass and morphological characteristics and analysis of their mineral content.

2.2. Biomass and morphological characteristics

The samples were weighed for Fresh weight (FW) and, after oven-drying at 60 °C until constant weight, Dry weight (DW). Leaf area (LA) was measured with a CI 2003 Laser Leaf Area Meter (CI-203) (CID, Inc., Camas, WA 98607, USA). Leaf mass per area (LMA) (corresponding to DW/LA), and its components (FW/LA) and [(DW/FW) · 100], as indicators of leaf thickness (T) and leaf density (D), respectively (Niinemets, 1999), were calculated.

2.3. Gas exchange and total chlorophyll content

Net photosynthesis (A), stomatal conductance (g_s), transpiration (E) and intercellular CO_2 concentration (C_i) were measured on 3–5 old and 3–5 young fully-expanded leaves from three plants per treatment with a gas-exchange system (LI-6400; Li-COR, Lincoln, Nebraska, USA).

Table 1
Biomass characteristics of poplar clones Eridano and I-214 plants submitted to different Zn concentrations in hydroponics. Different letters correspond to significant differences ($P<0.05$) between Zn treatments (A,B), clones (a,b) or kinds of leaf (α, β). Values are mean ± SE of 3–4 plants per treatment.

	Treatment	Clone			
		Eridano		I-214	
Old leaf number	Control	5.67 ± 0.33	Aa	8.33 ± 1.76	Aa
	Zn [1 mM]	5.67 ± 0.88	Aa	6 ± 3.00	Aa
	Zn [5 mM]	5 ± 0.58	Aa	5.00 ± 2.52	Aa
Young leaf number	Control	9.33 ± 0.67	Aa	10 ± 0.00	Aa
	Zn [1 mM]	7.67 ± 1.20	Aa	5 ± 1.00	Bb
	Zn [5 mM]	3.67 ± 0.33	Ba	2.67 ± 0.33	Ca
Stem length (cm)	Control	71.47 ± 2.86	Ab	96.33 ± 11.17	Aa
	Zn [1 mM]	66.17 ± 6.58	Aa	57.40 ± 2.70	Ba
	Zn [5 mM]	41.43 ± 2.39	Ba	49.73 ± 7.12	Ba
Stem diameter (cm)	Control	0.73 ± 0.03	Aa	0.78 ± 0.04	Aa
	Zn [1 mM]	0.7 ± 0.06	Aa	0.6 ± 0.00	Ba
	Zn [5 mM]	0.6 ± 0.06	Aa	0.45 ± 0.05	Ba
Old leaf biomass (g DW)	Control	2.24 ± 0.30	Aaβ	1.97 ± 0.36	Aaβ
	Zn [1 mM]	2.29 ± 0.13	Aaα	1.24 ± 0.35	ABbα
	Zn [5 mM]	2.76 ± 0.25	Aaα	0.83 ± 0.19	Bbα
Young leaf biomass (g DW)	Control	7.18 ± 0.62	Aaα	6.26 ± 0.48	Aaα
	Zn [1 mM]	4.04 ± 0.53	Baα	2.13 ± 0.21	Bbα
	Zn [5 mM]	2.1 ± 0.43	Caα	0.90 ± 0.26	Baα
Stem biomass (g DW)	Control	5.05 ± 0.45	Aa	7.18 ± 1.53	Aa
	Zn [1 mM]	3.56 ± 0.77	ABa	2.56 ± 0.49	Ba
	Zn [5 mM]	1.74 ± 0.15	Ba	1.84 ± 0.64	Ba
Woody cutting biomass (g DW)	Control	8.92 ± 0.08	Ab	15.93 ± 0.80	Aa
	Zn [1 mM]	8.47 ± 0.72	Aa	7.93 ± 1.14	Ba
	Zn [5 mM]	4.50 ± 0.52	Bb	8.88 ± 0.70	Ba
Aerial biomass (g DW)	Control	23.38 ± 1.24	Ab	31.34 ± 3.10	Aa
	Zn [1 mM]	18.36 ± 1.36	Aa	13.86 ± 0.51	Ba
	Zn [5 mM]	11.10 ± 0.95	Ba	12.44 ± 1.78	Ba
Root biomass (g DW)	Control	3.72 ± 0.14	Aa	4.95 ± 0.679	Aa
	Zn [1 mM]	3.42 ± 0.73	Aa	2.54 ± 0.61	Ba
	Zn [5 mM]	1.62 ± 0.09	Ba	0.77 ± 0.10	Ca
Aerial/root biomass (g DW)	Control	6.27 ± 0.11	Aa	6.42 ± 0.41	Ba
	Zn [1 mM]	5.69 ± 0.77	Aa	5.82 ± 1.58	Ba
	Zn [5 mM]	6.91 ± 0.70	Ab	16.22 ± 0.59	Aa
Total biomass (g DW)	Control	27.11 ± 1.37	Ab	36.29 ± 3.71	Aa
	Zn [1 mM]	21.78 ± 2.02	Aa	16.41 ± 0.01	Ba
	Zn [5 mM]	12.72 ± 0.95	Ba	13.21 ± 1.88	Ba

The environmental conditions in the leaf-measuring chamber were established as: PPFD: 1100 μmol·m^{-2}·s^{-1}, sample CO$_2$ (C_a): 370 μmol·mol^{-1}, water mole fraction: 14 ± 1 mol H$_2$O·mol air^{-1}, leaf temperature: 27 °C, VPD: 2 ± 0.3 kPa and air flux: 400 μmol·s^{-1}. Total chlorophyll content of 4 old and 4 young leaves per plant was measured with a leaf chlorophyll metre (SPAD, Minolta, Osaka, Japan).

2.4. Mineral content determination

Dried plant parts were carefully washed in distilled and MilliQ water. Leaves, stems and roots were ground to a fine powder in an agate mortar, and woody cuttings were milled in a cutting mill Pulverisette 15 (Fritsch). 0.8–1 g of plant material was pre-digested in 5 mL of HNO$_3$ overnight at room temperature. Digestion was performed in a digestor block (JP Selecta) at 105 °C for 3 h. A second digestion was done with 2.5 mL HClO$_4$ at 155 °C for 2 h and thereafter at 180 °C for 2 h. Zn, S, K, Mg, P, Fe, Ca, Cu and Mn were measured in the various plant parts by inductively coupled plasma mass spectrometry (ICP-MS PerkinElmer, model Elan-6000) at the Scientific and Technical Services of the University of Barcelona (*UB Serveis Científico-Tècnics*).

2.5. Tolerance index calculation and Zn uptake ratio

The tolerance index (Ti) was calculated as the ratio of the dry mass of plants (aerial parts and roots) grown in the solutions with Zn to that of plants grown in the control solution (Wilkins 1978). The Zn

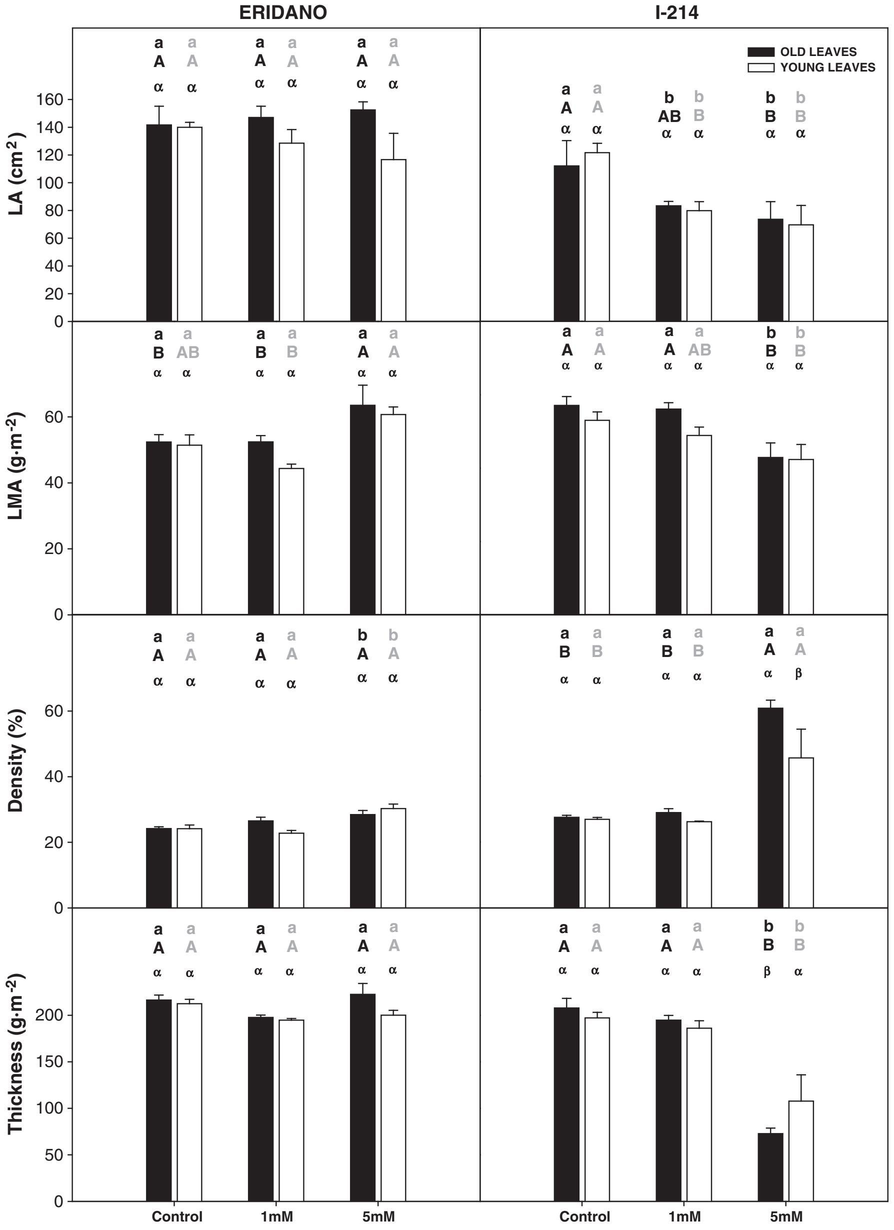


Fig. 1. Morphological leaf characteristics. Leaf area (LA), Leaf Mass per Area (LMA), Leaf density (D) and Leaf thickness (T) in old and young leaves of Eridano and I-214 clones submitted to 0 (control), 1 mM or 5 mM ZnCl$_2$. Different letters correspond to significant differences ($P<0.05$) between Zn treatments (A, B), clones (a, b) or kinds of leaf (young, old) in the same clone (α, β). Values are mean ± SE of measurements on 3–10 full expanded leaves on three plants per treatment.

uptake ratio (Sebastiani et al., 2004) was calculated as the ratio of the total amount of the metal in the whole plant to the total amount value of the metal in the different solutions.

2.6. Statistical analysis

All statistical procedures were performed using SPSS for Windows (v. 15.0, SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to test the main effects and interactions; differences were considered significant where $p \leq 0.05$.

3. Results

3.1. Visual symptoms

Visual symptoms of the toxic effects induced by 1 mM and 5 mM Zn concentration were observed in both clones (not shown). In both clones, young leaves of plants treated with 1 mM [Zn] showed marked chlorosis and some necrosis in sprouts, whereas plants treated with 5 mM [Zn] showed disrupted growth of aerial parts, slight chlorosis in young leaves and a lower development of roots, with a browning appearance compared to control. One third of I-214 plants treated with 5 mM [Zn] showed signs of severe damage (chlorotic and wilted leaves), as a result of which some gas-exchange and total chlorophyll measurements could not be performed.

3.2. Plant biomass characteristics

The plant biomass parameters (Table 1) showed that the number of old leaves was similar in both clones and in all treatment groups, whereas the number of young leaves stem length and stem diameter declined in both clones with increasing Zn concentration, particularly in I-214. The old leaf biomass declined with increasing [Zn] in I-214, whereas no effect was observed in Eridano. Young leaf biomass was affected by [Zn] in both clones. Stems, woody cuttings and root biomass showed slight differences between clones and declined with increasing [Zn]. Aerial, root biomass and total plant biomass declined at 1 mM in I-214 whereas the decline was observed at 5 mM in Eridano. No effect of zinc treatment was observed for the aerial to root biomass ratio in Eridano, whereas a marked increase at 5 mM [Zn] was observed in I-214.

3.3. Leaf morphological characteristics

Leaf area (LA) was not affected by Zn treatment in old or young Eridano leaves whereas it declined markedly in I-214 (Fig. 1). In Eridano leaf mass per area (LMA) showed highest values at 5 mM [Zn] in both old and young leaves, whereas in I-214 LMA decreased in old and young leaves at this concentration. Leaf density (D) and leaf thickness (T) were similar in old and young leaves of Eridano in all treatments whereas in I-214, D increased and T decreased markedly at 5 mM [Zn] in both kinds of leaf, especially old leaves. Young leaves showed differences between clones at 5 mM, with lower values of LMA and T and higher values of D in I-214.

3.4. Gas-exchange and chlorophyll content

In old leaves, net photosynthesis (A), stomatal conductance (g_s) and transpiration rate (E) declined in Eridano at 5 mM [Zn] whereas the decline in A began at 1 mm in I-214 (Table 2). Young leaves showed the effect of [Zn] reducing photosynthesis in both clones at 1 mM, whereas g_s and E were only affected in I-214. Intercellular

Table 2
Net photosynthesis (A), stomatal conductance (g_s), transpiration rate (E), intercellular CO_2 concentration (C_i), and total clorophyll content in old and young leaves of Eridano and I-214 clones submitted to different Zn concentrations. Different letters correspond to significant differences ($P<0.05$) between treatments (A,B), clones (a,b) or kinds of leaf (α, β). Values are mean ± SE of three plants per treatment. Severe damage in I-214 individual of 5mM $ZnCl_2$ treatment permitted the measurement of Total Chlorophyll content in only two plants (*).

	Treatment	Leaf	Clone			
			Eridano		I-214	
A ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Control	Old	11.85 ± 1.10	Abα	18.74 ± 2.51	Aaα
		Young	**15.74 ± 2.23**	**Aaα**	**13.95 ± 2.49**	**Aaβ**
	Zn [1 mM]	Old	14.03 ± 0.71	Aaα	9.1 ± 1.36	Bbα
		Young	**9.09 ± 0.76**	**Baβ**	**2.61 ± 0.85**	**Bbβ**
	Zn [5 mM]	Old	2.2 ± 0.71	Baα	2.81 ± 0.26	Caα
		Young	**3.78 ± 0.56**	**Caα**	**3.24 ± 0.06**	**Baα**
g_s ($mmol \cdot m^{-2} \cdot s^{-1}$)	Control	Old	244.13 ± 44.57	Aaα	320.56 ± 58.41	Aaα
		Young	**324.22 ± 9.47**	**Aaα**	**295.51 ± 28.52**	**Aaα**
	Zn [1 mM]	Old	333.56 ± 48.2	Aaα	349.11 ± 107.23	Aaα
		Young	**239.47 ± 32.75**	**Aaα**	**101.52 ± 33.63**	**Bbβ**
	Zn [5 mM]	Old	0.11 ± 0.09	Baα	6.02 ± 1.51	Baα
		Young	**12.841 ± 4.87**	**Baα**	**3.93 ± 1.17**	**Baα**
E ($mmol \cdot m^{-2} \cdot s^{-1}$)	Control	Old	4.48 ± 0.61	Baα	4.36 ± 0.57	Aaα
		Young	**5.82 ± 0.1**	**Aaα**	**3.99 ± 0.27**	**Abα**
	Zn [1 mM]	Old	6.22 ± 0.71	Aaα	4.45 ± 1.00	Abα
		Young	**4.69 ± 0.59**	**Aaβ**	**1.59 ± 0.48**	**Bbβ**
	Zn [5 mM]	Old	0.00 ± 0.002	Caα	0.14 ± 0.03	Baα
		Young	**0.3 ± 0.11**	**Baα**	**0.09 ± 0.03**	**Baα**
C_i ($\mu mol \cdot mol^{-1}$)	Control	Old	266.33 ± 16.5	Aaα	251.67 ± 6.56	Baα
		Young	**269.72 ± 14.18**	**Aaα**	**271.9 ± 12.61**	**Aaα**
	Zn [1 mM]	Old	274.78 ± 15.13	Aaα	308 ± 9.43	Aaα
		Young	**285.44 ± 13.97**	**Aaα**	**304.97 ± 20.63**	**Aaα**
	Zn [5 mM]	Old	0.00 ± 0.00	Baα	22.56 ± 22.56	Caα
		Young	**12.06 ± 11.73**	**Baα**	**0.00 ± 0.00**	**Baα**
Total chlorophyll (SPAD units)	Control	Old	40.46 ± 0.49	Aaα	40.74 ± 1.05	Aaα
		Young	**40.25 ± 0.36**	**Aaα**	**36.54 ± 2.49**	**Aaα**
	Zn [1 mM]	Old	39.94 ± 1.71	Aaα	35.44 ± 2.01	Aaα
		Young	**23.69 ± 2.26**	**Caβ**	**20.46 ± 2.66**	**Caβ**
	Zn [5 mM]	Old	37.57 ± 1.20	Aaα	33.93* ± 0.10	Aaα
		Young	**28.86 ± 0.81**	**Baβ**	**28.24* ± 0.19**	**Baβ**

CO_2 concentration (C_i) increased in I-214 old leaves at 1 mM [Zn], but was very low in both clones at 5 mM [Zn].Total chlorophyll content was maintained in old leaves of both clones with increasing [Zn] (Table 2). Young leaves of both clones showed a decrease at 1 mM [Zn] but an increase at 5 mM [Zn]. Differences between clones were not observed, although 5 mM [Zn] affected the survival of I-214 individuals, so chlorophyll content could be measured in only two of them.

3.5. Zinc and other nutrient content

In both clones, zinc was mainly accumulated in roots (Fig. 2). Zinc concentration in the various organs increased with increasing [Zn]; differences between clones were slight at 1 mM, whereas at 5 mM Eridano showed higher accumulation in stems, woody cuttings and roots. With increasing [Zn], S content showed an increasing trend at 1 mM in the different plant parts in both clones, declining at 5 mM to the control values. K content did not change except in old leaves and roots of clone I-214, which showed a decrease. Mg decreased in both clones in all plant parts except young leaves. P content was maintained in the roots of both clones and showed a decline at 5 mM [Zn] in aerial structures. Fe was maintained in all structures except leaves. Fe decreased at 1 mM in the young leaves of both clones and in old leaves of Eridano. Ca content was maintained in the roots, woody cuttings and young leaves of both clones and declined in old leaves only in Eridano. Cu content increased in the roots of both clones; in the other structures of I-214 Cu content was maintained, and in Eridano it decreased in both types of leaves. Mn content was maintained in the plant parts of I-214 whereas in Eridano a declining trend was observed in the aerial structures (Table 3). The total Zn per plant part (Fig. 3) was notable in roots. In Eridano, it was higher in young leaves and roots both at 1 and 5 mM [Zn] and in old leaves at 5 mM [Zn] with respect to I-214. The whole plant Zn was also higher in Eridano at both 1 and 5 mM [Zn]. Young leaves showed a higher amount of Zn than old leaves.

3.6. Tolerance index and Zn uptake ratio

At 1 mM [Zn], I-214 showed lower Ti in the total biomass due to lower Ti in young leaves, woody cutting and roots (Fig. 4). Eridano showed a decline of Ti at 5 mM [Zn] in young leaves, woody cutting and roots and no differences with I-214 were observed at this concentration except in old leaves, with a decrease in the latter. Young leaves showed lower Ti than old leaves. The Zn uptake ratio declined at 5 mM [Zn], and was higher in Eridano at both 1 and 5 mM [Zn] (Fig. 5).

4. Discussion

The effects of excess Zn on the physiological characteristics of *Populus* clones Eridano and I-214 were marked, with strong differences between them. Higher tolerance to Zn stress was observed in Eridano, since all plants survived the treatments, whereas in I-214, one third of the plants were so severely damaged that some measurements could not be performed. Visible symptoms of Zn toxicity were observed in the leaves of both clones at both [Zn]. In contrast, Di Baccio et al. (2010) did not report visible symptoms in the leaves of clone I-214 treated with 0.1 and 1 mM [Zn].

The effects of Zn treatment on plant growth were different for each clone, as highlighted by the biomass results (Table 1). The EC50, substrate Zn concentration resulting in 50% biomass reduction (Paschke et al., 2000) was higher in Eridano plants, since it occurred at 5 mM [Zn] (growth decline (53%) whereas in I-214, a strong decline (around 55%) was observed at 1 mM [Zn]). This finding was in accordance with Di Baccio et al. (2003). Although Zn and Cd tend to occur together at many contaminated sites, Cd rarely inhibits plant

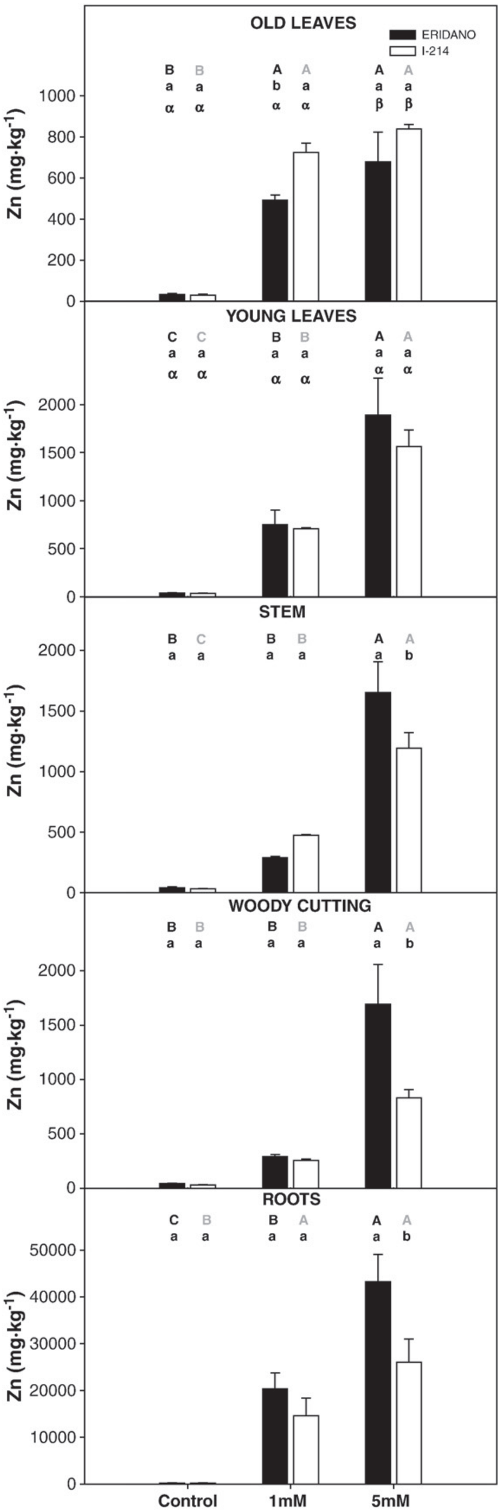
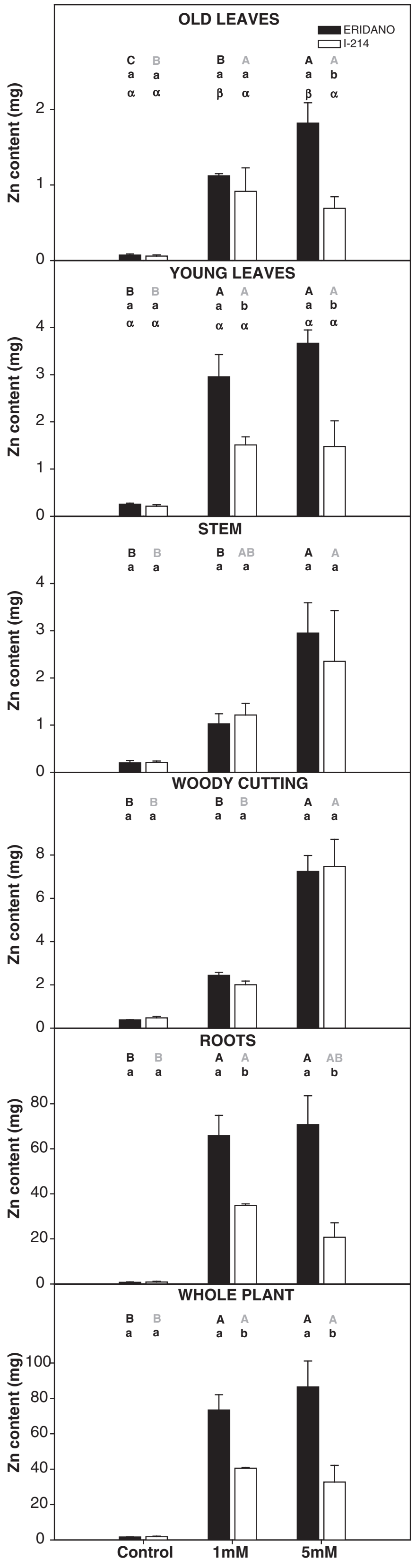


Fig. 2. Zinc concentration in plant structures. Zinc concentration in old and young leaves, stem, woody cutting and roots of clones Eridano and I-214 submitted to (control), 1 mM or 5 mM $ZnCl_2$. Different letters correspond to significant differences ($P<0.05$) between Zn treatments (A, B), clones (a, b) or kinds of leaf (young, old) in the same clone (α, β). Values are mean ± SE of measurements on three plants per treatment.

Table 3
Q.6.S, K, Mg, P, Fe, Ca, Cu and Mn content in different plant structures of *Populus* clones Eridano and I-214. Different letters correspond to significant differences ($P < 0.05$) between treatments (A,B), clones (a,b) or kinds of leaf (α, β). Values are mean ± SE of three plants per treatment.

			S		K		Mg		P		Fe		Ca		Cu		Mn	
Old leaves	Eridano	Control	4429.99 ± 51.99	Aaα	12,891.77 ± 118.38	Baα	3531.1 ± 185.23	Aaα	2501.12 ± 234.82	Abα	120.34 ± 5.31	Aaα	17,239.4 ± 929.19	Aaα	6.52 ± 0.12	Aaα	110.23 ± 10.75	Aaα
		1 mM	4165.69 ± 180.03	Aaβ	19,568.51 ± 1970.13	Aaα	3076.71 ± 165.55	ABaα	2420.35 ± 158.03	ABbβ	81.35 ± 1.2	Baα	14,591.18 ± 717.02	Aaα	5.4 ± 0.78	ABaα	85.75 ± 4.88	ABaα
		5 mM	3223.68 ± 430.64	Baα	14,720.09 ± 1438.22	ABaα	2274.99 ± 308.15	Baα	1889.88 ± 242.84	Baα	94.38 ± 19.23	ABaα	9508.65 ± 1004.6	Bbα	3.9 ± 0.32	Baα	65.81 ± 16.69	Baα
	I-214	Control	3754.83 ± 258.03	Aaα	13,560.04 ± 904.65	Aaα	3267.88 ± 443.93	Aaα	3058.18 ± 27.7	Aaα	66.02 ± 7.11	Abα	15,064.76 ± 1940.63	Aaα	3.7 ± 0.74	Abα	69.33 ± 3.31	Abα
		1 mM	3766.73 ± 3.42	Aaα	18,702.88 ± 1711.98	Aaα	3222.36 ± 129.31	ABaα	3528.26 ± 162.4	Aaβ	61.79 ± 6.91	Aaα	16,605.98 ± 556.99	Aaα	3.72 ± 0.64	Aaα	80.22 ± 5.44	Aaα
		5 mM	2533.53 ± 36.74	Baβ	4393.46 ± 2277.26	Bbβ	2366.81 ± 33.8	Baβ	1988.62 ± 52.54	Baα	93.55 ± 2.38	Aaα	14,374.75 ± 1341.44	Aaβ	5.29 ± 0.46	Aaα	75.43 ± 6.67	Aaα
Young leaves	Eridano	Control	4379.39 ± 305.23	ABaα	14,516.16 ± 2446.91	Aaα	2855.47 ± 138.82	Aaα	2902.27 ± 289.97	Baα	81.83 ± 5.33	Aaβ	10,985.31 ± 739.26	Aaβ	5.12 ± 0.86	Aaα	57.67 ± 5.37	ABaβ
		1 mM	5855.63 ± 144.9	Aaα	18,854.87 ± 111.86	Aaα	3361.15 ± 145.31	Aaα	4113.15 ± 231.5	Aaα	67.93 ± 7.49	ABaα	14,167.11 ± 1391.94	Aaα	3.78 ± 0.41	Aaα	82.55 ± 9.94	Aaα
		5 mM	3657 ± 589.16	Baα	14,472.66 ± 1063.81	Aaα	2801.91 ± 633.31	Aaα	1923.14 ± 349.72	Caα	51.48 ± 14.5	Baβ	9029.47 ± 2797.07	Aaα	3.65 ± 0.54	Aaα	51.25 ± 10.23	Baα
	I-214	Control	3766.14 ± 334.51	Aaα	14,443.05 ± 1743.69	Aaα	2777.6 ± 221.6	Aaα	2962.79 ± 181.83	Baα	61.17 ± 1.44	ABaα	12,384.44 ± 651.44	Aaα	4.09 ± 0.67	Aaα	56.93 ± 10.47	Aaα
		1 mM	4418.94 ± 165.83	Aaα	20,192.65 ± 309.12	Aaα	3079.75 ± 46.81	Aaα	4211.5 ± 102.75	Aaα	40.55 ± 1.41	Baα	9558.22 ± 377.49	Aaβ	2.06 ± 0.14	Baα	43.41 ± 3.15	Abβ
		5 mM	4393.84 ± 898.93	Aaα	11,875.95 ± 4813.32	Aaα	3528.82 ± 444.36	Aaα	2436.84 ± 221.13	Baα	73.23 ± 16.76	Aaα	13,857.02 ± 1069.22	Aaα	4.23 ± 0.07	Aaα	66.34 ± 6.55	Aaα
Stem	Eridano	Control	1269.57 ± 227.57	ABa	13,076.68 ± 1103.63	Aa	1425.72 ± 45.01	Ab	2436.8 ± 191.24	Aa	21.24 ± 1.74	Aa	7704.28 ± 99.89	Ba	2.91 ± 0.27	Ba	9.62 ± 1.1	ABa
		1 mM	1328.47 ± 55.13	Aa	16,441.12 ± 783.99	Aa	1684.37 ± 22.66	Ab	2791.3 ± 166.76	Aa	16.42 ± 1.99	Aa	8723.61 ± 526.19	ABb	2.23 ± 0.12	Ba	13.69 ± 0.34	Aa
		5 mM	838.73 ± 59.74	Ba	14,730.14 ± 2092.99	Aa	871.61 ± 64.89	Ba	2636.77 ± 277.79	Aa	71.82 ± 48.82	Aa	10,299.13 ± 791.67	Aa	3.87 ± 0.29	Aa	7.17 ± 1.11	Ba
	I-214	Control	874.45 ± 104.54	Ba	13,957.77 ± 364.21	Aa	2024.51 ± 122.54	Ba	2372.09 ± 26.91	Ba	13.89 ± 4.79	Aa	10,105.67 ± 473.74	ABa	2.56 ± 0.16	Aa	9.17 ± 1.43	ABa
		1 mM	1609.48 ± 158.5	Aa	13,305.63 ± 1518.04	Aa	2619.07 ± 171.21	Aa	3350.03 ± 215.71	Aa	6.38 ± 0.47	Aa	12,023.68 ± 0.46	Aa	2.4 ± 0.28	Aa	13.89 ± 0.93	Aa
		5 mM	755.32 ± 164.61	Ba	12,574.83 ± 3065.05	Aa	1201.26 ± 236.71	Ca	2596.65 ± 352.43	ABa	7.06 ± 1.19	Aa	7823.65 ± 1237.76	Bb	2.6 ± 0.33	Ab	8.35 ± 2.74	Ba
Woody cutting	Eridano	Control	384.93 ± 61.78	Aa	6589.14 ± 645.68	Aa	786.95 ± 30.87	Aa	1273.4 ± 44.79	Aa	14.62 ± 8.72	Aa	9857.62 ± 964.14	Aa	4.38 ± 0.46	Aa	10.72 ± 1.13	Aa
		1 mM	309.18 ± 33.68	Aa	5837.53 ± 456.66	Aa	776.75 ± 34.64	Aa	1141.95 ± 34.1	ABb	8.92 ± 1.61	Aa	11,183.3 ± 917.68	Aa	3.21 ± 0.15	Aa	13.08 ± 2.24	Aa
		5 mM	512.99 ± 113.25	Aa	5626.3 ± 830.23	Aa	551.8 ± 50.66	Ba	992.8 ± 142.43	Ba	7.57 ± 0.93	Aa	11,399.37 ± 1318.26	Aa	5.64 ± 1.42	Aa	7.21 ± 1.42	Aa
	I-214	Control	356.11 ± 93.32	Aa	5101.78 ± 12.21	Aa	775.56 ± 48.11	ABa	1255.06 ± 21.53	Aa	15.49 ± 2.73	Aa	6260.38 ± 119.97	Ab	3.69 ± 0.2	Aa	27.71 ± 19.33	Aa
		1 mM	331.77 ± 5.74	Aa	4811.27 ± 430.94	Aa	897.87 ± 89.4	Aa	1440.78 ± 144.32	Aa	20.24 ± 5.76	Aa	7729.64 ± 980.13	Ab	6.62 ± 3.3	Aa	11.95 ± 2.65	Aa
		5 mM	310.49 ± 28.92	Aa	4463.91 ± 60.23	Aa	662.23 ± 29.43	Ba	1228.5 ± 43.52	Aa	25.72 ± 8.17	Aa	6107.53 ± 67.99	Ab	3.85 0.23	Aa	5.59 ± 0.94	Aa
Roots	Eridano	Control	3125.15 ± 1092.14	Ba	12,399.76 ± 1802.28	Aa	2266.28 ± 680.92	Aa	22,796.47 ± 6376.78	Aa	202.84 ± 58.44	Aa	45,637.57 ± 13,418.4	Aa	11.49 ± 3.01	Ba	600.16 ± 192.46	Aa
		1 mM	5320.64 ± 250.70	Aa	16,001.48 ± 1535.32	Aa	2628.63 ± 54.63	Aa	17,378.11 ± 2322.15	Aa	355.17 ± 40.67	Aa	23,320.26 ± 3475.55	Aa	15.1 ± 1.38	ABa	322.03 ± 65.44	ABa
		5 mM	2918.11 ± 102.31	Ba	11,498.34 ± 1419.86	Aa	807.71 ± 17.73	Ba	21,007.21 ± 3915.83	Aa	434.25 ± 57.51	Aa	28,372.48 ± 6191.52	Aa	26.76 ± 3.36	Aa	216.05 ± 42.25	Ba
	I-214	Control	2903.52 ± 626.42	ABa	16,919.05 ± 2455.5	Aa	2296.14 ± 455.31	Aa	14,283.91 ± 4549.33	Aa	195.17 ± 48.12	Aa	26,617.06 ± 9610.36	Aa	9.31 ± 2.21	Ba	320.04 ± 91	Aa
		1 mM	4474.11 ± 526.85	Aa	16,697.66 ± 711.81	Aa	2353.93 ± 115.46	Aa	15,764.02 ± 4772.49	Aa	281.21 ± 0.67	Aa	17,542.98 ± 7582.48	Aa	13.99 ± 0.53	ABa	253.34 ± 101.16	Aa
		5 mM	1866.88 ± 640.1	Ba	6839.58 ± 2813.22	Ba	646.14 ± 200.54	Ba	9727.43 ± 1731.57	Aa	339.53 ± 132.04	Aa	11,044.1 ± 1588.71	Aa	24.06 ± 8.14	Aa	179.97 ± 72.57	Aa



growth, whereas excess Zn reduces growth (Sagardoy et al., 2009). The effect of increasing [Zn] on root biomass was especially strong at 5 mM [Zn] in clone I-214, with a 70% decline. Root growth is a more sensitive endpoint marker of metal toxicity than chlorophyll assays (Morgan et al. 2002), and inhibition of root growth and changes in root morphology have been reported (Broadley et al., 2007). In both clones, the lower biomass production at 5 mM [Zn] points to a lower tolerance at this [Zn] with respect to 1 mM [Zn].

The greater ability of Eridano to tolerate Zn toxicity than I-214 was highlighted by their higher respective Tolerance index (Ti) in roots and leaves at both [Zn] (Fig. 4), which indicates the ability of these plants to grow in the presence of a given concentration of metal (Wilkins, 1978; Zacchini et al., 2009).

A stronger effect of Zn exposure on I-214 with respect to Eridano plants was also ascertained at leaf morphological level (Fig. 2) and from the gas-exchange results (Table 2). In fact, I-214 plants showed a leaf area (LA) reduction and a marked T decrease and D increase in leaves. D increase is associated with thicker cell walls and smaller and more tightly packed cells (Niinemets, 2001). The morphological changes in response to heavy metals may impair species ability to withstand drought (Barceló and Poschenrieder, 1990).

The T decline observed accounts for reduced mesophyll layers, implying lower photosynthetic protein accumulation per unit leaf area and lower photosynthetic capacity. In fact, at 1 mM and especially in young leaves (developed during the Zn treatment), net photosynthesis declined markedly (82%) in I-214, whereas the decline was much lower (42%) in Eridano (Table 2). The unaffected photosynthesis in old leaves of Eridano at 1 mM [Zn] indicated that the lack of metal toxicity in these leaves may be due to the higher ability of this clone to protect cells from the oxidative attack, commonly induced in plants by metals (Cuypers et al., 2001). It may also be due to the low mobility of this metal which accumulates mainly in the younger structures. g_s and E in I-214 young leaves also decreased at 1 mM [Zn], probably reducing nutrient uptake. Decreased growth and photosynthesis under Zn excess have been described for other species (Caldelas et al., 2011; Mateos-Naranjo et al., 2008; Sagardoy et al., 2009; Vaillant et al., 2005). Moreover, the effect of toxic Zn reducing root biomass is reported to inhibit the photoassimilates translocation from leaves to roots as observed in bush beans (Ruano et al., 1988) leading to a relative increase in shoot growth as also observed in our work from the aerial/root biomass results at 5 mM [Zn] in I-214.

Zinc has been reported to inhibit or damage almost every point of the photosynthetic apparatus, *i.e.* chlorophyll synthesis, PSII, the oxygen-evolving complex, the plastoquinone pool, PSI, and Rubisco (Prasad, 2004). Accordingly, in the present study, chlorophyll content showed a marked decline in young leaves of both clones especially at 1 mM [Zn]. Reductions of the leaf area in plants exposed to 5 mM [Zn] may account for the higher chlorophyll contents observed in these plants with respect to those treated with1mM [Zn], and it was also a clear visual symptom. It has been suggested that young leaf chlorosis in plants grown at high [Zn] may be caused by deficiencies in Fe or Mg (Marschner, 1995; Sagardoy et al., 2009). The replacement of Mg by Zn may damage the chlorophyll synthesis system (Prasad and Strzalka, 1999). In our plants, Mg content in young leaves was maintained with increasing [Zn]; in contrast Fe content in young leaves showed the same trend as chlorophylls, decreasing at 1 mM [Zn] (Table 3). A decrease in Fe in mulberry plants with Zn toxicity (Tewari et al., 2008) has been reported.

Fig. 3. Zinc accumulation in plant structures. Zn total amount in different plant parts of clones Eridano and I-214 submitted to 0 (control), 1 mM or 5 mM $ZnCl_2$. Different letters correspond to significant differences ($P<0.05$) between Zn treatments (A, B), clones (a, b) or kinds of leaf (young, old) in the same clone (α, β). Values are mean ± SE of measurements on three plants per treatment.

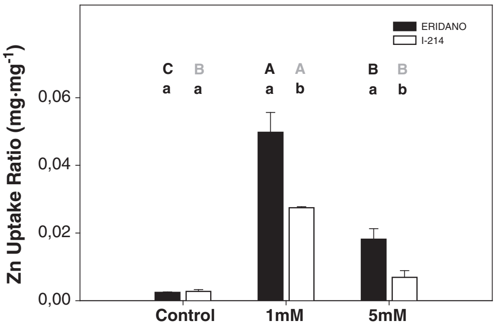


Fig. 5. Zinc uptake ratio. Total amount of Zn in the plant with respect to the amount in the solution in plants of clones Eridano and I-214 submitted to 0 (control), 1 mM or 5 mM $ZnCl_2$. Values are mean ± SE of measurements on three plants per treatment.

At 1 mM [Zn], C_i was maintained in Eridano and increased in I-214; these results indicated that CO_2 availability was not the limiting factor at this [Zn], suggesting non-stomatal limitation of photosynthesis for I-214. Monnet et al. (2001) found an increase in C_i in *Lolium perenne*, which they explained as a modification of RuBisCO activities. At 5 mM, the low Ci can be a consequence of the higher decreases in the observed g_s and also mesophyll conductance (g_{mes}), as reported in sugar beet grown in the presence of excess Zn by Sagardoy et al. (2010).

In both clones, an increase of [Zn] in the growth medium resulted in a higher [Zn] in all plant structures, especially the roots (one order of magnitude higher) (Fig. 2) as reported for *Populus tremula* (Vollenweider et al. 2011).The accumulation of Zn in roots could be due to a reduction in the Zn translocation to leaves, which are more sensitive to metals, and can be described as an avoidance response that confers increased tolerance to Zn excess (Kabata-Pendias and Pendias, 2001). At 5 mM [Zn] Eridano showed much higher [Zn] in roots than I-214. The capacity of these plants to accumulate metal ions particularly at root level could be exploited for the phytofiltration of metal-polluted waters, as a result of originated by industrial and agricultural activities. Moreover, the increase in metal accumulation in aerial parts of Eridano at 5 mM [Zn] (Fig. 3) may be an interesting selection characteristic for phytoextraction, especially when metal is accumulated in stem and woody cuttings. In fact, these plant parts will originate the woody non-renewable organs that could be more easily harvested and stored, thus avoiding loss of metals on the top soil due to leaf shedding.

Zn toxicity in crop plants occurs when leaf concentrations reach 400–500 mg·kg^{-1} dry weight (Marschner, 1995) although toxicity thresholds can vary widely, even within the same species (Broadley et al., 2007). In our study, at 1 and 5 mM [Zn] leaves (especially young leaves), showed metal concentrations that were above the toxicity threshold, affecting their growth capacity (as evidenced by the Ti index and biomass parameters) and gas-exchange results.

The total Zn accumulated in the whole plants of both clones was higher in Eridano (around 50%) and did not change from 1 mM to 5 mM in both clones, indicating that plants could not store more Zn in their structures above this latter concentration, which induced a strong decline in the efficiency of uptake of Zn from the medium (Fig. 5).

The nutrient balance in plant cells was disturbed by Zn toxicity; in fact changes in the concentration of the nutrients analysed were

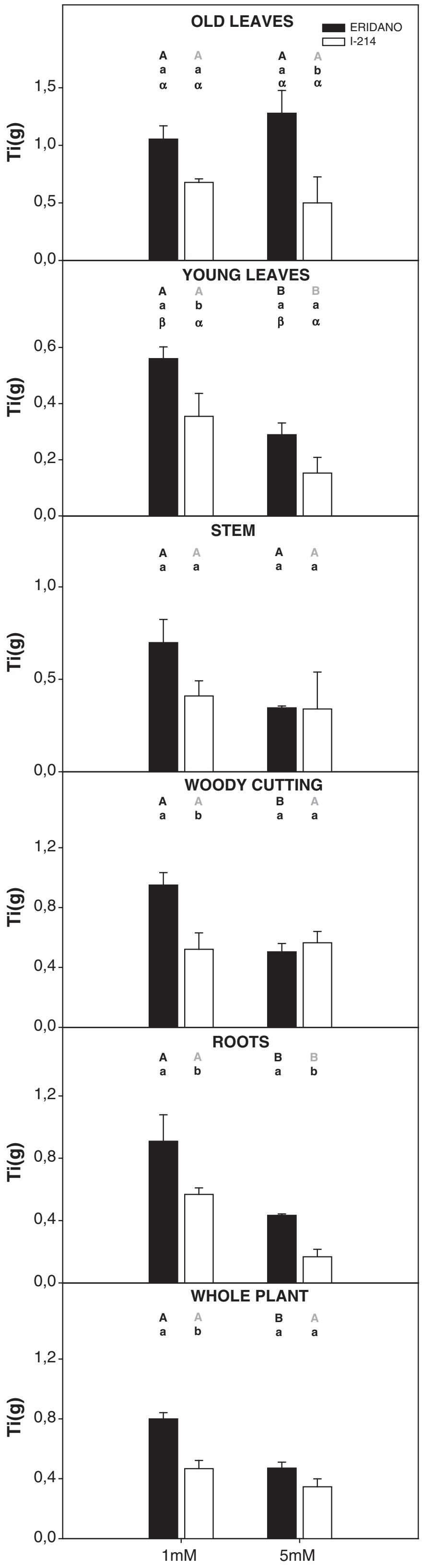


Fig. 4. Tolerance index in plant structures. Tolerance index (Ti) in old and young leaves, stem, woody cutting and roots of clones Eridano and I-214 submitted to 0 (control), 1 mM or 5 mM $ZnCl_2$. Different letters correspond to significant differences ($P<0.05$) between Zn treatments (A, B), clones (a, b) or kinds of leaf (young, old) in the same clone (α, β). Values are mean ± SE of measurements on three plants per treatment.

observed, depending on the clone, Zn concentration and plant part (Table 3). With increased Zn supply, differences in nutrient uptake among plants have been reported elsewhere (Bonnet et al., 2000; Mateos-Naranjo et al., 2008; Sagardoy et al., 2009; Tewari et al., 2008; Wang et al., 2009b). These differences might be attributable to differences in element-uptake, translocation mechanisms or interactions between elements (Sinha et al., 2006). Besides the changes in Mg and Fe content and their effect on chlorophyll content discussed above, the P decline observed mainly in aerial structures at 5 mM [Zn] might enhance the Zn toxicity, since treatment with phosphorus alleviated Zn stress (Jiang et al., 2007). Under Zn stress, decreased P uptake and lower P content in leaves has been reported in *Pisum sativum* (Päivöke, 2003), rapeseed (Wang et al., 2009a) and *P. tremula* (Vollenweider et al. 2011). Moreover, the declining trends in the concentrations of Mg, Cu, Fe, and Mn in response to increasing [Zn] may affect the activities of superoxide dismutases (SOD) and the antioxidant responses (Bonnet et al., 2000; Marschner, 1995).

5. Conclusions

The effect of increasing [Zn] was observed in the physiological responses of I-214 and Eridano clones; 1 mM [Zn] was tolerated by Eridano whereas 5 mM [Zn] led to metabolic impairment in both clones. Eridano showed a greater capacity of Zn accumulation especially in roots, and milder alterations occurred in the development of plants biomass, structural parameters and photosynthetic activity. A possible utilisation of this clone for phytoremediation of metal polluted substrates appears very promising and may be confirmed in further experiments at a larger scale.

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Capítol 5: Bescanvi de gasos, respostes fotoprotectores i antioxidants i acumulació de metalls en els clons de *Populus* I-214 i Eridano sotmesos a altes concentracions de Zn

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Gas-exchange, photo- and antioxidant protection, and metal accumulation in I-214 and Eridano *Populus* sp. clones subjected to elevated zinc concentrations

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ABSTRACT

Rooted cuttings of Eridano and I-214 *Populus* clones were treated in hydroponics with high [Zn] to establish their phytoextraction capacity and physiological responses for phytoremediation. The Bioconcentration factor, Uptake ratio and Translocation factor revealed that the highest Zn accumulation occurred in roots. Lower accumulation at 1 mM [Zn] in Eridano aerial parts limited Zn toxicity on photosynthetic machinery. Increasing [Zn] negatively affected growth, net photosynthesis, stomatal conductance, maximum quantum yield, chlorophyll content and hydric parameters. The physiological impairment in I-214 at 1 mM [Zn] indicated a greater sensitivity to high [Zn] than Eridano. At 5 mM [Zn], high toxicity for both clones occurred. Upregulation of photoprotective and antioxidant responses was a consequence of Zn stress rather than a Zn tolerance mechanism. Increased de-epoxidation state of the xanthophyll-cycle, α-Tocopherol and reduced glutathione and decreased total phenolics in I-214 at 1 mM [Zn] suggested that it responded earlier to oxidative stress when compared to Eridano.

1. Introduction

Levels of heavy metals in soils have increased in recent decades as a consequence of contamination due to anthropogenic activities such as the application of pesticides, sewage sludge in soils, smelter and incineration emissions and mining activities, among many others (Chaney, 1993). Accumulation of heavy metals such as zinc (Zn) leads to severe damage of vegetation (Ernst and Joosse-van Damme, 1983). Furthermore, there are many sources of Zn contamination in soils and these are often associated with Pb, Cu, and Cd (Pedler et al., 2004).

Zinc is an essential micronutrient in plants and can act as a functional, structural and regulatory co-factor in a large number of enzymes (Barak and Helmke, 1993) such as RuBisCo (Brown et al., 1993). Zn plays an important role in plant metabolism (e.g. nitrogen metabolism, photosynthesis, transpiration and auxin synthesis) (Marschner, 1995) and has an essential function in the stability of plant cell membranes (Welch et al., 1982). Appropriate Zn concentrations in tissues increase plant productivity and growth however, becomes toxic at supraoptimal concentrations (Broadley et al., 2007). Symptoms of Zn toxicity in plants are similar to those found with Cd or Pb (Fodor et al., 2005). Excess Zn can directly affect plant growth, photosynthetic activity, water relations and metabolism (Apel and Hirt, 2004).

Zinc toxicity can affect water relations at multiple levels: impairment of water transport into root cells and through roots (Poschenrieder and Barceló, 1999), a decrease in leaf water content (Barceló and Poschenrieder, 1990; Bonnet et al., 2000) or impairment of stomatal conductance (g_s) through alterations of guard cell development and guard cell function which in turn decrease rates of transpiration and photosynthesis (Sagardoy et al., 2010).

Excess Zn inhibits photosynthesis at various stages and through different mechanisms. Zn has been shown to have a specific effect on the Calvin cycle (Chaney, 1993) and photosystem activities (Van Assche and Clijsters, 1986). Alterations in electron transport as a consequence of stress exposure result in the production of reactive oxygen species (ROS) (Asada, 1994). Also, excess Zn generates oxidative damage (Cakmak and Marshner, 1993) producing ROS (Kappus, 1985) and concomitantly induces the disruption of photosynthetic electron transport and the antioxidant defence system

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(Cakmak, 2000). When ROS production exceeds the scavenging ability of a plant, oxidative damage can take place resulting in the degradation of cell membranes, nucleic acids and other cellular functions (Asada, 2006). In order to minimize ROS formation and prevent photoinhibition, plants increase thermal energy dissipation through the conversion of violaxanthin pigments to zeaxanthin in the xanthophyll-cycle (Demmig-Adams and Adams, 2006). On the other hand, ROS can be directly detoxified through several enzymatic pathways and antioxidant molecules such as ascorbate, α-Tocopherol and carotenoids (Foyer and Shigeoka, 2011). The antioxidant capacity of plants can be related to the tolerance to oxidative stress induced by high heavy metal exposure as reported (Zhu et al., 1999; Freeman et al., 2004; Liu et al., 2007). Information on the response of woody species to heavy metals is needed in order to optimize their use in the restoration of contaminated areas (Fernàndez et al., 2012; Romeo et al., 2014). Zn tolerant plants can be used to remove this metal by accumulating it in harvestable parts which can later be used to fertilize certain cereal crops, thereby increasing cereal grain yield (Zhang et al., 2013). Poplars are ideal candidates for the phytoremediation of contaminated substrates because of their fast growth, elevated water and nutrient usage, extensive root system (Mughini et al., 2013), high biomass production, and their capacity to absorb metals concentrate and tolerate them in their organs (Dos Santos Utmazian et al., 2007; Pietrini et al., 2010a). Plant capacity to accumulate Zn or Cd (unlike Cu, Pb or Ni) is dependent in part on their concentrations in soil and is probably due to their high solubility (Landberg and Greger, 1994). Zn uptake and translocation rates in some plant species is higher than those for Cd (Ali et al., 2000). *Populus* × *canadensis euramericana* Mönch.-I-214 clone and *P. deltoides* × *maximowiczii* – Eridano clone are largely used in poplar plantations in Italy for their adaptability to different environmental conditions. Their response and tolerance to heavy metals, especially Cd and Zn, have been reported (Sebastiani et al., 2004; Di Baccio et al., 2003; Zacchini et al., 2009; Romeo et al., 2014). In the present study, we have gone into greater depth in the study of the physiological effects of high [Zn] in order to expand our knowledge of this area, and especially in relation to the protection responses to stress at the chloroplast pigment and antioxidant level.

The general aims of this study were to assess (1) the Zn accumulation capacity of the *Populus* clones, I-214 and Eridano, and (2) their tolerance to Zn toxicity in order to provide relevant baseline information for future improvements in the field of phytoremediation techniques. A specific aim was to ascertain the contribution of photoprotective pigments and antioxidants to the tolerance of high Zn concentrations.

Populus cuttings were grown in hydroponic systems and exposed to 1 mM or 5 mM [$ZnCl_2$] threshold concentrations which have been described as inducing toxicity symptoms in poplar clones (Di Baccio et al., 2009). Measurements were performed on fully expanded leaves which had already developed before the application of Zn treatments and on fully expanded leaves developed after the application of treatments in order to evaluate the metal effect on plant development.

2. Materials and methods

2.1. Plant material and zinc treatments

Poplar woody cuttings (20-cm-long) obtained from Eridano (*Populus deltoides* × *maximowiczii*) and I-214 (*Populus* × *canadensis euramericana* Mönch.) clones were collected from adult plants grown since 2001 in the IBAF experimental fields near Rome. Cuttings were rooted in spring (April) in pots filled with 6 L of water for 3 weeks. Thirty-six rooted cuttings of a similar size were selected and were placed in pots with 6 L of nutrient solution (Moore, 1974) at pH = 6.5. Cuttings were growing for 3 weeks in nutrient solution, which was replaced twice weekly. At the end of this period, groups of 6 cuttings were selected at random and placed in pots filled with nutrient solution containing 0 mM (control), 1 mM or 5 mM zinc chloride ($ZnCl_2$) for each clone. Hydroponic culture was performed in a greenhouse (Serveis de Camps Experimentals (UB)) under 65–75% relative humidity, 26 °C maximum air temperature and maximum photosynthetic photon flux density (PPFD) of approximately 1000 $\mu mol\ m^{-2}\ s^{-1}$ at midday. Fully developed leaves before the Zn treatments were designated as old leaves, while fully developed leaves after the beginning of the treatments were designated as young leaves. After three weeks of treatment, sampling and measurements were carried out around midday (11:30 am–16:00 pm). Gas-exchange and chlorophyll fluorescence measurements were performed on uncut leaves of three plants per treatment. After these measurements were taken, plant parts were cut and separated into young leaves, old leaves, stems, woody cuttings and roots for biomass and Zn concentration determination of the dried material. Leaf samples from another three plants per treatment were selected and immediately frozen in liquid nitrogen, stored at −80 °C and lyophilized (Virtis Lyophiliser, Freezemobile 6EL, Gardiner, NY, USA). Lyophilized leaves were milled in a Cyclotec 1093/Foss Sample Mill (Tecator, Höganäs, Sweden) and thereafter all samples were stored until analyses of their chloroplast pigment, antioxidant content and carbon isotope composition were carried out.

2.2. Biomass determination and Relative Water Content

The samples of the different plant parts were weighed both for fresh weight (FW) and, after oven-drying at 60 °C until constant weight, for dry weight (DW). The relative water content (RWC) was calculated as [(FW − DW)/(FSW − DW) × 100], where FSW is fresh saturated weight (after rehydrating samples for 24 h in darkness at 4 °C). Leaf area (LA) was measured with a CI 2003 Laser Leaf Area Meter (CI-203) (CID, Inc., Camas, WA 98607, USA).

2.3. Zinc determination

Dried plants were carefully washed in distilled and MilliQ water. Leaves, stems and roots were ground to a fine powder in an agate mortar and woody cuttings were milled in a cutting mill Pulverisette 15 (Fritsch GmbH, Germany). Approximately 1 g of plant material was pre-digested in 5 mL of HNO_3 overnight at room temperature. Digestion was performed in a digestor block (JP Selecta) at 105 °C for three hours. A second digestion was done with 2.5 mL $HClO_4$ at 155 °C for two hours and afterwards at 180 °C for two hours. Zn content of different plant parts was determined by an Inductively Coupled Plasma Mass Spectrometry (ICP-MS PerkinElmer, model Elan-6000) at the Serveis Científico-Tècnics (UB). The Translocation factor (Tf), the Bio-concentration factor (BCF) and the Uptake ratio were calculated. Tf is defined as the ratio of metal concentration in aboveground parts to that in roots, and provides information about the capability of plants to translocate metals from the roots to the aerial parts. The BCF is defined as the ratio of metal concentration in the aerial parts and roots to that in the nutrient solution. The Uptake ratio provides information on the capability of the plant to extract the metal from the growing medium and to accumulate it in its structures. It was calculated as the total Zn content in each plant structure to the total Zn content in the different growth solutions at the end of the experiment. The Uptake ratio takes into account the metal content and the dry weight of each plant part, whereas the BCF only considers the metal concentration in each organ.

2.4. Gas-exchange parameters and chlorophyll fluorescence

Net photosynthesis (A) and stomatal conductance (g_s) were measured with a gas-exchange system (LI-6400; Li-COR, Lincoln, Nebraska, USA). The environmental conditions in the leaf-measuring chamber were established as: PPFD: 1100 μmol m^{-2} s^{-1}, sample CO_2 (C_a): 370 μmol mol^{-1}, water mole fraction: 14 ± 1 mol H_2O mol air^{-1}, leaf temperature: 27 °C, VPD: 2 ± 0.3 kPa and air flux: 400 μmol s^{-1}.

The maximum quantum yield of PSII photochemistry (F_v/F_m) was obtained with a Mini–Pam (Walz, Effeltrich, Germany) fluorometer after 20 min dark-adaptation of the leaves (which was observed to be a sufficient adaptation time for the complete oxidation of PSII reaction centers). Images of chlorophyll fluorescence were recorded with an Imaging-PAM, MICRO-version (Walz, Effeltrich, Germany) operated with Imaging Win v.2.21d software (Heinz Walz).

2.5. Leaf carbon isotope discrimination and nitrogen content

Carbon isotope composition (δ^{13}C) and total nitrogen (N) content were determined in approximately 1 mg of lyophilized organic matter with an elemental analyzer (EA1108, Series 1, Carlo Erba Instrumentazione, Milan, Italy) coupled to a mass spectrometer (Delta C, Finnigan MAT, Bremen, Germany) at the Serveis Científico-Tècnics (UB) and used to estimate carbon isotope discrimination (Δ^{13}C). δ^{13}C values were determined using a standard that was calibrated against Pee Dee Belemnite (PDB) carbonate and used to estimate carbon isotope discrimination (Δ^{13}C) as: $\Delta^{13}C = 1000(\delta_a - \delta_p)/(1 + \delta_p)$), where δ_a and δ_p are values for air (−10.5%) and the plant respectively (Farquhar et al., 1989). The ambient partial concentration of CO_2 (C_a) at the study site was 370 ppm.

2.6. Chloroplast pigments and α-Tocopherol

Chloroplast pigments and α-Tocopherol (α-Toc) were extracted from 50 mg of lyophilized material with pure acetone buffered with $CaCO_3$ and centrifuged twice at 12,000 × g. The supernatants were filtered through 0.2-Ym PTFE filters (Teknokroma, Barcelona, Spain). The pigments were separated by HPLC on a reversed-phase C18 column (Waters Spherisorb ODS1, 4.6 mm × 250 mm, Milford, Massachusetts, USA) and detected with a photodiode array detector according to García-Plazaola and Becerril (1999, 2001). α-Toc detection and quantification were performed with a Scanning Fluorescence Detector (SRD, Waters 474) that was operated in series with the photodiode array detector according to García-Plazaola and Becerril (1999, 2001). The relative de-epoxidation state of the xanthophyll-cycle pigments was estimated by the ratio (A + Z)/(V + A + Z).

2.7. Ascorbate, glutathione and total phenolics

Fifteen mg of lyophilized samples were extracted with metaphosphoric acid (6 w/v%) and centrifuged at 10,000 × g at 4 °C. Reduced ascorbate (ASA) content was determined according to Foyer et al. (1983) and measured by the change in A_{265} using a spectrophotometer (Cecil Aquarius, CE7400, Cecil Instruments, Cambridge, UK). Glutathione content was obtained according to Noctor and Foyer (1998). Reduced glutathione (GSH) was determined and measured by the change in A_{412}. (Cecil Aquarius, CE7400, Cecil Instruments, Cambridge, UK). Total phenolics (TPhe) content was determined according to Singleton and Rossi (1965) from lyophilized material homogenized with 20 volumes of an 80% methanol solution, extracted twice and centrifuged at 14,000 × g for 10 min and measured at A_{735} (with a spectrophotometer Perkin Elmer, Norwalk, CT, USA).

2.8. Statistical analysis

All statistical procedures were performed using SPSS for Windows (v. 15.0, SPSS Inc., Chicago, IL, USA). Analysis of Variance (ANOVA) was used to test the main effects against the appropriate error terms of the clone, the treatment (control, 1 mM [Zn], 5 mM [Zn]) and the kinds of leaf (young, old) on the parameters measured. A multiple comparison test of the means using the Duncan post hoc test was applied. Statistical significance was set at $p \leq 0.05$.

3. Results and discussion

3.1. Toxicity symptoms, growth and Zn accumulation in tissues

Toxic effects of Zn treatments on both poplar clones were visually detected at the tested concentrations. At 1 mM [Zn], young leaves exhibited diffused chlorosis and the presence of necrosis in sprouts. Plants exposed to 5 mM [Zn] showed a severe reduction in growth, roots exhibited a browning appearance and only a few new leaves developed, which, in addition, showed some symptoms of chlorosis. Zinc toxicity causes chlorosis in younger leaves, which may extend to older leaves after prolonged exposure to high Zn levels (Ebbs and Kochian, 1997). In I-214, one third of 5 mM [Zn] treated plants showed signs of very severe damage (chlorotic and wilted leaves) and did not allow for the performance of several measurements.

Biomass production decreased at high Zn concentrations; at 1 mM [Zn], I-214 showed approximately 50% biomass reduction (Table 1), whereas the total biomass of Eridano was similar to control plants, thus indicating it had a higher tolerance to the Zn treatment than did I-214. At 5 mM [Zn] high toxicity levels in both clones, according to the observed growth decrease, occurred. No effect of the metal was observed in the ratio of aerial to root biomass in Eridano, whereas I-214 showed a marked increase at 5 mM [Zn] as a result of a low root biomass (Table 1). Both clones showed a decrease in the development of roots, mainly at 5 mM [Zn] (data not shown), indicating a high sensitivity of these structures to excess Zn concentrations. The decrease of root biomass can negatively affect their absorbance capacity and in turn, Zn accumulation.

The Uptake ratio and the Bio-concentration factor (BCF) results reflected that in both clones the highest Zn extraction from the growth medium occurred at 1 mM [Zn] (Table 1). Eridano exhibited larger Zn extraction at 5 mM [Zn] than I-214. Furthermore, roots were the structures with the highest Zn accumulation. The highest metal accumulation in both roots and leaves was seen for both clones at 1 mM [Zn]. The lower Zn accumulation in roots and leaves at 5 mM [Zn] was associated with a lower metal extraction and biomass production. At 5 mM [Zn], roots and leaves of Eridano presented a higher metal content than those of I-214, as displayed in the Uptake ratio results.

BCF and the Tf provided details about the Zn distribution pattern in the different organs (Table 1). Metal translocation from roots to aerial parts decreased at 1 mM [Zn] and 5 mM [Zn] with respect to the control, and mainly in Eridano. In fact, the Tf ratio displayed values lower than 1. These facts, in conjunction with the large Zn concentration in roots, points to high metal uptake but low transfer to aerial parts (root accumulator species), this allowing for the enhanced protection of structures involved in photosynthesis as described for poplar clones exposed to other heavy metals in hydroponics (Dos Santos Utmazian et al., 2007; Zacchini et al., 2009; Romeo et al., 2014). Eridano would therefore possess a higher level of protection in aerial parts than I-214 especially at 1 mM [Zn] due

Table 1
Biomass and Zn accumulation in different organs. Aerial to root biomass ratio, total biomass, translocation factor (TF) (mg kg^{-1}), Bio-concentration factor (BCF) (mg kg^{-1}) and Uptake ratio (mg g^{-1}) measured on three plants per treatment and clone. Values are mean ± SE. Different letters correspond to significant differences at $p \leq 0.05$ between treatments (A–C), clones (α, β) or kinds of leaf (a, b).

		ERIDANO		I-214	
Aerial/root biomass (g Dw)	Control	6.27 ± 0.11	A α	7.03 ± 0.68	B α
	Zn [1 mM]	5.69 ± 0.77	A α	5.82 ± 1.58	B α
	Zn [5 mM]	6.91 ± 0.70	A β	16.22 ± 0.59	A α
Total biomass (g Dw)	Control	27.11 ± 1.37	A α	31.76 ± 5.23	A α
	Zn [1 mM]	21.78 ± 2.02	AB α	16.41 ± 0.10	B α
	Zn [5 mM]	12.72 ± 0.95	B α	13.21 ± 1.88	B α
Translocation factor (TF)	Control	1.00 ± 0.44	A α	0.88 ± 0.29	A α
	Zn [1 mM]	0.09 ± 0.02	B α	0.16 ± 0.04	A α
	Zn [5 mM]	0.14 ± 0.01	B α	0.18 ± 0.02	A α
BCF old leaves	Control	4.86 ± 0.81	Ca α	5.36 ± 0.94	Caα
	Zn [1 mM]	33.35 ± 1.71	Aa β	49.1 ± 3.08	Aaα
	Zn [5 mM]	14.27 ± 3.06	Bb α	17.63 ± 0.47	Bbα
BCF young leaves	Control	5.45 ± 10.77	Ba α	5.5 ± 0.49	Baα
	Zn [1 mM]	50.88 ± 10.22	Aa α	47.99 ± 0.59	Aaα
	Zn [5 mM]	39.81 ± 8.10	Aa α	32.87 ± 3.68	Aaα
BCF shoot	Control	5.98 ± 1.50	C α	4.96 ± 0.50	B α
	Zn [1 mM]	19.57 ± 0.67	B β	32.1 ± 0.36	A α
	Zn [5 mM]	34.79 ± 5.34	A α	25.08 ± 2.70	A β
BCF woody cutting	Control	6.48 ± 0.24	C α	4.53 ± 0.31	B α
	Zn [1 mM]	19.66 ± 1.45	B α	17.27 ± 0.98	A α
	Zn [5 mM]	35.59 ± 7.69	A α	17.5 ± 1.60	A β
BCF aerial parts	Control	20.54 ± 2.01	B α	21.02 ± 2.34	C α
	Zn [1 mM]	123.46 ± 11.81	A α	146.46 ± 3.83	A α
	Zn [5 mM]	124.47 ± 19.99	A α	99.98 ± 5.38	B α
BCF root	Control	29.31 ± 8.23	C α	29.05 ± 6.33	C α
	Zn [1 mM]	1380.82 ± 229.31	A α	988.95 ± 253.88	A α
	Zn [5 mM]	908.95 ± 124.11	B α	547.4 ± 104.83	B α
Uptake ratio old leaves	Control	0.11 ± 0.02	Cbα	0.09 ± 0.02	Cbα
	Zn [1 mM]	0.76 ± 0.02	Abα	0.62 ± 0.21	Aaα
	Zn [5 mM]	0.38 ± 0.06	Bbα	0.15 ± 0.03	Baβ
Uptake ratio young leaves	Control	0.38 ± 0.04	Baα	0.32 ± 0.05	Baα
	Zn [1 mM]	2.00 ± 0.32	Aaα	1.03 ± 0.12	Aaα
	Zn [5 mM]	0.77 ± 0.06	Baα	0.31 ± 0.12	Baβ
Uptake ratio shoot	Control	0.30 ± 0.08	A α	0.32 ± 0.05	A α
	Zn [1 mM]	0.70 ± 0.15	A α	0.82 ± 0.17	A α
	Zn [5 mM]	0.62 ± 0.14	A α	0.50 ± 0.23	A α
Uptake ratio woody cutting	Control	0.58 ± 0.02	B α	0.72 ± 0.10	B α
	Zn [1 mM]	1.65 ± 0.10	A α	1.36 ± 0.12	AB α
	Zn [5 mM]	1.52 ± 0.16	A α	1.57 ± 0.26	A α
Uptake ratio roots	Control	1.07 ± 0.27	C α	1.30 ± 0.48	B α
	Zn [1 mM]	44.67 ± 6.04	A α	23.64 ± 0.48	A α
	Zn [5 mM]	14.88 ± 2.69	B α	4.35 ± 1.35	B β

to the lower Zn BCF in shoots and old leaves, whereas at 5 mM [Zn] this clone presented higher Zn accumulation in shoots and woody cuttings.

The Zn accumulation observed in both clones exposed to different treatments is in accordance with Sebastiani et al. (2004), even though in their work, poplar plants were grown on soil amended with industrial wastes and therefore likely to have a different metal bioavailability. This metal bioavailability can change notably depending on the physico-chemical characteristics of the soil in question, and therefore, this problem can be overcome through the use of quick hydroponic screenings (Dos Santos Utmazian et al., 2007). Special attention must be paid to the metal accumulation in shoots and woody cuttings, which are not renewable tissues unlike foliage, and can be collected during harvesting. Uptake ratio in shoots and woody cuttings indicated lower accumulation in these structures than in roots; however, since the woody part of poplar adult plants represents a much more significant proportion of total biomass than roots, the metal content of the woody part may account for the majority of metal accumulated in a tree (Pietrini et al., 2010a).

3.2. Gas-exchange, water relations and carbon isotope discrimination

Increasing Zn concentration caused a notable decline in gas-exchange rates (Fig. 1). In both clones, young leaves displayed higher functional impairment than old leaves at 1 mM [Zn]. A and g_s results obtained for 1 mM [Zn] treatment indicated that Zn toxicity affected photosynthetic processes before affecting water relations, probably starting in young leaves which are growing tissues with metabolically active cells and which are more sensitive to metal excess (Ernst, 2006). At 1 mM [Zn], I-214 was the most affected clone, exhibiting a decline of A and g_s in young leaves and a reduction in A in old leaves with respect to the control, while Eridano showed signs of toxicity only in young leaves with a reduction in A with respect to the control. The observed lower toxicity symptoms

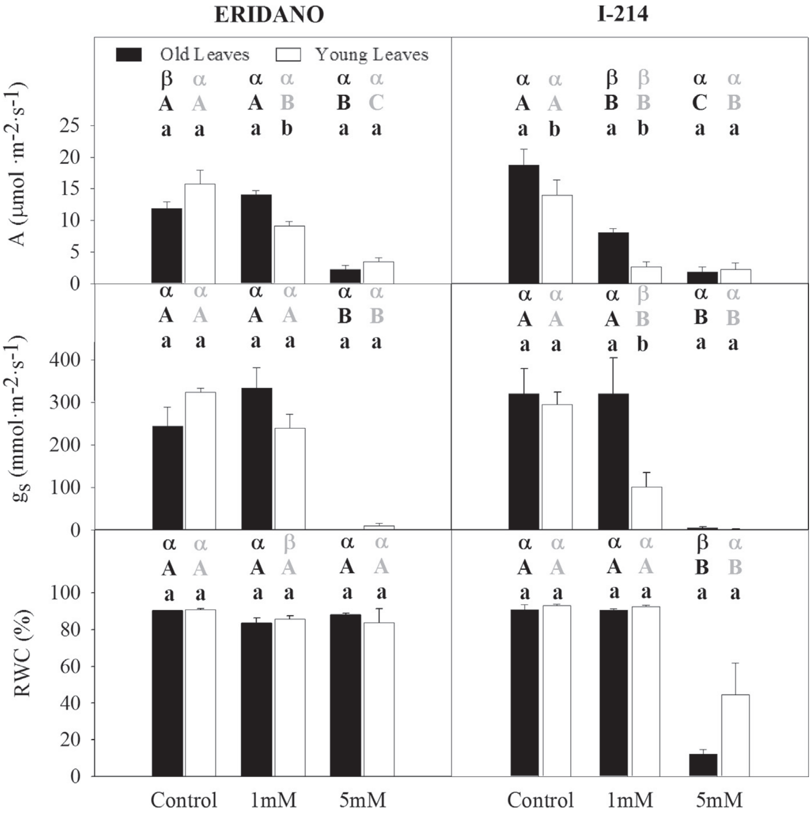

Fig. 1. Gas-exchange parameters and relative water content of leaves. Net photosynthesis (A), stomatal conductance (g_s) and relative water content (RWC) measured on a minimum of three leaves (young and old) of three plants per treatment and clone. Values are a mean ± SE. Different letters correspond to significant differences at $p \leq 0.05$ between treatments (A–C), clones (α, β) or kinds of leaf (a, b).

in Eridano leaves at 1 mM [Zn] could be related to the lower Zn accumulation in aerial parts with respect to I-214 as previously mentioned. At 5 mM [Zn], *A* and g_s decreased markedly in old and young leaves of both clones which exhibited high metal toxicity.

G_s is an integrative indicator of the degree of water stress (Flexas et al., 2002) and can also be a useful characteristic to assess effective phytoextraction in woody plants (Stomp et al., 1993). Plants can be defined as either suffering mild, moderate, severe, or extreme drought stress when g_s values are above 70%, between 70% and 40%, between 40% and 10%, and less than 10% of control values respectively (Galmés et al., 2013). At 1 mM [Zn], old leaves of both clones displayed similar g_s values to controls, while in Eridano young leaves experienced mild water stress ($g_s > 200\ \text{mmol m}^{-2}\ \text{s}^{-1}$), and in I-214 severe water stress ($g_s \geq 100\ \text{mmol m}^{-2}\ \text{s}^{-1}$). At 5 mM [Zn] plants in both clones experienced extreme water stress.

At 1 mM [Zn], both clones did not show alterations in leaf water content as demonstrated by high RWC values (between 80% and 90%) which were similar to the control (Fig. 1). At 5 mM [Zn], stomata closure allowed for the maintenance of RWC values between 80% and 90% in Eridano (Fernàndez-Martínez et al., 2013), whereas in I-214 RWC was lower than 40% reflecting a notable water deficit.

Δ^{13}C is negatively correlated with time-integrated water use efficiency (WUE) in C3 plants (Knight et al., 1994) which is defined as the ratio between plant biomass accumulation and plant transpiration. Δ^{13}C results (Fig. 2) showed that long-term WUE was maintained in both clones at 1 mM [Zn] with respect to the control, whereas at 5 mM [Zn] WUE increased in Eridano as a result of stomatal closure, a fact which is related to the high RWC observed. Δ^{13}C values vary with respect to the relative contribution of the enzymatic and diffusional fractionation processes, and follow a linear function of C_i/C_a (Farquhar and Richards, 1984), where C_i is the intercellular [CO$_2$] and C_a the ambient [CO$_2$]. Taking into account that Δ^{13}C results reflect those of, C_i, Δ^{13}C maintenance at 1 mM [Zn] with respect to the control pointed to a sustained C_i in young and old leaves of both clones. At 5 mM [Zn] the reduction of C_i in Eridano was a result of a notable stomatal closure. On the contrary, at 1 mM [Zn] C_i maintenance and the sustained or decreased g_s values with respect to the control in both clones indicated that the observed reduction in *A* was related to a non-stomatal limitant. In fact, in young leaves a decrease in *A* was related to a reduction in electron transport and photoinhibition occurrence as shown by F_v/F_m values (Fig. 4). Moreover, in young leaves of Eridano, a decrease in N content on an area basis (Fig. 2) would result in a lower rate of RuBisCO synthesis. In old leaves at 1 mM [Zn], a decrease in *A* only occurred in I-214 and was not related to electron transport or N availability, thereby indicating possible changes in mesophyll conductance of CO$_2$ (Di Baccio et al., 2003; Sagardoy et al., 2010) and/or modifications in RuBisCO activities. Indeed, if Zn is favored in local competition over Mg for the formation and catalytic function of the ternary RuBisCO–CO$_2$–metal^{2+} complex, then the affinity of RuBisCO for CO$_2$would decrease (Monnet et al., 2001).

3.3. Photo- and antioxidant protection

Chlorophyll fluorescence images provided information on the effect of Zn toxicity on the photosynthetic apparatus along the leaf surface. At 5 mM [Zn], photochemistry was affected in areas

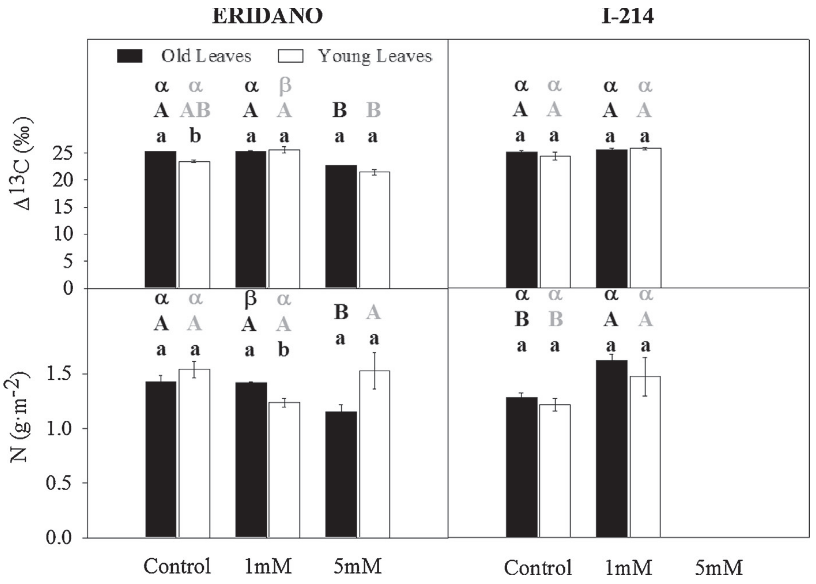

Fig. 2. Carbon isotope discrimination ($\Delta^{13}C$) and nitrogen content (N) in young and old leaves. Values are a mean ± SE of three plants per treatment and clone. Different letters correspond to significant differences at $p \leq 0.05$ between treatments (A–C), clones (α, β) or kinds of leaf (a, b). Measurements on I-214 leaves treated with 5 mM [Zn] were not possible due to severe damage.

close to the vein, which displayed an F_v/F_m of less than 0.75 indicating severe photoinhibition (Fig. 3). The typical range of values for non-severely stressed leaves lies between 0.75 and 0.85 (Björkman and Demmig, 1987). Leaf areas that were especially affected became chlorotic and necrotic (patches of dark color), and exhibited remarkably low F_v/F_m values, suggesting a large accumulation of metals (Pietrini et al., 2010b).

Chlorophyll fluorescence results showed that both clones experienced severe photoinhibition at 1 mM [Zn] in young leaves (F_v/F_m around 0.5) (Fig. 4). At 5 mM [Zn], Eridano presented slight photoinhibition in both kinds of leaves ($F_v/F_m = 0.75$) while I-214 showed severe photoinhibition (F_v/F_m around 0.35).

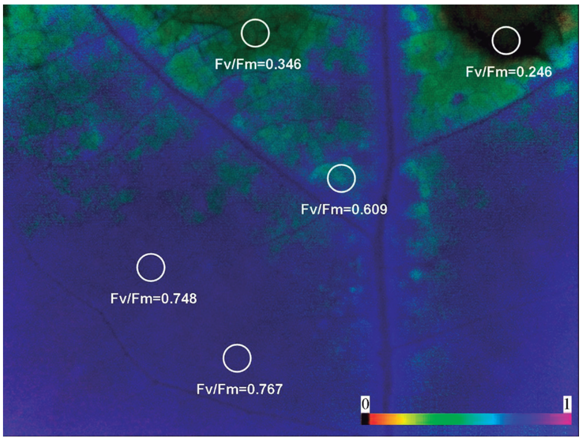

Fig. 3. Chlorophyll fluorescence image of the effect of 5 mM [Zn] on the maximum PSII quantum yield (F_v/F_m) of an old I-214 leaf. F_v/F_m values of different points within the image are given. F_v/F_m was obtained for one squared image area of 26 mm × 34 mm per leaf. The false color code depicted at the bottom ranges from 0 (black) to 1 (purple). (For interpretation of the references to color in this text, the reader is referred to the web version of the article.)

Zn can replace Mn at the site of water photolysis and can lead to an inhibition of oxygen production and electron transfer (Ralph and Burchett, 1998). However, the observed increase of the de-epoxidation index (AZ/VAZ) pointed to an electron transfer to reaction centers and indicated the augmentation in the dissipation of excitation excess energy as heat at high [Zn] thus protecting leaves from light-induced damage (Fig. 4). The decline of F_v/F_m and the increase of AZ/VAZ might indicate that Zn reacted with reaction centers (Monnet et al., 2001).

Photoprotective and antioxidant responses augmented with increasing [Zn], however under high levels of Zn, the photochemical process and most physiological functions were severely impaired leading to the wilting of plants. Indeed, it should be mentioned that the severe damage in I-214 leaves with the 5 mM [Zn] treatment did not allow for the determination of the chloroplast pigment and antioxidant content.

The initial responses to Zn toxicity were detected in young leaves at 1 mM [Zn] in both clones. I-214 exhibited enhanced responses in young leaves at 1 mM [Zn] when compared to Eridano and also showed earlier oxidative stress episodes. Photoprotection responses were described by AZ/VAZ and VAZ/Chl which highlight the current and the potential capacity to dissipate excess excitation energy respectively. Control plants of I-214 presented increased mechanisms when compared to Eridano control plants in the protection of the functional state of the photosynthetic apparatus as reflected by a high AZ/VAZ (Fig. 4). This could indicate different sensitivities to oxidative stress, and therefore to high [Zn], between clones.

Values for AZ/VAZ at 1 mM [Zn] in young leaves of both clones and mainly in I-214, with respect to the control tended to be higher, and increased notably at 5 mM [Zn] in both kinds of leaf of Eridano (Fig. 4). The VAZ/Chl ratio increased only in young leaves (Fig. 4) at 1 mM [Zn] and mainly in I-214 as a result of low Chl a + b.

The chlorophyll synthesis system can be damaged by the substitution of Mg by Zn (Prasad and Strzalka, 1999) or by the competition between Fe and Zn which can result in the destruction of antennae pigments (Abadía et al., 1999). At 1 mM [Zn], the severe

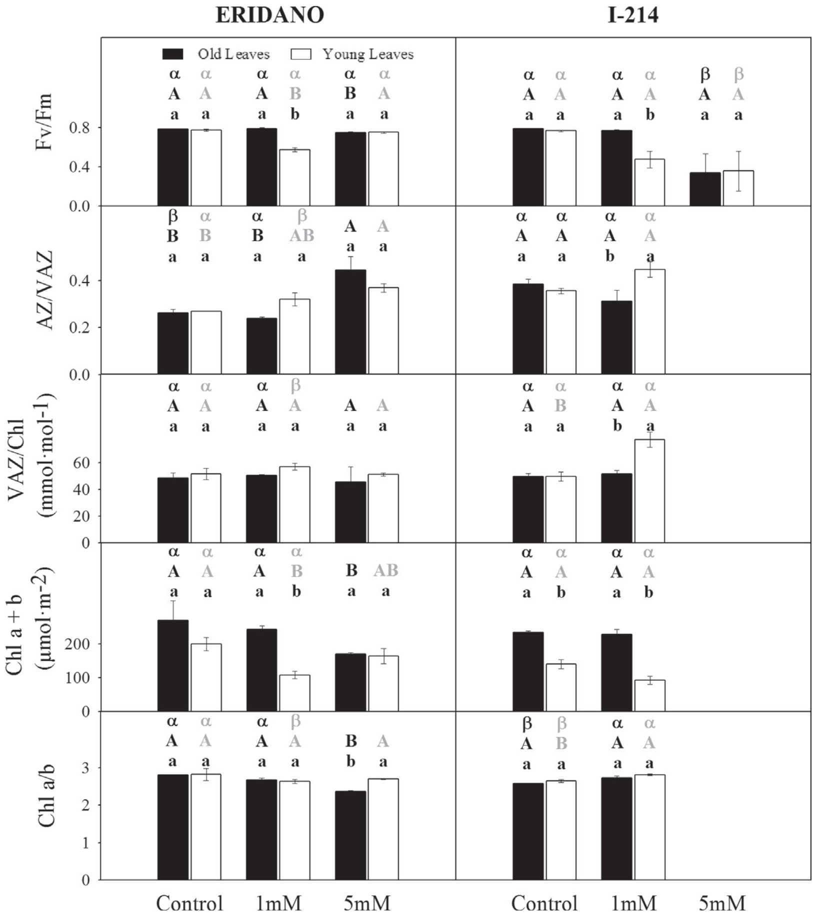

Fig. 4. PSII maximum quantum yield and chloroplast pigments. Maximum quantum yield of PSII photochemistry (F_v/F_m), de-epoxidation degree of xanthophyll cycle pigments (AZ/VAZ), total content of xanthophyll cycle pigments (VAZ/Chl), total Chlorophyll content (Chlorophyll *a* + *b*) and Chlorophyll *a*/*b* ratio measured from young and old leaves of three plants per treatment and clone. Values are a mean ± SE. Different letters correspond to significant differences at $p \le 0.05$ between treatments (A–C), clones (α, β) or kinds of leaf (a, b). Measurements on I-214 leaves treated with 5 mM [Zn] were not possible due to severe damage.

photoinhibition observed in young leaves can be related to the decrease in total chlorophyll content (Fig. 4) (responsible for the diffused chlorosis) and a decreased photosynthetic electron sink. Decreases in Chl a + b with increasing [Zn] occurred especially in Eridano. Control plants of I-214 presented lower Chl a/b with respect to Eridano, this being related to a reduced proportion of reaction center core complexes to light-harvesting antenna complexes in PSI and PSII (Nie and Baker, 1991) (Fig. 4). At 5 mM [Zn], the slight decrease of Chl a/b in old Eridano leaves could be associated to ongoing senescence processes (Sestak, 1985).

Among the chloroplast lipophilic antioxidants, α-Toc is an important molecule that can deactivate $^1O_2$, reduce $O_2^{\bullet-}$ and stop lipid peroxidation (Takenaka et al., 1991; Munné-Bosch, 2005) while β-carotenes (β-Car) are efficient quenchers of triplet chlorophyll Chl^3 and $^1O_2$ (Gill and Tuteja, 2010). Trends for both clones showed that as [Zn] increased, so did α-Toc content (Fig. 5) as reported in *Lemna minor* (Artetxe et al., 2002).

No changes in β-Car content were observed among different Zn treatments (Fig. 5). Ascorbate and glutathione are the most abundant water-soluble antioxidants. Ascorbate can provide protection to membranes by directly scavenging $O_2^{\bullet-}$ and OH^- and by regenerating α-Toc from tocopheroxyl radical (Smirnoff, 2000). Reduced glutathione (GSH) is a potential scavenger of $^1O_2$, H_2O_2 (Noctor and Foyer, 1998) and the most dangerous types of ROS such as OH^- (Larson, 1988). Phytochelatin formation in Zn treated plants also drains GSH from antioxidant defenses, thereby exacerbating oxidative stress (Scheller et al., 1987). Ascorbate was found only in its reduced form (ASA) in all Zn treatments (Fig. 5) indicating an adequate regeneration by GSH. Old I-214 leaves from the control treatment demonstrated greater ASA levels than those of Eridano indicating higher detoxification activities and that there was greater sensitivity to oxidative stress. ASA content increased in Eridano at 5 mM [Zn] showing higher oxidative stress with respect to 1 mM [Zn]. Increasing [Zn] did not provoke alterations in GSH levels when compared to its oxidized form (data not shown). These results indicated an active stress acclimation of the glutathione pool. In Eridano, GSH levels were maintained with increasing [Zn] (Fig. 5). At 1 mM [Zn], I-214 showed enhanced GSH levels in young leaves with respect to the control and to Eridano, which allowed for the maintenance of ASA levels. GSH enhancement was also observed in *L. minor* (Artetxe et al., 2002).

Phenolics (TPhe) are secondary metabolites that have a major role in antioxidant defence in plant cells (Grace and Logan, 2000) and act as metal chelators (Sakihama et al., 2002). TPhe increased in Eridano at 5 mM [Zn] in old leaves (Fig. 5) while no alterations were observed in young leaves among different treatments, which would indicate a high antioxidative capacity. I-214 exhibited higher oxidative stress in both kinds of leaf at 1 mM [Zn] as showed by the

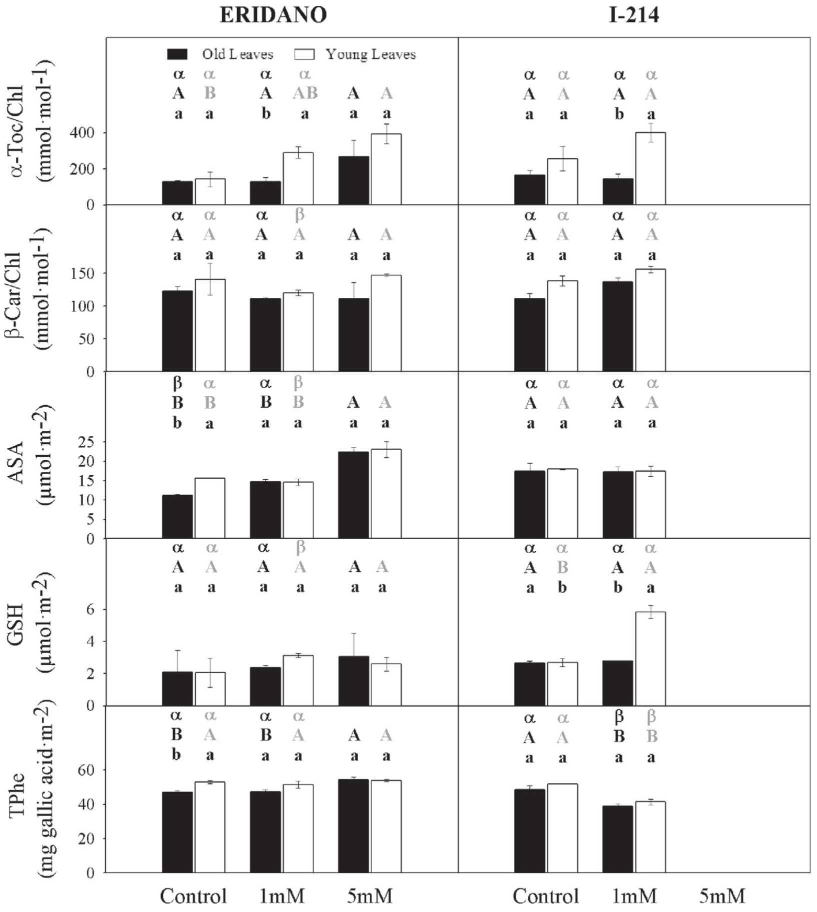

Fig. 5. Leaf antioxidant content. α-Tocopherol (α-Toc/Chl), β-Carotene (β-Car/Chl), reduced ascorbate (ASA), reduced glutathione (GSH) and total phenolics (TPhe) content measured in young and old leaves of three plants per treatment and clone. Values are a mean ± SE. Different letters correspond to significant differences at $p \leq 0.05$ between treatments (A–C), clones (α, β) or kinds of leaf (a, b). Measurements on I-214 leaves treated with 5 mM [Zn] were not possible due to severe damage.

decline in TPhe which could be related to the degradation of the TPhe pool under Zn toxicity. TPhe was the only group of antioxidants which had a reduced content at toxic levels of Zn. This fact could be related to a decrease in its' *de novo* synthesis and suggests that TPhe could have a more direct interaction with Zn at toxic levels.

4. Conclusions

Both poplar clones showed the highest Zn accumulation in roots. In Eridano, lower Zn accumulation in aerial parts when compared to I-214 were observed at 1 mM [Zn], allowing it to limit the toxic effects of this metal on photosynthetic performance. Eridano exhibited greater tolerance to high [Zn] than did I-214, especially at 1 mM [Zn], as was demonstrated by the improved growth, photosynthesis and stomatal conductance. 5 mM [Zn] produced high toxicity levels for both clones and resulted in impaired biomass production, photochemical processes and water relations. Wilted leaves of I-214 at 5 mM [Zn] revealed higher susceptibility to toxicity by excess Zn than in the case of Eridano. Photoprotective and antioxidant responses were enhanced with increasing [Zn], especially in I-214 and indicated a higher sensitivity to excess Zn. These results show the different reactions of both poplar genotypes to high [Zn] exposure at both the morpho-physiological and biochemical level. For phytoremediation purposes, the remarkable ability of the Eridano clone to concentrate [Zn] in the rooting system together with a notable tolerance to high levels of this metal demonstrate its' suitability for both the phytostabilisation of polluted soils and the phytodepuration of metal-contaminated wastewaters in natural decontamination systems.

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DISCUSSIÓ

1. EFECTE DEL DÈFICIT HÍDRIC, DEL CO_2 ELEVAT I DE LES INFECCIONS PER PATÒGENS EN LA FISIOLOGIA DE TRES ESPÈCIES DE L'ESTATGE SUB-ALPÍ DINS EL CONTEXT DE CANVI CLIMÀTIC

A l'alta muntanya, les condicions de sequera no són habituals, es limiten sobretot a l'estiu, on la probabilitat de que se'n donin és del 10% (Pfister i Rutishauser 2000, Calanca 2007). Les espècies vegetals que conformen les comunitats sub-alpines estan adaptades a condicions d'abundància d'aigua, de manera que una disminució en la disponibilitat hídrica, i no necessàriament unes condicions de sequera, pot afectar negativament la seva fisiologia fins el punt de causar-les un estrès i de limitar el seu desenvolupament. Els resultats obtinguts en aquest estudi han permès conèixer les respostes del bedoll, el neret i el pi negre davant d'aquestes condicions, i la informació extreta pot ésser aplicada a l'elaboració de prediccions de la capacitat d'aquestes espècies per tolerar els episodis de sequera previstos pel futur, que esdevindran cada cop més freqüents i intensos com a conseqüència del canvi climàtic. Partint de les condicions que es donen actualment a l'alta muntanya, en aquest estudi no parlem de condicions pròpiament de sequera sinó que fem referència a la baixa disponibilitat d'aigua o al dèficit hídric.

S'han obtingut diferents respostes en les tres espècies estudiades en front de les condicions de dèficit hídric, d'alta irradiància, de concentracions elevades de CO_2 i d'estrès biòtic. Aquestes respostes han vingut condicionades, en part, per les característiques pròpies de cada espècie, com ara el fet que el bedoll és un arbre caducifoli, el pi negre una conífera i el neret un arbust perennifoli. A més, aquestes espècies presenten trets estructurals i funcionals diversos que els permeten adaptar-se de manera diferent a les múltiples interaccions amb el medi. Per una banda, hem vist que els diferents sistemes d'arrels de les tres espècies no els permeten tenir el mateix accés a les reserves d'aigua, fet que s'ha vist reflectit en les diferents respostes fisiològiques: el pi negre i el bedoll disposen d'un sistema d'arrels més desenvolupat en relació al neret, tal i com mostrà el seu menor N/P (Poorter i Nagel 2000), i que els permet accedir a les reserves d'aigua existents a major profunditat, tractant-se d'una característica important durant les condicions de dèficit hídric. El sistema d'arrels del neret, però, és menys desenvolupat i es distribueix per les capes més superficials del sòl, fet que el fa tenir menys accés a les reserves d'aigua i estar més exposat a les variacions en el règim de precipitacions. Tot i això, el neret és una espècie adaptada a l'ombra, per tant, en estar menys temps exposada a altes intensitats de llum i a altes temperatures, les pèrdues d'aigua a través de la transpiració són menors (Larcher i Wagner 2004).

S'ha observat que l'estructura de la fulla també té una funció destacada en la capacitat d'adaptació de la planta a les condicions restrictives del medi, intervenint en el manteniment de l'estat hídric i nutricional de la fulla i en l'eficiència de la fotosíntesi, entre d'altres. El pi negre és l'espècie que presentà fulles més escleròfil·les, seguit del neret. Aquestes fulles s'han caracteritzat per tenir un major pes específic foliar (LMA), un major nombre de capes de cèl·lules del mesòfil, cèl·lules més compactades i amb parets més gruixudes, fet que els ha permès mantenir en major grau el contingut hídric i reduir la seva pèrdua per transpiració. Aquestes característiques estan associades amb el fet que es tracta d'espècies amb fulles de llarga durada i necessiten mantenir la funcionalitat d'aquestes durant més temps, per tant, han de mostrar majors adaptacions a estressos mecànics i a la pressió de l'herbivoria. Per altra banda, el bedoll presentà fulles amb menor pes específic foliar (LMA) relacionat amb un major contingut en proteïnes i amb una menor inversió en components com lípids o lignina. En ser caducifoli presentà fulles de poca durada que es caracteritzen per tenir altes taxes de fotosíntesi, associades a a un creixement ràpid de la planta, durant el període de primavera-estiu (Wright *et al.* 2004); per això hem vist que el bedoll disposà, entre altres trets característics, d'una major superfície foliar en comparació amb les altres espècies, fet que li permetè captar una major llum incident. En condicions de dèficit hídric hem vist que es donaren modificacions en l'estructura de les fulles d'aquesta espècie, com ara un increment del pes sec i una disminució de l´àrea foliar, i que els permetria mantenir en major mesura el contingut hídric, reduint però la superfície per captar la llum incident i per realizar la fotosíntesi.

Les respostes de les plantes davant les diferents condicions ambientals estudiades van ser, en general, més favorables a principis d'estiu que no pas a finals, en part perquè les plantes es trobaven en la seva fase més productiva. En canvi, a finals d'estiu es van evidenciar processos relacionats amb la senescència, moment en que es dóna la remobilització de nutrients de les fulles cap a altres estructures (brots, tija, arrels, etc.) (Millard and Grelet 2010), i esdevenen menys eficients per tolerar les restriccions ambientals. Per altra banda, s'observà un avançament en la senescència foliar com a conseqüència de l'estrès causat pel dèficit hídric.

1.1. Efecte de la disminució de la disponibilitat hídrica estival

1.1.1. Respostes en el bescanvi de gasos i paràmetres hídrics

Es van observar diferents règims de precipitacions al llarg dels anys 2011 i 2012, diferències que van ser més acusades sobretot a l'estiu i que ens han permès estudiar en les diferents espècies estudiades l'efecte del dèficit hídric a curt termini és a dir, de la restricció de pluges dins d'un període de temps comprès entre dies i setmanes afectant sobretot als nivells d'aigua presents en les capes més superficials del sòl; i a llarg termini és a dir, associat amb precipitacions escasses al llarg de setmanes o mesos arribant a afectar les reserves d'aigua de les capes més profundes del sòl. A l'estiu del 2011 es van donar condicions de dèficit hídric a llarg termini, mentre que a l'estiu del 2012 es van donar condicions de dèficit hídric a curt termini.

Al llarg de l'estiu del 2011 es van observar poques variacions en el dèficit de pressió de vapor (VPD), fet que indicà que les alteracions observades en la conductància estomàtica de les plantes estigueren bàsicament relacionades amb la disminució de disponibilitat d'aigua en el sòl. Els resultats obtinguts mostraren un efecte de la disminució de la disponibilitat hídrica a llarg termini de l'estiu del 2011 sobre la fisiologia del bedoll, coincidint en aquest cas amb l'inici de la senescència foliar, un estadi de la fenologia on les fulles esdevenen més susceptibles a les condicions ambientals. El neret també va veure's afectat per la disminució de la disponibilitat hídrica a llarg termini de l'any 2011, tot i que les alteracions més importants en condicions de dèficit hídric les va presentar a curt termini, com va succeir a l'estiu del 2012. En aquesta època es van donar variacions més marcades en el VPD al llarg de l'estiu i poden haver sigut causants, juntament amb el dèficit hídric a curt termini, de les alteracions observades en la conductància estomàtica (g_s) del neret. El pi negre va ser l'única espècie que no va presentar limitacions hídriques.

S'ha descrit que el paràmetre que millor reflecteix l'estat hídric de la planta és la conductància estomàtica (g_s) (Flexas *et al.* 2002). Altres paràmetres com la discriminació isotòpica del ^{13}C (Δ^{13}C), el contingut relatiu hídric de la fulla (RWC) i la hidratació foliar (H), també aporten informació sobre les relacions hídriques i foren determinats al llarg de l'estudi.

El pi negre no va mostrar alteracions en la g_s ni en els altres paràmetres esmentats, evidenciant que al llarg dels períodes de dèficit hídric estival no va presentar problemes hídrics tal i com s'ha comentat anteriorment. En condicions de baixa disponibilitat hídrica el bedoll i el neret van presentar característiques d'estrès hídric sever.

El pi negre i el neret presenten fulles escleròfil·les que van mostrar poques alteracions morfològiques al llarg de l'època de creixement en els anys 2011 i 2012.

Aquest fet els ha permès mantenir, sobretot al pi negre, una alta eficiència en l'ús de l'aigua (WUE) al llarg de l'estiu i que està inversament relacionada amb la $\Delta^{13}C$. En el cas del bedoll, el seu menor LMA li va conferir una menor WUE. La morfologia de la seva fulla, a diferència de les altres dues espècies, va experimentar modificacions al llarg de l'estiu del 2011 associades al dèficit hídric a llarg termini. Les variacions en l'estructura de la fulla del bedoll es correlacionaren amb la WUE (correlació negativa LMA-$\Delta^{13}C$) observant-se una relació entre les adaptacions morfològiques i el manteniment del contingut hídric foliar. En el pi negre i el neret, la variació d'un dels dos paràmetres no condicionà la variació de l'altre, fet que s'explica per les seves fulles escleròfil·les.

Les alteracions de les relacions hídriques observades en el bedoll i en el neret afectaren els seus processos fotosintètics donant-se una disminució de la disponibilitat de CO_2 als cloroplasts degut a la disminució de la g_s (i possiblement a una disminució en la conductància del mesòfil). El pi negre és l'espècie que mostrà els valors més alts de fotosíntesi neta (*A*) i de capacitat fotosintètica màxima (A_{max}). A finals d'estiu es va donar una disminució en A_{max} però no degut a una limitació estomàtica, si no com a conseqüència d'una disminució en l'activitat de la RuBisco, tal i com mostrà la disminució de la velocitat de carboxilació màxima ($V_{c,max}$). El bedoll també va presentar valors alts de *A* i de A_{max} a l'inici de l'estiu, no obstant, aquests van disminuir de manera important a finals d'estiu coincidint amb les condicions de poca disponibilitat hídrica a llarg termini. La disminució de la fotosíntesi en el bedoll es va donar com a conseqüència d'una limitació estomàtica. El neret va presentar una disminució de *A* en condicions de dèficit hídric a curt termini i una tendència a disminuir en condicions de dèficit hídric a llarg termini.

1.1.2. Respostes davant altes intensitats de llum

Les tres espècies estudiades van presentar diferents respostes davant altes intensitats lumíniques, les quals afecten el procés de captació de la llum per part de les clorofil·les i la posterior transmissió de l'energia d'excitació a la cadena de transport electrònic i finalment el procés de fixació del CO_2. En pi negre, les altes intensitats de llum no van donar lloc a modificacions en l'assimilació del CO_2. En el bedoll, en condicions de dèficit hídric a llarg termini la fotosíntesi es saturà a irradiàncies més baixes i es donà una menor fixació de CO_2 en comparació amb les condicions de major disponibilitat d'aigua, tal i com van mostrar la disminució del rendiment quàntic màxim aparent de fixació de CO_2 (Φ_{CO2}) i de la saturació lumínica de la fotosíntesi (A_{sat}) al setembre del

2011. Aquesta limitació en la fotosíntesi del bedoll es donà com a conseqüència del tancament estomàtic, fet que provocà alteracions en el transport electrònic. El neret va presentar saturació de la fotosíntesi a baixes intensitats de llum, fet que està relacionat amb la seva adaptació a les condicions d'ombra ja que inverteix més recursos en la síntesis de clorofil·les per captar la llum incident i menys en la síntesi de RuBisco.

L'exposició a altes intensitats de llum pot donar lloc a un excés d'energia absorbida per les clorofil·les que no és dissipada de manera eficient i que pot causar limitacions en la maquinària fotosintètica, originant episodis de fotoinhibició i, fins i tot, danys oxidatius. El pi negre, no experimentà alteracions en el transport electrònic ni episodis d'estrès tal i com reflectiren els valors òptims de F_v/F_m. En el bedoll, les condicions de dèficit hídric a llarg termini afectaren negativament el transport electrònic del PSII, donant lloc a processos de fotoinhibició. En el neret, tant en condicions de baixa disponibilitat d'aigua a curt termini com a llarg termini, la fotoquímica esdevingué més limitada per l'excés de llum, ja que el transport electrònic es saturà a baixes intensitats de llum, experimentant així fotoinhibició.

1.1.3. Respostes de fotoprotecció i antioxidants

Davant de condicions de dèficit hídric la maquinària fotosintètica de les tres espècies utilitzà mecanismes de fotoprotecció i sistemes antioxidants per prevenir la formació d'espècies reactives d'oxigen (ROS) i/o per detoxificar-les, ja que podrien donar lloc a danys oxidatius. Al llarg de l'estiu del 2011 i del 2012, el pi negre va ser l'espècie que mostrà major participació de mecanismes de fotoprotecció i d'antioxidants; així, s'observà un alt contingut en carotens i xantofil·les en relació al contingut en clorofil·les (tal i com mostrà l'índex radiomètric SIPI) que participaren en la dissipació de l'excés d'energia d'excitació mitjançant el cicle de les xantofil·les i un contingut alt en ascorbat reduït (AscA), que van contribuir a mantenir uns alts valors de F_v/F_m, indicatius de molt poca fotoinhibició. També l'arquitectura de la capçada del pi i la morfologia de les seves fulles li conferiren una major fotoprotecció amb menor exposició de la superfície foliar a la llum incident degut, en el cas de la fulla, a la combinació de diferents característiques com ara la seva forma cilíndrica, els seus angles inclinats i el fet de que les fulles es proporcionen un ombrejat mutu (Valladares 1999). Tots aquests factors contribuïren a un manteniment més eficient dels processos fotosintètics sota situacions d'estrès.

El bedoll, per altra banda, va destinar més recursos a la síntesis de clorofil·les que no pas a mecanismes de fotoprotecció i va presentar els menors valors d'AscA durant tot l'estiu essent l'espècie amb una menor capacitat antioxidant. Aquesta menor inversió en la protecció dels mecanismes fotosintètics possiblement està relacionada amb la seva naturalesa caducifòlia, invertint més recursos en la productivitat i menys en la preservació de la fulla. En conseqüència, en períodes de dèficit hídric, la maquinària fotosintètica manifestà episodis de fotoinhibició, i contribuïren a l'avançament de la senescència foliar.

El neret va presentar signes d'estrès oxidatiu en condicions de dèficit hídric a llarg termini, mostrant una disminució en els processos de fotoprotecció i en els nivells d'AscA i experimentant una fotoinhibició severa. A l'hivern, el neret està recobert per una capa de neu que li el permet protegir-se de les altes irradiàncies i de la pèrdua d'aigua per transpiració, no obstant, una futura pèrdua d'aquesta capa com a conseqüència de l'increment de les temperatures podria causar una major recurrència dels processos de fotoinhibició i de danys oxidatius.

1.1.4. Variacions en el contingut nutricional foliar

Al llarg de l'estiu s'observaren variacions en el contingut foliar d'alguns minerals, en part com a conseqüència de les condicions de dèficit hídric estival, en part degut a la senescència foliar. Per a la preservació del contingut en nutrients a les plantes, durant la senescència es dóna una remobilització d'aquests des de la fulla cap a altres estructures com ara la tija, les arrels, etc., en el cas d's espècies caducifòlies com el bedoll; o bé, cap a brots joves com és el cas d'espècies perennifòlies com el pi negre i el neret (Millard *et al.* 2001). L'inici de la senescència i, per tant, de la remobilització de nutrients, pot avançar-se en el cas de que les condicions ambientals siguin més estressants, fet que afectaria a l'eficiència dels processos fotosintètics. La senescència a finals de l'estiu es va avançar en el bedoll i el neret com a conseqüència del dèficit hídric, tal i com mostraren els alts valors dels quocients C/N i, en el cas del neret, de C/P. La baixa disponibilitat hídrica a llarg termini a l'estiu del 2011 van condicionar al bedoll a reutilitzar nutrients destinats a la fotosíntesi, com ara el N, per a ésser utilitzats en l'estructura foliar, limitant així l'eficiència en els processos fotosintètics, tal i com s'ha comentat anteriorment. En el cas del neret, el contingut en N disminuí en les fulles velles degut a la seva translocació cap als brots joves, afectant negativament la fotosíntesi. El pi negre no va mostrar signes de senescència avançada, no obstant es va donar una disminució dels nivells de N soluble (que reflecteix en gran mesura la quantitat i l'activitat de la RuBisco). La disminució del N soluble es donà com a conseqüència de la translocació cap als brots joves, afectant la seva capacitat de carboxilació tal com s'observà en els valors decreixents de $V_{c,max}$ i A_{max}. La fotosíntesi en pi negre, per tant, es va veure limitada per la disponibilitat en N.

Quan es donen condicions de dèficit hídric, hi ha una menor translocació de nutrients provinents d'estructures com les arrels cap a les parts aèries degut a la menor transpiració i a la menor absorció d'aigua i elements minerals (Pinkerton i Simpson 1986). Com a conseqüència poden donar-se deficiències d'alguns elements. Tant el pi negre com el neret van presentar dèficit de N i P al llarg de l'estiu, fet que pot estar relacionat amb el seu possible desenvolupament en sòls pobres en nutrients, tret característic d'espècies amb fulles de llarga durada (Mediavilla i Escudero 2003). Durant els períodes de dèficit hídric el bedoll va presentar dèficit en N i en Fe (aquest últim element també en neret), nutrients importants per al bon funcionament de l'aparell fotosintètic, la deficiència dels quals pot haver contribuït a la senescència prematura de les fulles.

1.2. Efecte de l' increment de la concentració de CO_2 ambiental

L'increment dels nivells de CO_2 ambiental a curt termini va donar lloc a un augment en les taxes fotosintètiques en les tres espècies, sobretot en el pi negre i en el bedoll, que van arribar a doblar-les en relació a les observades a nivells actuals de CO_2 ambiental. Aquest fet està relacionat amb una major difusió del CO_2 cap el cloroplast degut al major gradient de CO_2 entre la superfície foliar i aquest, donant lloc a un increment en la carboxilació de la RuBisco. En altres estudis (Ainsworth i Rogers 2007) s'ha vist que les altes concentracions de CO_2 ambiental provocaren una disminució en la g_s, sobretot a exposicions a llarg termini, no obstant, en aquest estudi no es va observar cap disminució en cap de les tres espècies, tan sols un manteniment de la g_s comportant un augment de la WUE_{int} de les tres espècies.

A altes concentracions de CO_2, la fotosíntesi en plantes C3 no es veu limitada per la carboxilació de la RuBisco sinò per la capacitat de regeneració de la RuBP que assoleix el seu màxim a altes intensitats de llum. En el cas del bedoll, en condicions de baixa disponibilitat hídrica i de concentracions de CO_2 elevat, tot i disminuir la g_s, el C_i va augmentar degut a la major difusió del CO_2 a través de l'estoma, donant lloc a una major carboxilació per part de la RuBisco, i provocant que la fotosíntesi es saturés a intensitats de llum més altes en comparació amb nivells CO_2 actuals. Per altra banda, també es va observar un augment en el transport electrònic. Els alts nivells de C_i van donar lloc a un major consum de NADPH i ATP, fet que va disminuir la probabilitat de que es donés un excés d'energia d'excitació en el PSII. Aquestes respostes en la fotosíntesi del bedoll mostren que una exposició a altes concentracions de CO_2 a curt termini podria ser-li beneficiosa en condicions de dèficit hídric. Com ja s'ha comentat anteriorment, el neret, en tractar-se d'una espècie adaptada a l'ombra, inverteix més recursos en captar la llum que arriba a les fulles que no pas en assimilar el CO_2 (RuBisco). A intensitats creixents de llum, no s'observaren diferències en la fotosíntesi en condicions de CO_2 elevat en comparació amb els nivells de CO_2 actuals, tal i com mostraren els valors de A_{sat}, Φ_{CO2} i $PPFD_{sat}$. Per altra banda, tampoc es va observar cap diferència en el transport electrònic, evidenciant que l'assimilació del CO_2 i la fotoquímica estan limitats per les baixes intensitats de llum. El pi negre va presentar valors dels paràmetres fotosintètics més alts que els de neret, no obstant, en cap de les dues espècies es van veure diferències en les corbes A/PPFD ni en la fotoquímica entre nivells de CO_2 actuals i de CO_2 elevats.

En termes generals, les altes concentracions de CO_2 ambientals a curt termini tingueren un efecte positiu en la fotosíntesi i en el transport electrònic en les tres espècies. En el cas de que es doni una exposició perllongada a altes concentracions de CO_2 possiblement aquestes respostes es donin en estadis inicials del seu desenvolupament, mentre no presentin limitacions per altres recursos. En el cas contrari, es pot donar una aclimatació de la capacitat fotosintètica i produir-se una disminució en la velocitat de carboxilació de la RuBisco i en el transport electrònic, fet que pot augmentar la probabilitat de que s'acumuli l'excés d'energia d'excitació i de que es produeixin danys en l'aparell fotosintètic, tal i com s'ha vist en altres estudis (Moore *et al.* 1999, Ainsworth i Rogers 2007).

1.3. Efecte de les infeccions per patògens

Les tres espècies van presentar diferents tipus d'infeccions, algunes, com en el cas del pi negre, no sembla que puguin tenir un efecte perjudicial per l'espècie. Els individus de pi negre presentaren alguns fascicles amb fulles senescents, els quals van mostrar la presència dels fongs *Cytospora pinastri* Fr., *Lophodermella sulcigena* (Link) Tubeuf i *Hendersonia acicola* Munch. et Tub. Es tracta de fongs oportunistes que infectaren els fascicles debilitats però que no es detectaren en fulles no senescents. En el neret s'observà la presència de les fumagines *Aureobasidium pullulans, Fumago vagans* i *Cladosporium* sp., així com d'àcars eriòfids (*Eriophyes* sp.).

Les infeccions més importants que es van detectar foren les produïdes pels fongs basidiomicets de l'ordre Uredinals coneguts popularment com rovells: *Melampsoridium betulinum* en bedoll i *Chrysomyxa rhododendri* en neret. En bedolls, no només es van trobar un major nombre d'individus infectats, sinó que aquests presentaren un grau de severitat de la infecció major que els nerets. Les pústules o uredinis del rovell afectaren la fotoquímica de la regió de la fulla infectada, produint-se fotoinhibició severa ($F_v/F_m < 0.75$). Tot i que els nerets presentaren fulles amb un menor grau d'infecció que els bedolls, els resultats del rendiment quàntic màxim (F_v/F_m) indicaren un major grau de fotoinhibició. Això també es va veure reflectit en la major disminució en el contingut en clorofil·les totals en fulles del neret on les infeccions tenien menor presència en comparació amb el bedoll.

1.4. Perspectives futures de les tres espècies

Els resultats obtinguts en aquest estudi aporten nova informació sobre les respostes del bedoll, el pi negre i el neret en front de condicions ambientals associades al canvi climàtic, que pot ésser aplicada en estudis sobre l'evolució de les seves poblacions i/o sobre els canvis en l'estructura de la comunitat sub-alpina en condicions futures.

La presència d'una major sequera estival en l'estatge sub-alpí en el futur pot limitar de manera important el desenvolupament del bedoll i del neret, ja que, tal i com mostren els resultats obtinguts, necessiten d'una alta disponibilitat de reserves hídriques en el subsòl, sobretot en el cas de la primera espècie, i d'una alta freqüència de precipitacions, sobretot en el cas de la segona espècie. La menor disponibilitat hídrica donarà lloc a que ambdues espècies adoptin estratègies per evitar la pèrdua d'aigua, com ara una disminució de la transpiració, un augment del pes sec de la fulla i una disminució de la seva àrea foliar. Com a conseqüència, l'assimilació de CO_2 disminuirà notablement i es donarà un excés d'energia provinent de la llum incident que no podrà ser dissipada de manera eficient, donant lloc a fotoinhibició, la formació de ROS i la generació de danys oxidatius a les cèl·lules. La menor disponibilitat hídrica estarà relacionada amb una major deficiència de nutrients en les fulles, com ara el N o el Fe, importants per al bon funcionament dels processos fotosintètics. L'augment de les temperatures en un futur pot donar lloc a una disminució de la capa de neu que protegeix el neret enfront les altes irradiàncies i les condicions de dèficit hídric. Les limitacions en la fotosíntesi del neret causades pel dèficit hídric i les altes intensitats de llum observades en aquest estudi, poden agreujar-se en el cas de que disminueixi la coberta de neu en l'alta muntanya (Larcher i Wagner 2004). Per altra banda, la major recurrència d'infeccions per patògens produiran majors limitacions en la fotosíntesi. L'efecte del rovell pot ser encara més agressiu ja que, per una banda, la prevalença i la severitat de la infecció poden augmentar en condicions de major dèficit hídric i, per una altra, l'impacte en el seus hostes pot ser major degut a que patiran més episodis d'estrès hídric i esdevindran més susceptibles a ser infectats (Niinemets 2001, Juroszek i von Tiedemann

2011).

Tots aquests processos donaran lloc a una senescència prematura de la fulla i probablement augmentarà la mortalitat, sobretot en individus juvenils. Aquestes condicions, més estressants pel bedoll i el neret, i que donaran majors limitacions en el seu creixement, poden implicar que altres espècies més tolerants acabin desplaçant les seves poblacions, com és el cas del pi negre, que ha mostrat unes respostes més favorables a les condicions de dèficit hídric i altes intensitats de llum. No obstant, hi ha models que prediuen una disminució de l'àrea geogràfica del pi negre entre el 75% i el 90% per finals de segle degut a la disminució en les precipitacions i a l'augment de les temperatures, i es desplaçaran a una alçada de 2.472 m quan actualment es troben a 2.340 m aproximadament (Benito-Garzón *et al*. 2008, Pérez *et al*. 2011).

2. RESPOSTES ECOFISIOLÒGIQUES DE DIFERENTS CLONS DE *Populus* DAVANT CONDICIONS DE DÈFICIT HÍDRIC ESTIVAL I D'ALTES CONCENTRACIONS DE Zn

2.1. Efecte del dèficit hídric estival en cinc clons de *Populus*

2.1.1. Efecte en les relacions hídriques i en l'estructura foliar

Tenint en compte que l'estudi es va realizar durant l'època de creixement dels 5 clons de pollancre, es va observar que el dèficit hídric estival va afectar les seves respostes fisiològiques de manera diferent segons el moment de l'etapa de creixement en que es produí. Al llarg de l' estudi es va donar un dèficit de precipitacions a finals de primavera - principis d'estiu, fet que va afectar les relacions hídriques dels pollancres en la fase més productiva del creixement. Per altra banda cal destacar les escasses precipitacions i les altes temperatures de finals d'estiu, moment en que les fulles es trobaren a l'inici de la senescència presentant així una major susceptibilitat als estressos ambientals.

Les diferències en la disponibilitat hídrica al llarg de l'estiu es van fer evidents en les relacions hídriques dels cinc clons de pollancre. Per una banda, aquests van presentar un elevat contingut relatiu hídric foliar (RWC) per sobre del 90% a finals de la primavera, fet que s'associà amb un major tancament estomàtic per evitar la pèrdua d'aigua per transpiració i mantenir així un major contingut hídric foliar. El major tancament estomàtic va donar lloc a un increment en l'eficiència en l'ús de l'aigua

(WUE). A mesura que avançà l'etapa de creixement, el RWC i l'índex radiomètric WI (associat a la g_s) van anar disminuint i va ser a finals d'estiu on les plantes van experimentar les condicions de major limitació hídrica. La seva productivitat també pot haver disminuït a finals d'estiu com a conseqüència d'una menor activitat i/o contingut en la RuBisco, tal i com mostrà la reducció en els nivells de N foliar i la degradació de les clorofil·les, i que estarien relacionats amb l'inici de la senescencia.

Davant les diferents condicions de disponibilitat d'aigua al llarg de l'estiu, els diferents clons de pollancre van mostrar diferents adaptacions en les fulles, ja sigui augmentant l'activitat de la RuBisco per realitzar més fotosíntesi i produir més biomassa en el cas que les condicions fossin més favorables, ja sigui invertint més recursos en estructura foliar en el cas d'haver-hi menor disponibilitat d'aigua. L'època en que es van donar majors precipitacions va ser a mitjans d'estiu (juliol), on es va donar un increment en l'àrea foliar, i un major gruix foliar, incrementant així el nombre de capes del mesòfil i augmentant la capacitat fotosintètica. La limitació hídrica a finals d'estiu va donar lloc a una disminució de l'àrea foliar, que és un bon indicador de la magnitud de la reducció de la biomassa de la part aèria de la planta i, per tant, de la sensibilitat del clon de pollancre a les condicions de dèficit hídric (Monclus *et al.* 2005); a un engruiximent en les parets cel·lulars i a una disminució de la mida de les cèl·lules, les quals van esdevenir més agrupades. Aquestes respostes van permetre evitar en major mesura la pèrdua del contingut hídric foliar. No s'observà cap relació entre els resultats de $\Delta^{13}C$ i els valors dels paràmetres de biomassa de la fulla, fet que indicà que l'augment de la WUE pot no estar lligat de manera necessària amb una disminució de la productivitat.

2.1.2. Respostes fotoprotectores i antioxidants

Normalment, l'estrès hídric estival indueix un major tancament estomàtic i una consegüent disminució de l'assimilació de CO_2. Aquest fet pot provocar un excés d'energia d'excitació en els tilacoides que pot donar lloc a fotoinhibició i que podria arribar a causar dany oxidatiu. El rendiment quàntic màxim del PSII (F_v/F_m) s'ha emprat com un indicador de l'estat funcional de l'aparell fotosintètic. Molts dels clons de pollancre d'aquest estudi no van experimentar un estrès sever durant la sequera estival, tal i com indicaren els valors de F_v/F_m, que es van mostrar sempre dins el rang entre 0.75 i 0.85. Aquest fet indicà que tot i la baixa disponibilitat hídrica, l'aparell fotosintètic es va mostrar funcional pràcticament al llarg de tota l'època de creixement. Per evitar que es donés dany oxidatiu, els pollancres dissiparen en forma de calor part de l'excés d'energia lumínica absorbida mitjançant el cicle de les xantofil·les. Els processos de major dissipació tèrmica es donaren a finals d'estiu, amb una major presència del contingut en xantofil·les totals en relació a les clorofil·les, indicatiu d'una major fotoprotecció en aquest període.

A més de la dissipació tèrmica mitjançant el cicle de les xantofil·les, el sistema d'antioxidants també va contribuir a segrestar i/o detoxificar ROS, tot evitant l'estrès oxidatiu en l'aparell fotosintètic. A finals d'estiu es va observar la major activitat antioxidant, incrementant-se els nivells en α-Toc, AscA, GSH i TPhe. Uns alts nivells d'AscA i GSH al setembre també poden estar relacionats amb la síntesis de zeaxantina (Z) mediant així en els processos de dissipació de l'excés d'energia d'excitació.

2.1.3. Susceptibilitat a les infeccions per patògens

Tots els clons van mostrar susceptibilitat a la infecció per rovell (*Melampsora* sp.) tot i que en diferent mesura en funció del clon. La severitat de la infecció va anar incrementant-se a mesura que avançava el període de creixement dels pollancres, arribant al seu màxim a finals d'estiu. Els clons més susceptibles al dèficit hídric estival van esdevenir els més sensibles a la infecció per rovell, mostrant altes incidències i severitats de la infecció. En un estudi en el que participà el doctorand, es va veure que en alguns d'aquests clons de pollancre el rovell tingué un efecte negatiu en les clorofil·les, les relacions hídriques i els processos fotoquímics en el cas dels clons més sensibles a la infecció (Elena *et al*. 2014, veure annex). Únicament en dos clons es va detectar una altra infecció, la causada pel tíngid *Monosteira unicostata*.

2.1.4. Resum sobre la susceptibilitat a l'estrès causada pel dèficit hídric i la infecció per patògens en els cinc clons de Populus

En general, davant les condicions de baixa disponibilitat hídrica al llarg de l'època de creixement, els cinc clons de pollancre van mostrar adaptacions estructurals i en l'obertura estomàtica per tal d'evitar la pèrdua d'aigua, i en els sistemes de fotoprotecció i aintioxidants per evitar l'estrès oxidatiu, tot plegat per intentar mantenir uns alts nivells de productivitat. No obstant, cada clon es caracteritzà per mostrar capacitats diferents per tolerar aquestes condicions així com diferents susceptibilitats a la infecció pel rovell.

2.1.4.1. Adige i Lux

Són els clons que van mostrar les millors respostes fisiològiques durant l'època de creixement. Durant els períodes de major dèficit hídric (maig i setembre) van presentar un RWC adequats i la major WUE en relació als altres clons, evitant en major mesura la pèrdua d'aigua per transpiració. El clon Lux va ser el que va presentar una major àrea foliar, fet que està relacionat amb una major productivitat. Aquests dos clons van ser els que van presentar les majors adaptacions en les seves fulles a les condicions ambientals, i ho van fer mitjançant l'increment de l'àrea foliar per tal d'optimitzar la captació de la llum per realitzar la fotosíntesi al juliol, o bé disminuint l'àrea foliar i augmentant el pes sec per tal d'evitar la pèrdua del contingut hídric al setembre.

Aquests dos clons presentaren les majors respostes fotoprotectores i antioxidants, mostrant els majors nivells de dissipació tèrmica i el major contingut en α- Toc, AscA, GSH i TPhe. Aquestes respostes van permetre mantenir el correcte funcionament de l'aparell fotosintètic tot evitant que es donés fotoinhibició. El clon Lux va presentar un menor contingut en pigments fotosintètics a l'inici de l'època de creixement, degut probablement al encara estat immadur de les fulles, no obstant, no va patir fenòmens de fotoinhibició degut a una alta dissipació tèrmica. Adige va ser el clon menys susceptible a la infecció pel rovell, tot mostrant la menor presència del patògen en les seves fulles i els menors graus de severitat. Lux va presentar una major presència de la infecció en els seus individus, no obstant, la seva severitat va ser baixa.

2.1.4.2. Luisa Avanzo i 58-861

Són els clons que van esdevenir més sensibles a les condicions de dèficit hídric. El clon 58-861 va presentar l'àrea foliar més petita, tret característic d'una menor productivitat. El clon L. Avanzo va ser el que va presentar una menor WUE, indicant una menor regulació de l'obertura estomàtica per evitar la pèrdua d'aigua per transpiració. A l'inici de l'estiu els dos clons van mantenir el contingut hídric foliar mitjançant el tancament estomàtic i a través de l'augment del pes sec de la fulla i la disminució de la seva àrea foliar, tal i com van mostrar els valors de RWC, WI, Δ^{13}C i paràmetres estructurals foliars. No obstant, quan es donaren condicions d'alta irradiància, els dos clons van presentar estrès oxidatiu, tal i com van mostrar els valors baixos de F_v/F_m. Així, 58-861 mostrà una alta activitat del cicle de les xantofil·les per dissipar l'excès d'energia d'excitació en forma de calor, però evidencià una major limitació en els seus mecanismes antioxidants, tal i com mostren els seus baixos continguts en AscA i GSH. L. Avanzo mostrà un alt contingut en TPhe i un increment en AscA durant aquest període que són indicatius del requeriment d'una major intervenció dels mecanismes de protecció per fer front a l'estrès oxidatiu, no obstant van operar de manera ineficient ja que els valors de F_v/F_m es mantingueren baixos.

A finals d'estiu els dos clons van presentar una disminució important del contingut hídric de la fulla, tot mostrant uns valors de RWC per sota del 80%, indicatius d' estrès hídric. L. Avanzo va experimentar una disminució en la seva àrea foliar, i ambdós clons van incrementar el LMA fet que els permetria evitar en major mesura la pèrdua del contingut hídric per transpiració. Els mecanismes de fotoprotecció i antioxidants en L. Avanzo es van mostrar més eficients en aquest període, no obstant, 58-861 va presentar més problemes en les respostes antioxidants. En aquest clon es va observar una disminució en el contingut en GSH tot i l'augment dels nivells de glutatió total, indicant que gran part del glutatió es trobava en estat oxidat i, per tant, que aquest clon presentà majors dificultats per mantenir el "pool" de glutatió en estat reduït durant les condicions d'estrès.

L. Avanzo i 58-861 van ser els clons més susceptibles a les infeccions, sobretot a finals d'estiu. Per una banda L.Avanzo va ser el clon que presentà major sensibilitat a la infecció per rovell, mostrant signes d'infecció severa en el 86% de les seves fulles. Per altra banda, 58-861 presentà una infecció moderada per rovell, no obstant, també va mostrar infecció pel tíngid *Monosteira unicostata*. La susceptibilitat d'aquests dos clons per les infeccions pot estar relacionada amb el seu menor "pool" de glutatió i/o amb la major formació de ROS a finals d'estiu fet que pot jugar un rol important en les reaccions bioquímiques contra el desenvolupament de la malaltia.

2.1.4.3. I-214

Aquest clon no va presentar símptomes importants d'estrès per les condicions de dèficit hídric al llarg de l'estiu, però tampoc va mostrar característiques destacades en la productivitat o en la capacitat de tolerar les condicions d'estrès. Per una banda, va ser el clon que va presentar menors variacions en la WUE al llarg del'estiu i, a més, va mostrar-ne uns valors alts, característiques que indiquen una regulació estomàtica per evitar la pèrdua d'aigua per transpiració. No obstant, a mitjans i finals d'estiu, el contingut hídric foliar va disminuir tal i com van mostrar els valors de RWC <80% indicatius d'estrès hídric. Aquest clon va mostrar, juntament amb Lux i Adige, les majors adaptacions morfològiques de la fulla que li permetrien mantenir en major grau el contingut hídric foliar.

Davant les altes intensitats de llum incident, I-214 no mostrà cap variació en els valors de F_v/F_m al llarg de l'estiu, indicant que no va patir fotoinhibició. No es va donar un augment en la dissipació de l'excés d'energia d'excitació, fent evident que la fotoquímica va funcionar de manera adequada. En quant al sistema d'antioxidants, I-214 va ser el clon que va presentar un menor contingut en AscA, no obstant, els seus nivells juntament amb els de GSH van incrementar-se a finals d'estiu, intervenint així en el manteniment de la funcionalitat de l'aparell fotosintètic.

En quant a la infecció per patògens, I-214 va ser, juntament amb 58-861 el més susceptible a ser infectat, ja que va mostrar presència del rovell amb un grau de moderat, i de *Monosteira unicostata*.

2.2. Efecte de les altes concentracions de Zn

Per determinar la susceptibilitat del clon I-214 a nivells elevats de Zn en el medi de creixement, s'han estudiat les seves respostes fisiològiques i s'han comparat amb les del clon Eridano, que es caracteritza per presentar bones respostes enfront condicions d'estrès com ara una alta capacitat de supervivència en l'estadi juvenil en condicions de camp (Coyle *et al.* 2006a), una alta resistència a les malalties (Coyle *et al.* 2006b), una bona tolerància a nivells elevats de metalls, com ara el Zn, i una elevada capacitat d'acumular-los en les seves estructures tal i com han mostrat alguns experiments de fitorremediació (Sebastiani *et al.* 2004).

Fins ara, s'han utilitzat diferents mètodes en els estudis sobre l'aclimatació dels arbres a l'estrès per metalls, com són el cultiu de suspensions cel·lulars (Dickinson *et al.* 1992) o els cultius de calls (Watmough i Dickinson, 1995), però el que més s'està fent servir és el de creixement de plàntules, tot i que, en comparació amb els arbres madurs, són més sensibles a les condicions adverses. Existeixen moltes diferències fisiològiques entre els arbres adults i les plàntules, com ara la distribució del carboni en la planta, l'estructura de la capçada i la fracció de teixit fotosintèticament inactiu, que va augmentant amb l'edat (Turner 1994). Tot i que les plàntules, o esqueixos, són més sensibles a les condicions adverses, la seva resposta a nivells elevats de metalls pesants permet determinar l'habilitat de diferents espècies o clons de la mateixa espècie per absorbir, tolerar i sobreviure a l'estrès causat per aquestes condicions. Així, per exemple, s'han dut a terme estudis de 10 clons de pollancres davant altes concentracions de Cd que van permetre veure diferències en la producció de biomassa i en la capacitat d'absorbir el metall (Zacchini *et al.* 2009, Pietrini *et al.* 2010a).

L'estudi realitzat sobre l'efecte del Zn en els clons I-214 i Eridano mitjançant un cultiu hidropònic ha permès conèixer els processos biològics que permeten tolerar i acumular el metall en el pollancre, i avaluar l'aplicabilitat dels dos clons en tasques de fitoremediació.

2.2.1. Símptomes visuals i producció de biomassa

Les dues concentracions de Zn (1mM i 5mM) amb les que es van treballar van esdevenir tòxiques per als dos clons. A nivell visual es van observar diferents símptomes en funció del grau de toxicitat. Així, a 1mM [Zn] les fulles joves presentaren una clorosi molt estesa i alguns brots van manifestar necrosi. A 5mM [Zn] les respostes van ser més severes: es va donar una forta disminució del creixement, les arrels van presentar un color marronós (en el tractament control les arrels presentaven un color blanquinós), i poques fulles van arribar a desenvolupar-se, i les que ho van fer van mostrar clorosi.

La menor producció de biomassa a 5mM [Zn] en comparació amb 1mM [Zn] va fer evident que els dos clons presentaren major toxicitat a aquestes concentracions. Les arrels van esdevenir un òrgan sensible a les altes concentracions de Zn observant-se, sobretot a 5mM [Zn], una disminució del seu creixement. La reducció de la biomassa de l'arrel pot afectar negativament la seva capacitat d'absorció d'aigua i d'elements minerals i, per tant, de Zn, fet que pot suposar un inconvenient a l'hora d'ésser utilitzat en tasques de fitoremediació. En experiments amb faveres s'ha vist que la reducció de la biomassa degut a la toxicitat per Zn inhibeix la translocació de fotoassimilats des de les fulles cap a les arrels (Ruano *et al*. 1988) donant lloc a un increment en el creixement de la tija en relació a les arrels, tal i com s'ha vist a I-214 a 5mM [Zn].

2.2.2. Absorció i acumulació de Zn

Per determinar l'aplicabilitat dels dos clons en tasques de fitoremediació, és important conèixer la seva capacitat per absorbir, acumular i tolerar les altes concentracions del metall. Un augment de les [Zn] en el medi de creixement donà lloc a majors [Zn] en totes les estructures de les plàntules, especialment a les arrels (un ordre de magnitud més gran). L'acumulació d'altes [Zn] a les arrels pot estar relacionada amb una limitació de la translocació del metall cap a les fulles, les quals són estructures especialment sensibles als metalls pesants ja que és on es donen els processos fotosintètics i, per tant, pot ésser considerada una resposta per evitar que es doni toxicitat en aquests òrgans. Aquest fet li confereix a la planta una major tolerància a les altes [Zn]. La capacitat d'aquestes plantes per acumular Zn, particularment a les arrels, és una característica que es podria aprofitar per a la rizofiltració d'aigües contaminades per metalls com a conseqüència de les activitats industrials i agrícoles. A 5mM [Zn], Eridano mostrà una major concentració de Zn en les arrels que no pas I-214. Per altra banda, l'augment en l'acumulació de Zn en les parts aèries d'Eridano a 5mM [Zn] en relació a 1mM [Zn] pot suposar una característica interessant per a la fitoextracció, especialment quan el metall és acumulat en la tija i en l'esqueix llenyós. De fet, aquests òrgans de la planta acabaran esdevenint estructures llenyoses sense capacitat de renovació (al contrari que les fulles) i amb una menor capacitat d’acumular metalls, però que poden ser extrets i emmagatzemats per així enretirar el metall del medi sense causar grans danys a l'arbre tenint en compte la seva capacitat de rebrot.

La toxicitat per Zn en les plantes cultivades es dóna quan els nivells d’aquest metall assoleixen una concentració en les fulles entre 400 i 500 $mg \cdot kg^{-1}$ de pes sec (Marschner 1995) tot i que els llindars de toxicitat poden variar àmpliament, fins i tot dins la mateixa espècie (Broadley *et al.* 2007). En aquest estudi, les fulles (sobretot les joves) van mostrar en ambdós tractaments de Zn concentracions del metall per sobre del llindar, afectant negativament el creixement (tal i com s’ha vist en l’índex de tolerància (T_i) i en els paràmetres de biomassa) i al bescanvi de gasos. La quantitat de Zn acumulada en la planta sencera va ser major en Eridano que no pas en I-214 (al voltant d’un 50%), i no es van observar diferències entre 1mM [Zn] i 5mM [Zn] en cap dels dos clons, fet que indicà que a 1mM [Zn] els dos clons van arribar al límit de la capacitat d’emmagatzemar el metall, donant lloc a una disminució de l’eficiència d’absorció de Zn provinent del medi de creixement.

Els dos clons van presentar una major capacitat d’extreure Zn del medi en el tractament d’1mM [Zn], tal i com van mostrar els resultats dels paràmetres “Uptake ratio” i “Bio-concentration factor” (BCF). Eridano va ser el clon amb una major capacitat d’extracció de Zn del medi a 5mM [Zn]. La major capacitat d’acumular el metall per part dels dos clons es va donar a 1mM [Zn], concretament a les fulles i les arrels. A 5mM [Zn], la menor acumulació en fulles i arrels està associada a una menor absorció del metall com a conseqüència d’una menor producció de biomassa. Eridano presentà un major contingut de Zn a fulles i arrels; la tija i l’esqueix van ser els òrgans que mostraren una menor acumulació de Zn. De totes maneres, ja que la fusta de l’individu adult representa una proporció més significativa de la biomassa total que no pas les arrels, el contingut en metall de la part llenyosa pot arribar a representar, en el cas de l’individu adult, la part majoritària del contingut de tot l’arbre (Pietrini *et al.* 2010a).

Els resultats de BCF i T_f (factor de translocació) ens han proporcionat informació sobre la distribució de Zn en els diferents òrgans. Així, es va veure que la translocació del metall de les arrels a les parts aèries va disminuir a 1mM [Zn] i a 5mM [Zn] respecte el control, sobretot en Eridano. De fet, el T_f mostrà valors inferiors a 1, fet que, juntament amb la major concentració de Zn a les arrels, indicà que es donà una major absorció del metall per les arrels però una menor transferència cap a les parts aèries característica de les espècies denominades “acumuladores a les arrels”. Aquest fet comporta una major protecció de les estructures relacionades amb la fotosíntesi, com s’ha comentat anteriorment, i d’acord amb el descrit en altres clons de pollancre exposats a altres tipus de metalls pesants en cultius hidropònics (Romeo *et al.* 2014). Per tant, Eridano mostrà una major capacitat de protecció de les parts aèries que no pas I-214, especialment a 1mM [Zn].

En relació a l’acumulació de Zn observada en els dos clons exposats als diferents tractaments, Sebastiani *et al.* (2004) van obtenir resultats similars, tot i que, en el seu treball, els pollancres van créixer en sòls contaminats amb residus industrials i, per tant, presentaren una diferent biodisponibilitat del metall. Aquesta pot variar en funció de les característiques físico-químiques del sòl en qüestió i, per tant, aquest problema pot ésser solucionat mitjançant els cultius hidropònics (dos Santos Utmazian *et al.* 2007).

2.2.3. Estructura de la fulla, relacions hídriques i bescanvi de gasos

Les altes concentracions de Zn van tenir efectes diversos en la morfologia de la fulla, en les relacions hídriques i en el bescanvi de gasos dels dos clons. Els canvis en la morfologia de la fulla en resposta als metalls pesants pot disminuir la capacitat de les espècies per tolerar les condicions de sequera (Barceló i Poschenrieder 1990). Les modificacions observades estan relacionades amb una major inversió dels recursos de la planta per evitar la pèrdua d’aigua per transpiració. L’augment de la densitat foliar (D) està relacionada amb l’engruiximent de les parets cel·lulars i amb unes cèl·lules més petites i agrupades (Niinemets 2001). La disminució del gruix foliar (T) indica que s’ha donat una reducció de la capa de cèl·lules del mesòfil i, per tant, una menor acumulació de la proteïna fotosintètica per unitat d’àrea foliar, i una menor capacitat fotosintètica.

Els resultats obtinguts de A i g_s a 1mM [Zn] mostraren que la toxicitat per Zn afectà els processos fotosintètics abans que les relacions hídriques. Per altra banda A i g_s van disminuir de manera molt marcada a 5mM [Zn], mostrant una alta toxicitat pel metall. La g_S és un indicador integratiu del grau d’estrès hídric (Flexas *et al.* 2002) i és una característica que pot permetre avaluar l’efectivitat de la fitoextracció en les plantes llenyoses (Stomp *et al.* 1993). A 1mM [Zn] es van observar episodis d’estrès hídric, no obstant va ser a 5mM [Zn] on van experimentar estrès hídric extrem ($g_s \leq 25$ mmol·m^{-2}·s^{-1}).

Els resultats de $\Delta^{13}C$ mostren que la WUE a llarg termini no va variar a 1mM [Zn] en relació al control. Tenint en compte que els resultats de $\Delta^{13}C$ reflecteixen els valors de

C_i, es pot deduir que el manteniment dels valors de $\Delta^{13}C$ a 1mM [Zn] en relació al control indicà un manteniment de C_i. Contràriament, a 1mM [Zn], el manteniment de C_i i el manteniment o la disminució de la g_s en relació al control indicaren que la reducció de *A* estigué relacionada amb la disminució del transport electrònic i els processos de fotoinhibició, tal i com mostren els valors de F_v/F_m.

2.2.4. Fotoquímica, pigments cloroplàstics i antioxidants

Les imatges de fluorescència de les clorofil·les aportaren informació sobre l'efecte de la toxicitat per Zn en l'aparell fotosintètic al llarg de la superfície foliar. A 5mM [Zn], la fotoquímica es va veure afectada en àrees properes a la vena principal, les quals van mostrar valors de F_v/F_m per sota de 0.75 indicant fotoinhibició severa. Les àrees de la fulla que es van mostrar especialment afectades per la toxicitat pel Zn van esdevenir cloròtiques i necròtiques (regions de color més fosc), i van mostrar valors de F_v/F_m especialment baixos, suggerint la presència d'una major acumulació de metalls (Pietrini *et al*. 2010b).

Els resultats de fluorescència de les clorofil·les mostraren fotoinhibició severa en els dos clons a 1mM [Zn] ($F_v/F_m \approx 0.5$). El Zn pot desplaçar el Mn en el lloc de protòlisis de l'aigua inhibint la producció d'oxigen i la transferència d'electrons (Ralph i Burchett 1998). Aquest fet, però, no s'observà ja que l'augment en l'índex de de- epoxidació (AZ/VAZ) indicà que es donà transferència d'electrons cap als centres de reacció i un augment de la dissipació de l'excés d'energia d'excitació en forma de calor a altes [Zn] (Monnet *et al*. 2001).

Les respostes de fotoprotecció i antioxidants van augmentar a mesura que les [Zn] al medi de creixement van ser majors. Tot i això, a altes [Zn] els processos fotoquímics i moltes funcions fisiològiques van veure's alterats de manera important donant lloc al marciment de la fulla. D'aquesta manera, en el cas de I-214, els danys causats a les fulles a 5mM [Zn] no van permetre determinar el contingut en pigments cloroplàstics ni d'antioxidants. El sistema de síntesis de clorofil·les pot haver patit danys degut a la substitució del Mg pel Zn (Prasad i Strzalka 1999) o per la competència entre el Fe i el Zn, comportant la destrucció dels pigments antena (Abadía *et al*. 1999).

Els dos clons van mostrar tendències dels nivells de α-Toc a augmentar a mesura que ho feien les concentracions de Zn, com també s'ha vist en altres estudis amb *Lemna minor* (Artetxe *et al*. 2002). L'Ascorbat únicament es va trobar en el seu estat reduït en tots els tractaments amb Zn indicant una adequada regeneració a partir del

GSH. Per altra banda, l'augment en les concentracions de Zn no va provocar alteracions en els nivells de GSH en comparació amb la seva forma oxidada. Aquests resultats mostren una aclimatació activa del "pool" de glutatió a l'estrès.

2.2.5. Balanç nutricional

El balanç nutricional a les cèl·lules vegetals es va veure alterat per la toxicitat per Zn, i es van donar diferències en l'absorció de nutrients a concentracions creixents de Zn (Wang *et al*. 2009). Aquestes diferències poden ser atribuïdes a als mecanismes de translocació o a les interaccions entre elements (Sinha *et al*. 2006). A més dels canvis en el contingut en Mg i Fe i el seu efecte en el contingut en clorofil·les, la disminució del P observada sobretot en les estructures aèries a 5mM [Zn], podria agreujar la toxicitat per Zn, (Jiang *et al*. 2007), com també s'ha vist en estudis amb *Populus tremula* (Vollenweider *et al*. 2011). A més, la tendència dels nivells de Mg, Cu, Fe i Mn a disminuir en resposta a les concentracions creixents de Zn podria afectar les activitats de la superòxid dismutasa (SOD) i a les respostes antioxidants (Bonnet *et al*. 2000).

2.2.6. Diferències en les respostes de les fulles joves i les fulles velles

Els efectes de la toxicitat del Zn es van manifestar de manera diferent en les fulles presents dins d'un mateix individu de pollancre, en funció de si aquestes es van desenvolupar abans de realitzar el tractament amb [Zn] (fulles velles) o bé, si es van desenvolupar després (fulles joves). En termes generals, com s'ha comentat anteriorment, a 1mM [Zn] les fulles joves van desenvolupar-se amb clorosi difusa, mentre que algunes fulles velles només en van presentar símptomes. Les altes concentracions de Zn no van causar diferències en la producció de biomassa entre fulles velles i joves. Les diferències, però, es van fer evidents a 1mM [Zn]. En les fulles joves s'observà que els alts nivells de Zn afectaren negativament la *A* i la g_s, mentre que en les fulles velles van afectar a la *A*, però en menor mesura que a les fulles joves. Aquests resultats suggereixen que la toxicitat per Zn es va fer evident, en primer lloc, en els processos fotosintètics que no pas en les relacions hídriques, probablement començant primer en les fulles joves, que constitueixen teixits en desenvolupament amb cèl·lules metabòlicament actives i que són més sensibles a l'excés de metall (Ernst 2006). Per altra banda, les fulles joves van experimentar estrès hídric lleu (g_s = 100-200 mmol·m^{-2}·s^{-1}) mentre que les fulles velles van presentar valors de g_S similars als del control. A 1mM [Zn], la disminució en el contingut en N per àrea foliar en les fulles joves pot estar relacionat amb una menor síntesis de la RuBisco. Per altra banda, en fulles velles, la disminució de *A* no estigué relacionada amb el transport electrònic o la disponibilitat de N, indicant, per tant, possibles canvis en la conductància de CO_2 a través del mesòfil (Di Baccio *et al*. 2003; Sagardoy *et al*. 2010) i/o modificacions en l'activitat de la RuBisco. De fet, si s'afavoreix el Zn en la competència respecte al Mg per la formació i per la funció catalítica del complexe ternari Rubisco- CO_2-metall $^{2+}$, llavors l'afinitat de la RuBisco pel CO_2 podria disminuir (Monnet *et al*. 2001). A 1mM [Zn], la fotoinhibició severa observada en fulles joves pot estar relacionada amb un descens en el contingut en clorofil·les totals (responsable de la clorosi difusa) i amb la disminució de la fotosíntesi. Van existir diferents sensibilitats a l'estrès oxidatiu entre els diferents tipus de fulla, ja que va ser en les fulles joves on es van detectar les respostes inicials dels sistemes de protecció a la toxicitat per Zn a 1mM [Zn], reflectit per un elevat quocient AZ/VAZ.

La major susceptibilitat de les fulles joves a les altes [Zn] es deu, en part, a que aquestes estructures acumulen un major contingut de Zn que no pas les fulles velles. Tenint en compte que les fulles velles mostren major tolerància a les altes [Zn], es suggereix que l'aplicació de plàntules de pollancres en tasques de fitoremediació, almenys en els casos de I-214 i Eridano, es realitzi amb esqueixos que presentin un mínim de biomassa foliar, ja que pot facilitar el seu desenvolupament en aquests medis estressants.

2.2.7. Resum sobre la diferent susceptibilitat a l'estrès causada per concentracions elevades de Zn a Eridano i I-214

Els efectes de l'excés de Zn en les característiques fisiològiques d'ambdós clons van ser evidents, però amb diferències remarcables entre ells: Eridano presentà una major tolerància a les altes concentracions de Zn que no pas I-214 ja que, en aquest darrer, un terç de les plàntules del tractament 5mM [Zn] van manifestar signes de danys molt severs (fulles cloròtiques, deshidratades i marcides), fet que no va permetre realitzar algunes mesures fisiològiques.

Les diferències entre clons també es van observar en els paràmetres de biomassa. A 1mM [Zn], la producció de biomassa en Eridano va ser similar a la del control, mentre I-214 mostrà una reducció del 55%. I-214 va mostrar un increment en la relació de la biomassa aèria respecte la de l'arrel a 5mM [Zn] degut a una menor biomassa d'aquesta darrera estructura. La major capacitat d'Eridano per tolerar la toxicitat per Zn en relació a I-214 es va fer evident pel seu major índex de tolerància (T_i) tant en les arrels com en les fulles a ambdues concentracions de Zn.

Les altes concentracions de Zn van tenir efectes diferents en la morfologia de la fulla, en les relacions hídriques i en bescanvi de gasos dels clons Eridano i I-214. De fet, I-214 va mostrar un increment de la densitat foliar (D) i una disminució del gruix foliar i de l'àrea foliar (LA), indicant que presentà majors dificultats per mantenir el contingut hídric foliar i que les fulles destinaven més recursos a l'estructura que no a la fotosíntesi. De fet, a 1mM [Zn] i, especialment en fulles joves, la fotosíntesi neta va disminuir de manera important (82%) en I-214, mentre que en Eridano la reducció va ser més moderada (42%). A 1mM [Zn], I-214 va ser el clon més afectat per la toxicitat per Zn, mostrant una disminució en *A* i, només en fulles joves, en g_s, mentre Eridano va mostrar signes de toxicitat únicament en fulles joves tal i com mostrà la disminució de *A* en relació al control. Els menors símptomes de toxicitat observats a les fulles d'Eridano a 1mM [Zn] podria estar relacionat amb la menor acumulació de Zn en les parts aèries en relació a I-214, tal i com s'ha esmentat anteriorment

A 1mM [Zn], Eridano va experimentar estrès hídric lleu ($g_s \leq 200$ mmol· $m^{-2} \cdot s^{-1}$), mentre que I-214 presentà estrès hídric sever ($g_s \leq 100$ mmol· $m^{-2} \cdot s^{-1}$). A 5mM [Zn] els dos clons van experimentar estrès hídric extrem, com ja s'ha comentat abans. A 1mM [Zn], cap dels clons no va mostrar alteracions en el contingut hídric de les fulles tal i com van mostrar els alts valors de RWC (entre 80% i 90%). A 5mM [Zn], el tancament estomàtic va permetre al manteniment dels valors de RWC entre 80% i 90% en Eridano (Fernàndez-Martínez *et al.* 2013), mentre que en I-214 el RWC va mostrar valors per sota del 40% mostrant un dèficit hídric important. A 5mM [Zn] la WUE augmentà en Eridano degut al tancament estomàtic, fet que està relacionat amb l'alt RWC observat. A aquestes [Zn] la reducció de C_i en Eridano va ser resultat d'un notable tancament estomàtic.

I-214 va presentar més problemes en la fotoquímica que no pas Eridano. A 5mM [Zn], Eridano va presentar fotoinhibició ($F_v/F_m = 0.75$) mentre I-214 va presentar fotoinhibició severa ($F_v/F_m \approx 0.35$). I-214 va mostrar més fotoprotecció a 1mM [Zn] en comparaciò amb Eridano, reflectit per un elevat AZ/VAZ, i també va ser el primer en mostrar episodis d'estrès oxidatiu, fet que pot indicar diferents susceptibilitats a les altes [Zn], entre clons. Per altra banda, a Eridano es donà una major disminució de clorofil·les a mesura que augmentaren les concentracions de Zn. Els individus de I-214 del tractament control van presentar un major contingut en AscA que els individus control d'Eridano, indicant una major activitat de detoxificació i, per tant, una major sensibilitat a l'estrès oxidatiu. Els nivells de TPhe van augmentar en Eridano a 5mM [Zn] tot mostrant una major capacitat antioxidant.

CONCLUSIONS

1. L'exposició del bedoll, neret i pi negre a altes concentracions de CO_2 a curt termini va donar lloc a un augment de les taxes fotosintètiques, no va causar variacions en la conductància estomàtica i va provocar un increment en la WUE en relació als resultats obtinguts a les concentracions ambientals de CO_2 actuals.

2. El pi negre no es va veure afectat per les condicions de dèficit hídric essent l'espècie que presentà les majors taxes de fotosíntesi, la capacitat fotoprotectora més alta, la major inversió de N en RuBisco, i uns alts nivells de K, Fe i Mg foliars.

3. Les característiques estructurals foliars del pi negre van contribuir a que presentès més tolerància a les condicions de dèficit hídric, mentre que la disponibilitat de N, l'activitat i/o els nivells de la RuBisco foren els principals limitants de la seva fotosíntesi.

4. El bedoll va ser l'espècie més vulnerable a condicions de dèficit hídric, tal i com mostraren els resultats de bescanvi de gasos, de $\Delta^{13}C$ i els paràmetres estructurals foliars.

5. El bedoll presentà limitacions estomàtiques de la fotosíntesi en condicions de dèficit hídric que van contribuir a un avançament de la senescència foliar tal i com mostraren els valors de PNUE, C/N i la disminució dels nivells de N.

6. El bedoll va ser l'espècie més susceptible a la infecció per rovell, però l'efecte d'aquesta infecció en l'aparell fotosintètic va ser més notable en neret.

7. El neret va mostrar susceptibilitat a una baixa disponibilitat hídrica. A altes intensitats de llum presentà limitacions de la fotosíntesi i una disminució en l'eficiència dels centres de reacció del PSII, donant lloc a una major susceptibilitat a la fotoinhibició.

8. Dels clons de pollancre estudiats, Adige i Lux, van presentar durant l'època de creixement, característiques estructurals, de fotoprotecció i antioxidants que permeteren preservar la funcionalitat del PSII en condicions d'estrès hídric considerant-se adequades pel seu ús en la restauració ambiental dins de la regió mediterrània, en canvi Luisa Avanzo i 58-861 foren els clons més susceptibles a patir estrès.

9. Adige i Lux van presentar una baixa susceptibilitat a les infeccions per *Melampsora* sp. i *Monosteira unicostata*, mentre que L. Avanzo i 58-861 foren els que presentaren una major susceptibilitat.

10. Les concentracions de Zn a 5mM en el medi de creixement van esdevenir molt toxiques pels clons de pollancre I-214 i Eridano donant lloc a una disminució en la producció de la biomassa i al deteriorament dels processos fotosintètics i de les relacions hídriques.

11. Les arrels foren les estructures de la planta que presentaren una major acumulació de Zn, sobretot en Eridano.

12. Eridano presentà menors [Zn] en les fulles en relació a I-214, fet que li va permetre limitar els efectes tòxics del metall en el procés fotosintètic.

13. Les respostes de fotoprotecció i antioxidants augmentaren a majors [Zn], sobretot en I-214, indicant una major sensibilitat a l’excés de Zn.

14. La destacable habilitat del clon Eridano per acumular Zn en les seves estructures junt amb una notable tolerància als alts nivells d’aquest metall demostren la seva aplicabilitat en tasques de fitorremediació.

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ANNEX

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Research paper

Stomatal patchiness in the Mediterranean holm oak (*Quercus ilex* L.) under water stress in the nursery and in the forest

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The evergreen holm oak *Quercus ilex* L. is the most representative tree in Mediterranean forests. Accurate estimation of the limiting factors of photosynthesis for *Q. ilex* and the prediction of ecosystem water-use efficiency by mechanistic models can be achieved only by establishing whether this species shows heterogenic stomatal aperture, and, if so, the circumstances in which this occurs. Here, we collected gas-exchange and chlorophyll fluorescence data in *Q. ilex* leaves from a nursery to measure the effects of stomatal oscillations on PSII quantum yield (Φ_{PSII}) under water stress. Stomatal conductance (g_s) was used as an integrative indicator of the degree of water stress. Images of chlorophyll fluorescence showed heterogeneous Φ_{PSII} when g_s was <50 mmol H_2O m^{-2} s^{-1}, representative of severe drought and corresponding to a container capacity <45%. Stomatal patchiness was related to a coefficient of variation (CV) of Φ_{PSII} values >2.5%. A parallel study in the forest confirmed heterogeneous Φ_{PSII} values in leaves in response to declining water availability. Three kinds of *Q. ilex* individuals were distinguished: those resprouting after a clear-cut (resprouts, R); intact individuals growing in the same clear-cut area as resprouts (controls, C); and intact individuals in a nearby, undisturbed area (forest controls, CF). Patchiness increased in C and CF in response to increasing drought from early May to late July, whereas in R, Φ_{PSII} values were maintained as a result of their improved water relations since the pre-existing roots were associated with a smaller aerial biomass. Patchiness was related to a % CV of Φ_{PSII} values >4 and associated in the summer with mean g_s values of 30 mmol H_2O m^{-2} s^{-1}. Under milder drought in spring, Φ_{PSII} patchiness was less strictly related to g_s variations, pointing to biochemical limitants of photosynthesis. The occurrence of heterogenic photosynthesis caused by patchy stomatal closure in *Q. ilex* during severe drought should be taken into account in ecosystem modelling in which harsher water stress conditions associated with climate change are predicted.

Keywords: imaging fluorescence, photosynthesis limitants, stomatal patchiness, water stress, ecosystem modelling.

Introduction

Stomatal and non-stomatal limitants of photosynthesis can be determined via an indirect calculation of the intercellular CO_2 concentration (C_i), which is obtained from gas-exchange measurements (Von Caemmerer and Farquhar 1981). These calculations assume uniform stomatal aperture in the whole leaf. However, under severe stress, C_i can be overestimated because of heterogeneity in stomatal closure (Laisk 1983), thereby leading to the misinterpretation of photosynthetic responses to C_i and invalidating the predictions of different photosynthetic limitants (Jones 1985, Takanashi et al. 2006). Moreover, stomatal patchiness would not only lead to C_i variations but would also determine photosynthetic rates.

Heterogeneity in the distribution of photosynthesis, which is attributed mainly to 'patchy' stomatal aperture, has been reviewed by Terashima (1992), Beyschlag and Eckstein (1998) and Mott and Buckley (2000), among others. The patches observed on a leaf (caused by differences in stomatal conductance) can be different each time they appear (e.g., size and shape) and can appear at different places on the surface (Cardon et al. 1994). Patchy stomatal aperture is short and arises in response to perturbation or stress factors, such as a decrease in humidity (Cardon et al. 1994), a reduction (Eckstein et al. 1996) or an increase in the photosynthetic photon flux rate (PPFR) (Bro et al. 1996), drought stress (Downton et al. 1988) and abscisic acid treatment (Terashima et al. 1988, Mott 1995). Patchiness can be stationary or may oscillate and move around the leaf (Cardon et al. 1994). It may also be unpredictable, showing changes in behaviour over time (Mott and Peak 2007). Hydraulic interactions between stomata depend on the interactions between epidermal turgor, stomatal aperture and transpiration, and it has been proposed that they coordinate stomata within a patch (Mott and Franks 2001, Marenco et al. 2006).

The evergreen holm oak (*Quercus ilex* L.) is a deep-rooted dominant species of Mediterranean forests. These ecosystems are characterized by episodes of drought stress during the summer. This stress induces a decrease in leaf stomatal conductance in *Q. ilex* hypostomatous leaves, thereby restricting the availability of CO_2 in chloroplasts and resulting in a midday depression of photosynthesis (Tenhunen et al. 1987). *Quercus ilex* presents a great capacity to resprout from its underground organs after a disturbance or in response to specific management practices (Retana et al. 1992). In both scenarios, resprouts exhibit similar enhanced rates of gas exchange and growth in the first few years with respect to the original vegetation (Fleck et al. 1996, 1998). This finding is attributed to the use of underground reserves (Malanson and Trabaud 1988) and the increased availability of water (Saruwatari and Davis 1989) or nutrients (Oechel and Hastings 1983), or both, to *Q. ilex* as a result of its extensive root system and small resprouting shoot. The increased probability of drought, heat stress and rising atmospheric CO_2 concentration during the coming decades is particularly relevant in the Mediterranean Basin (Christensen et al. 2007) as vegetation structure and plant productivity will be markedly affected.

Under simulated Mediterranean summer conditions, *Arbutus unedo* L. (Beyschlag and Pfanz 1990) and *Quercus suber* L. leaves (Beyschlag et al. 1992) show a pronounced patchy distribution of stomatal conductance. In young olive leaves, patchiness caused by exposure to large vapour pressure deficit (VPD) has been also reported (Loreto and Sharkey 1990). In addition, the deciduous oak *Quercus petraea* Matt. Liebl. also shows non-uniform gas exchange in response to increasing drought (Epron and Dreyer 1993). Heterogenic stomatal closure has also been described in other woody angiosperms, such as *Quercus alba* L., *Quercus stellata* Wangenh and *Acer saccharum* Marsh., but in these cases not under severe drought (Ni and Pallardi 1992).

Although patchy stomatal conductance is not detectable with gas-exchange measurements, chlorophyll fluorescence imaging provides a non-destructive technique to determine the spatial distribution of photosynthesis, and the images obtained can be interpreted in terms of stomatal conductance.

The aim of the study was to determine the occurrence of patchy photosynthetic behaviour, its relation with the heterogeneity of stomatal aperture and whether this behaviour occurs in holm oak under drought stress. To the best of our knowledge this is the first study to address these issues in *Q. ilex*, the predominant species in Mediterranean forests.

For this purpose, we performed studies in the forest and in the nursery. The former was conducted from spring (mild drought) to summer (severe drought). Here, we also compared resprouting individuals with intact individuals growing either in the same area or in a nearby undisturbed area. Canopy transpiration and thus, soil water availability was therefore expected to differ between these two areas. A complementary study in the nursery was performed to simulate conditions that are not readily characterized in the forest. In the nursery, we gradually exposed potted plants to increasing water stress, thereby simulating a wide range of soil water content values (under the same environmental conditions).

Materials and methods

Plant material and experimental design

Nursery study

Six 3-year-old holm oak plants (*Quercus ilex* L.) were lifted from a nursery and transferred to 3.5 l pots filled with peat and perlite (2 : 1, v/v). The pots were placed in a covered nursery in natural Mediterranean conditions at the Experimental Fields of the University of Barcelona, NE Spain (41°22′59′N, 2°6′44′E, altitude 60 m above sea level). Plants were irrigated daily with water to container capacity (CC). After two weeks, irrigation was stopped to achieve ~30% CC, thereby inducing a gradual drought. The duration of each drought cycle lasted ~5 weeks (Figure 1). Leaf gas-exchange and

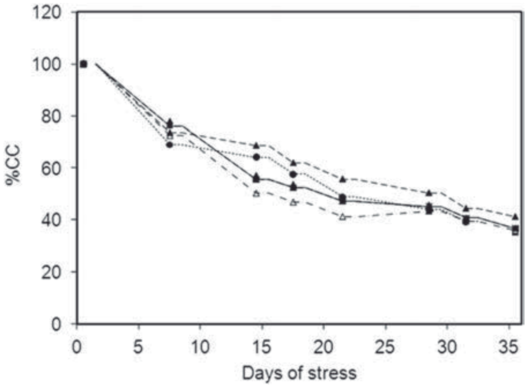

Figure 1. Container capacity percentage of five individual plants used in the study during the drought treatment in the nursery.

chlorophyll fluorescence were determined at three different CC of the substrate: 100% CC, 75–50% CC and 50–35% CC.

Forest study

Forest studies were carried out in Can Coll, Serra de Collserola forest, Barcelona, Spain; 41°28′28′N, 2°7′32′E at an elevation of 140 m and oriented north–northeast. The climate is Mediterranean, with cold winters, cool wet springs and autumns, and hot dry summers. This area has a mean annual temperature of 13–14 °C and an annual rainfall of 500–700 mm. The 35-year-old forest is dominated by *Q. ilex* and *Pinus halepensis* Mill. Here we distinguished between three kinds of *Q. ilex* individuals: those resprouting after a clear-cut (resprouts, R), intact individuals in the same clear-cut area as resprouts (controls, C) and intact individuals in a nearby undisturbed area (forest controls, CF). Measurements were taken in May (11 May 2010) and July (26 July 2010), respectively (Table 1).

Gas exchange

In the nursery study, net photosynthesis (*A*) and stomatal conductance (g_s) were determined on two attached, fully developed, young leaves from five plants at distinct %CC using a gas-exchange system (LI-6400; Li-COR, Lincoln, NE, USA) equipped with a Li-COR light source (6400-02B LED). Environmental conditions in the leaf measuring chamber were established as: PPFR: 800 μmol m^{-2} s^{-1}; relative humidity: 50–55%; chamber temperature 25 °C; sample CO$_2$ (C_a): 400 μmol mol^{-1} and air flux: 150 μmol s^{-1}. Previous analyses of *A*/PPFR curves with PPFR established as 0, 50, 100, 200, 400, 600, 800, 900, 1000, 1500 μmol m^{-2} s^{-1} on one leaf from four plants allowed the determination of the light-saturated rate of net CO$_2$ assimilation at ambient CO$_2$ (A_{sat}), which was around 750 μE m^{-2} s^{-1}.

Table 1. Climatological data from the meteorological station nearest to the study site. Data shown correspond to the month before the measurement days.

	Spring (11 April 2010–11 May 2010)	Summer (26 June 2010–26 July 2010)
Mean daily temperature (°C)	13.51 ± 0.61	24.54 ± 0.38
Mean daily maximum temperature (°C)	17.80 ± 0.77	30.28 ± 0.50
Mean daily minimum temperature (°C)	10.50 ± 0.54	20.71 ± 0.32
Mean daily relative humidity (%)	65.65 ± 2.49	53.23 ± 2.16
Mean daily precipitation (mm)	0.08 ± 0.04	0.00 ± 0.00
Mean daily atmospheric pressure (HPa)	1012.87 ± 1.00	1015.93 ± 0.52
Solar irradiance (W m^{-2})	211.11 ± 16.23	309.12 ± 8.24

In the forest study, *A* and g_s were determined on six leaves from each of the three experimental groups (CF, C, R) with the same gas-exchange system. The following conditions were thereby achieved, which are similar to atmospheric conditions on the days of the measurements: May: PPFR 800 μmol m^{-2} s^{-1}; relative humidity: 50%; chamber temperature 25 °C; sample CO$_2$ (C_a): 400 μmol mol^{-1} and air flux: 150 μmol s^{-1}. July: PPFR 1000 μmol m^{-2} s^{-1}; relative humidity: 45%; chamber temperature 30 °C; sample CO$_2$ (C_a): 400 μmol mol^{-1} and air flux: 150 μmol s^{-1}.

All measurements (nursery and forest) were performed between 13:30 and 16:30 h local time. Steady-state conditions inside the leaf chamber were achieved after ~5 min.

Imaging fluorescence

Immediately after gas-exchange measurements, chlorophyll fluorescence images were recorded on the same leaves (attached leaves in the nursery and detached leaves in the forest study) by means of an Imaging-PAM, MICRO-version (Walz, Effeltrich, Germany), a pulse-amplitude-modulated (PAM) chlorophyll fluorometer that measures images of chlorophyll fluorescence and provides all relevant chlorophyll fluorescence parameters using the saturation pulse method. After 30 min of dark adaptation of the leaves, minimum (F_o) and maximum fluorescence (F_m) and maximum quantum yield of PSII photochemistry (F_v/F_m) (equivalent to (F_m–F_o)/F_m) were obtained. Subsequently, the light-adapted components of chlorophyll fluorescence (steady-state yield (*F*), maximum fluorescence yield (F'_m) and the effective quantum yield of photosystem II (PSII) photochemistry (Φ_{PSII}; equivalent to (F'_m–*F*)/F'_m); Genty et al. 1989) were obtained with an incident actinic light of 735 μmol m^{-2} s^{-1}. An image for each chlorophyll fluorescence parameter was obtained. These images were shown with a standard false colour code ranging from black via red, orange, yellow, green, blue and violet to purple. These colours code in numbers between 0 and 1. Hence, all measured or calculated parameters were normalized to values between 0 and 1. One image per leaf was taken on each measurement day. In the nursery study, approximately the same delimited area was studied throughout the drought process. The measurement area of the Imaging-PAM MICRO-version corresponds to a 3.5 × 4.5 mm; this corresponds to one image. The values of the chlorophyll fluorescence parameters (F_v/F_m and Φ_{PSII}) given correspond to the mean of 10 points, randomly selected, per image, with standard error.

Statistical analyses

Statistical analyses were performed using SPSS for Windows (v. 15.0, SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to test the main effects and interactions, against appropriate error terms, of the three kinds of *Q. ilex* individuals (C, CF and R) and season (May and July) on

Table 2. Variation in gas exchange: net photosynthesis (A), stomatal conductance (g_s) and chlorophyll fluorescence parameters (maximum PSII quantum yield (F_v/F_m) and effective PSII quantum yield (Φ_{PSII})) during gradual drought stress of two leaves from five plants. The A and g_s values correspond to one measurement per leaf on each sampling day. The F_v/F_m and Φ_{PSII} values represent the mean ± s.e. of 10 zones per leaf within the image obtained with the Imaging-PAM. % CV corresponds to the coefficient of variation of Φ_{PSII} data. The last column (P) shows the presence (+) or absence (–) of patchiness, as determined visually on the basis of the change of colour of several zones of the chlorophyll fluorescence images. Bold rows correspond to g_s values <50 mmol m^{-2} s^{-1}.

Plant	Leaf	CC (%)	A (µmol m^{-2} s^{-1})	g_s (mmol m^{-2} s^{-1})	F_v/F_m	Φ_{PSII}	CV (%)	P
1	**1**	100.00	7.49	79.5	0.702 ± 0.004	0.372 ± 0.003	0.89	–
		77.90	10.50	127.0	0.702 ± 0.002	0.383 ± 0.003	0.68	–
		53.76	7.46	97.4	0.695 ± 0.003	0.317 ± 0.004	1.42	–
		48.62	8.85	105.0	0.696 ± 0.005	0.307 ± 0.005	1.58	–
		45.77	**4.42**	**37.9**	**0.628 ± 0.010**	**0.304 ± 0.009**	**2.89**	+
		40.63	6.40	64.5	0.686 ± 0.006	0.339 ± 0.006	1.63	–
		35.43	**0.14**	**9.1**	**0.560 ± 0.010**	**0.165 ± 0.008**	**5.12**	**Necrotic**
1	**2**	100.00	8.77	104.1	0.729 ± 0.006	0.370 ± 0.003	0.88	–
		77.90	7.87	95.7	0.744 ± 0.004	0.376 ± 0.004	1.01	–
		53.76	8.37	81.9	0.681 ± 0.008	0.301 ± 0.005	1.50	–
		48.62	6.10	66.5	0.703 ± 0.012	0.281 ± 0.010	3.60	+
		45.77	**2.34**	**17.5**	**0.610 ± 0.009**	**0.267 ± 0.012**	**4.54**	+
		40.63	**4.46**	**25.8**	**0.679 ± 0.004**	**0.299 ± 0.014**	**4.55**	+
		35.43	**1.60**	**9.4**	**0.631 ± 0.012**	**0.2430.009**	**3.58**	+
2	**1**	**43.92**	**3.08**	**18.0**	**0.586 ± 0.014**	**0.230 ± 0.012**	**5.28**	+
		39.38	**1.93**	**22.2**	**0.537 ± 0.023**	**0.211 ± 0.016**	**7.76**	+
		37.32	**1.12**	**8.8**	**0.569 ± 0.017**	**0.195 ± 0.010**	**5.16**	+
2	**2**	**43.92**	**1.56**	**9.2**	**0.678 ± 0.006**	**0.236 ± 0.010**	**4.35**	+
		39.38	**0.84**	**7.1**	**0.558 ± 0.014**	**0.181 ± 0.006**	**3.11**	+
		37.32	**–0.62**	**1.8**	**0.432 ± 0.018**	**0.140 ± 0.008**	**5.51**	+
3	**1**	**50.35**	**5.03**	**26.2**	**0.689 ± 0.005**	**0.340 ± 0.008**	**2.47**	–
		44.38	**4.21**	**21.8**	**0.698 ± 0.004**	**0.336 ± 0.006**	**1.87**	+
		41.18	**2.83**	**13.3**	**0.681 ± 0.004**	**0.310 ± 0.004**	**1.26**	–
3	**2**	**50.35**	**5.15**	**28.6**	**0.771 ± 0.004**	**0.372 ± 0.005**	**1.40**	–
		44.38	**1.79**	**15.2**	**0.554 ± 0.007**	**0.225 ± 0.008**	**3.57**	+
		41.18	**1.20**	**1.02**	**0.640 ± 0.009**	**0.249 ± 0.012**	**4.83**	+
4	**1**	100.00	8.10	89.5	0.729 ± 0.003	0.398 ± 0.006	1.46	–
		72.40	10.00	112.0	0.731 ± 0.003	0.381 ± 0.003	0.67	–
		46.87	7.95	85.1	0.721 ± 0.003	0.323 ± 0.007	2.05	–
		41.28	**3.25**	**38.4**	**0.695 ± 0.003**	**0.349 ± 0.013**	**3.66**	–
		43.44	**2.46**	**14.0**	**0.515 ± 0.013**	**0.183 ± 0.013**	**7.34**	+
		39.53	**4.77**	**31.6**	**0.691 ± 0.009**	**0.324 ± 0.013**	**3.98**	+
		36.01	**–0.25**	**3.19**	**0.647 ± 0.005**	**0.247 ± 0.006**	**2.32**	+
4	**2**	41.28	7.34	68.5	0.726 ± 0.002	0.322 ± 0.008	2.59	–
		43.44	**4.30**	**22.5**	**0.690 ± 0.007**	**0.389 ± 0.003**	**0.78**	+
		39.53	**3.67**	**13.8**	**0.703 ± 0.005**	**0.324 ± 0.009**	**2.93**	+
		36.01	**–0.67**	**0.64**	**0.599 ± 0.020**	**0.245 ± 0.010**	**4.04**	+
5	**1**	100.00	10.20	105.0	0.756 ± 0.002	0.365 ± 0.003	0.76	–
		76.25	9.90	100.0	0.721 ± 0.005	0.351 ± 0.002	0.66	–
		52.39	10.40	114.0	0.732 ± 0.001	0.325 ± 0.008	2.48	–
		47.19	10.80	96.6	0.750 ± 0.002	0.375 ± 0.003	0.69	–
		45.29	11.10	71.0	0.732 ± 0.002	0.396 ± 0.003	0.82	–
		40.79	12.80	114.0	0.695 ± 0.004	0.347 ± 0.004	1.16	–
5	**2**	100.00	10.50	99.3	0.730 ± 0.006	0.377 ± 0.004	1.02	–
		76.25	8.68	102.0	0.768 ± 0.004	0.343 ± 0.004	1.17	–
		52.39	7.50	67.9	0.770 ± 0.004	0.303 ± 0.010	3.27	–
		47.19	7.58	95.3	0.699 ± 0.008	0.330 ± 0.005	1.41	–
		45.29	7.51	53.6	0.676 ± 0.007	0.348 ± 0.006	1.71	–
		40.79	9.41	68.1	0.703 ± 0.005	0.324 ± 0.009	2.93	–

gas-exchange and chlorophyll fluorescence results in the forest. Post-hoc comparisons were checked using Duncan's test at a significance of $P < 0.05$.

Results and discussion

To study the occurrence of patchy stomatal behaviour in *Q. ilex*, the predominant tree in the Mediterranean forests, we examined changes in the chlorophyll fluorescence of leaves from trees subjected to a gradual water stress in the nursery and in leaves from trees under increasing drought in the forest. Fluorescence images, together with thermal imaging, have been used to evaluate the stomatal patchiness in response to drought in *Xanthium strumarium* L. (Mott et al. 1993, West et al. 2005), *Phaseolus vulgaris* L. (Genty and Meyer 1995), *Helianthus annuus* L. (Cardon et al. 1994), *Gossypium hirsutum* L. (Marenco et al. 2006) and *Tradescantia virginiana* L. (Nejad et al. 2006). This method provides clear images in heterobaric leaves, which, due to their anatomy, show no lateral diffusion of gases (Terashima 1992). Fluorescence imaging is a powerful non-destructive method to relate the spatial changes in stomatal conductance (g_s) with chlorophyll fluorescence parameters (Omasa and Takayama 2003). The values of Φ_{PSII} obtained by fluorescence images can be considered representative of the relative activity of linear electron transport.

In our study, increasing drought was reflected in the gas-exchange and chlorophyll fluorescence values in plants grown in the nursery and in the forest. In the nursery, *A* decreased from 8–10 μmol $m^{-2} s^{-1}$ at 100% CC, which is an optimum value for *Q. ilex* (Fleck et al. 1998) to 1–5 μmol $m^{-2} s^{-1}$ at 50–35% CC (equivalent to around a 50–80% decrease) at the end of the experiment (Table 2). In parallel, a decrease in g_s was observed; g_s is an integrative indicator of the degree of water stress (Flexas et al. 2002, Galmes et al. 2007). Values of 50–100 mmol $m^{-2} s^{-1}$ correspond to moderate water stress, net photosynthesis being limited by stomata, while values <50 mmol $m^{-2} s^{-1}$ are considered representative of severe drought and are accompanied by non-stomatal limitations (Medrano et al. 2002). In most cases, at 50–35% CC, g_s values were consistently <50 mmol $m^{-2} s^{-1}$.

Parallel to changes in gas exchange, alterations in the chlorophyll fluorescence parameters F_v/F_m and Φ_{PSII} were also observed during the nursery drought (Table 2). It is known that a drought stress can increase the rate of photorespiration (Medrano et al. 2002) and, as reported on cotton leaves, higher rates of photorespiration correspond to higher PSII efficiency (Massacci et al. 2008). Besides these considerations, in our work we observed that leaves under severe drought showed higher heterogeneity of Φ_{PSII} values with respect to less stressed leaves. In a single leaf, the presence of patchiness (+) was determined visually on the basis of the change of colour of several zones of the chlorophyll fluorescence images (Figure 2). Patchiness was detected mainly in leaves with a g_s <50 mmol H_2O $m^{-2} s^{-1}$ (marked in bold in the table), which was registered in 83% of all the leaves measured. A comparison of the images of leaves with a low g_s with those with a high g_s and *A* rates showed a similar colour in all points (areas) in the latter, thereby indicating similar values of Φ_{PSII} (Figure 2). The

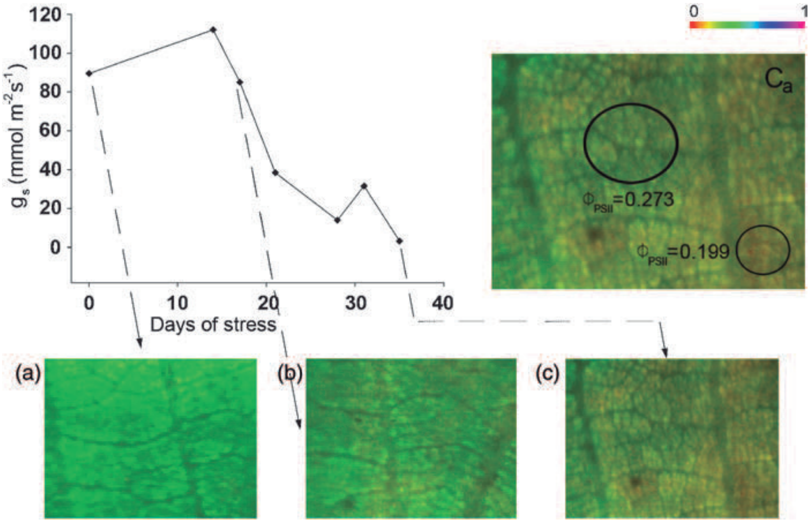

Figure 2. Images of the PSII quantum yield (Φ_{PSII}) of *Q. ilex* generated by the Imaging-PAM during the drought treatment (individual 4, leaf 1). (a) and (b) images correspond to a well-watered plant (g_s: 130–75 mmol $m^{-2} s^{-1}$) and to a plant subjected to moderate water stress (g_s: 50–75 mmol $m^{-2} s^{-1}$), respectively. Image (c) corresponds to a drought-stressed leaf (g_s <50 mmol $m^{-2} s^{-1}$). The real area of the images was 3.4 × 4.5 mm. Image c_a is an enlargement of image (c) with two marked points with different Φ_{PSII} values corresponding to the average of all Φ_{PSII} values inside each point generated by the Imaging-PAM software.

visual criterion was confirmed by the % coefficient of variation (CV) of Φ_{PSII} values, calculated as the ratio of the standard deviation to the mean of the 10 points selected. Images with areas of different colour showed a higher % CV. In fact, in 90% of all cases, a % CV >2.5 corresponded to visual patchiness. The CV of Φ_{PSII} was not statistically higher than system repetitiveness (around 9%). *Quercus ilex* stomatal patchiness might be a consequence of its heterobaric leaf anatomy (Beyschlag and Eckstein 1998, Nikolopoulos et al. 2002), caused by the presence of independent small compartments, termed areoles, which have distinct light regimes and water status (Terashima 1992, Pieruschka et al. 2010). In earlier studies, we reported that patchiness was not observed in *Q. ilex* plants submitted to a very gradual water stress (plants were watered daily) (Peña-Rojas et al. 2004, 2005). This observation is consistent with the findings of Cornic and Masacci (1996), who described that these conditions minimize or remove heterogenic stomatal aperture.

We performed a parallel study in the forest to ascertain whether patchiness is observed in *Q. ilex* in the field. Although several studies have revealed that plants in natural environments show heterogeneous stomatal closure during certain periods of the day (Beyschlag and Pfanz 1990, Beyschlag et al. 1992, Kamakura et al. 2011), relatively few examined the effects of patchiness in natural settings. It has been proposed that patchiness does not occur in these settings because dehydration can be slow (Gunasekera and Berkowitz 1992, Kubiske and Abrams 1993). In addition, the relevance of patchiness has been questioned (Lawlor and Cornic 2002). Moreover, it was proposed that the absence of patchiness response in field plants of cotton was caused by the adaptability of plants to drought (Wise et al. 1992).

In the forest study, gas-exchange rates differed between the three kinds of plants examined and sampling date (Figure 3); in some cases these changes were associated with changes in water availability. The three kinds of individuals (C, CF and R) were expected to show differences in water availability as demonstrated in an earlier study (Peña-Rojas et al. 2005). The density of individuals in the undisturbed area (CF) was much higher than those growing in the clear-cut area (C and R), which affected transpiration and soil water availability. Moreover, the soil water availability for resprouting individuals (R) was higher than for C individuals, both growing in the same area, due to the lower shoot/root ratio of R. Soil water availability also declines in Mediterranean forest from spring to summer due to the scarcity of precipitation and increased leaf temperature; furthermore, increased DPV in the summer results in higher transpiration rates.

In May and July, R showed higher *A* and g_s with respect to C or CF, with small differences between these two controls. In early May, milder climatic constraints with respect to July values, as evidenced by lower vapour pressure deficits (May VPD:

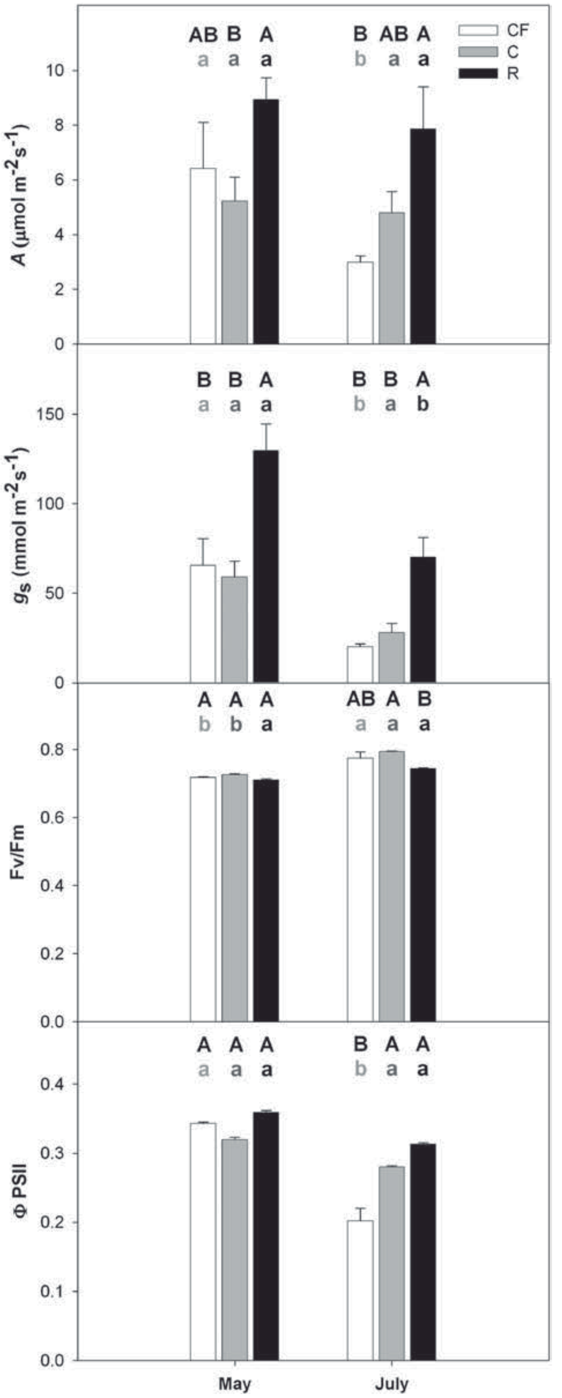

Figure 3. Net photosynthesis (*A*), stomatal conductance (g_s), maximum PSII quantum yield (F_v/F_m) and effective PSII quantum yield (Φ_{PSII}) determined on *Q. ilex* plants: control (C), forest control (CF) and resprouts (R). Significant differences ($P < 0.05$) are indicated by different letters (lower case (a, b) indicate seasonal differences in each kind of plant and upper case (A, B) show differences between kinds of plant on the same measuring date (May, July).

1.52 ± 0.04 KPa in CF, 1.51 ± 0.04 KPa in C and 1.28 ± 0.03 KPa in R; July VPD: 2.52 ± 0.01 KPa in CF, 2.44 ± 0.05 KPa in C and 2.39 ± 0.04 KPa in R), and higher precipitation before the summer drought (Table 1) account for higher g_s rates with respect to those obtained in late July. The improved water status in R (smaller total leaf area results in a lower transpirative demand even at the same soil water availability) with respect to the controls would explain the stimulated gas exchange avoiding a midday depression of photosynthesis (Fleck et al. 1998, Peña-Rojas et al. 2004).

With respect to chlorophyll fluorescence parameters (Figure 3), the F_v/F_m values in July were within the range 0.75–0.85, which is normal for well-watered plants (Björkman and Demmig 1987). In contrast, in May, slight photoinhibition was detected in all plants. The CF plants showed a marked decline in A, g_s and Φ_{PSII} from May to July, but no change in F_v/F_m was observed, indicating that these plants reversed photoinhibition before dawn. Although most of these results are not novel, they confirm that our forest experimental set-up was consistent with earlier work (Fleck et al. 1998, El Omari et al. 2001).

In May, heterogenic Φ_{PSII} were observed in 33% of C, 40% of CF and 42.3% of R individuals, whereas in July, under increasing drought, it was observed in 100% of C and CF individuals and 50% of R individuals (Table 3). Chlorophyll fluorescence images from forest *Q. ilex* leaves (Figure 4) confirmed these results with R plants maintaining values while C and CF individuals showed increased patchiness from May to July. The presence of patchiness was related to a CV of Φ_{PSII} higher than 4% in 95.5% of all cases (Table 3). In the nursery, the occurrence of patchiness corresponded mainly to leaves with a g_s <50 mmol H_2O $m^{-2}\,s^{-1}$; in the forest in July, the g_s of controls was around 25 mmol $m^{-2}\,s^{-1}$ and accounted for patchiness in 92% of individuals. The R plants were less affected by drought and in July they showed g_s values around 75 mmol $m^{-2}\,s^{-1}$ and Φ_{PSII} patchiness was detected in only 50% of these plants. Higher water availability in May in the forest was reflected in mean g_s over 60 mmol $m^{-2}\,s^{-1}$ and the presence of patchiness was markedly reduced in all kinds of leaves.

Biochemical limitants of photosynthesis may account for the observed Φ_{PSII} patchiness under milder drought conditions in spring in the forest, even at high A and g_s. It has been demonstrated that stomatal patchiness is responsible for the heterogeneity of Rubisco capacity in *Rosa rubiginosa* L. (Meyer and Genty 1999). These results, together with others (Wise et al. 1992, Sassenrath-Cole and Pearcy 1994, Bro et al. 1996), question whether the changes observed in the chlorophyll fluorescence parameters were caused by stomatal heterogeneity or whether this heterogeneity was partly produced by metabolic parameters.

In conclusion, our results in nursery and in natural conditions in the forest indicate that heterogenic photosynthesis in *Q. ilex* occurs as a result of patchy stomatal closure during severe drought. This finding should be taken into account when considering the limiting factors of photosynthetic and ecosystem modelling during severe water stress, a scenario that is expected to increase, as predicted in climatic models. Although the patchiness of stomatal conductance may not significantly influence the carbon–water balance (Buckley et al. 1999), it may contribute to lower water-use efficiency than that predicted by mechanistic ecosystem models (Reichstein et al. 2002).

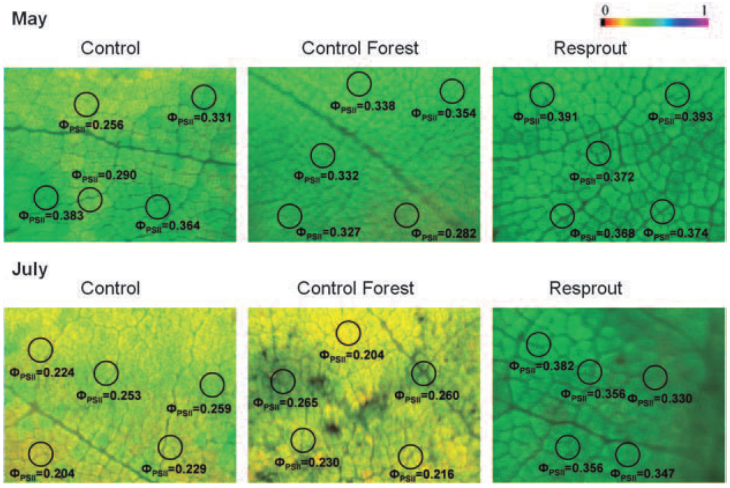

Figure 4. Images of the PSII efficiency (Φ_{PSII}) of *Q. ilex* generated by the Imaging-PAM in forest individuals (control, forest control and resprouts) in May and July. The real area of the images was 3.5 × 4.5 mm. Marked points with different Φ_{PSII} values corresponded to the average of all the Φ_{PSII} values inside each point generated by Imaging-PAM software.

Table 3. Variation in gas exchange (net photosynthesis (*A*) and stomatal conductance (g_s)) and chlorophyll fluorescence parameters (maximum PSII quantum yield (F_v/F_m) and effective PSII quantum yield (Φ_{PSII})) between two sampling dates (May and July) for individual *Q. ilex*: control (C), forest controls (CF) and resprouts (R). The *A* and g_s values correspond to one measurement per leaf on each sampling day. F_v/F_m and Φ_{PSII} values represent the mean ± s.e. of 10 zones per leaf within the image obtained with the Imaging-PAM. % CV corresponds to the coefficient of variation of Φ_{PSII} data. The last column (P) shows the presence (+) or absence (–) of patchiness, as determined visually on the basis of the change of colour of several zones of the chlorophyll fluorescence images. Bold rows correspond to g_s values <50 mmol m^{-2} s^{-1}.

Sampling date	Sample	*A* (µmol m^{-2} s^{-1})	g_s (mmol m^{-2} s^{-1})	F_v/F_m	Φ_{PSII}	CV (%)	P
May	C	6.94	95.3	0.779 ± 0.003	0.374 ± 0.003	2.09	–
May	C	1.51	32.8	0.66 ± 0.011	0.193 ± 0.002	2.82	–
May	C	5.45	67.4	0.757 ± 0.003	0.37 ± 0.003	2.86	–
May	C	4.61	59.5	0.733 ± 0.003	0.314 ± 0.014	13.58	+
May	C	7.64	58.6	0.708 ± 0.013	0.308 ± 0.014	14.46	+
May	**C**	**5.16**	**40.9**	**0.719 ± 0.019**	**0.359 ± 0.003**	**2.28**	–
May	**CF**	**4.13**	**34.2**				
May	CF	14.1	129.0	0.757 ± 0.005	0.326 ± 0.007	7.14	+
May	CF	5.89	73.4	0.728 ± 0.002	0.346 ± 0.005	4.69	+
May	CF	4.15	48.8	0.708 ± 0.010	0.38 ± 0.003	2.35	–
May	CF	2.63	31.4	0.696 ± 0.008	0.366 ± 0.005	4.36	–
May	CF	7.56	76.6	0.717 ± 0.004	0.338 ± 0.003	2.61	–
May	R	6.34	144.0	0.659 ± 0.002	0.342 ± 0.009	8.09	+
May	R	11.7	178.0	0.749 ± 0.003	0.388 ± 0.004	3.01	–
May	R	7.51	77.7	0.751 ± 0.004	0.335 ± 0.003	3.08	–
May	R	8.22	130.0	0.687 ± 0.005	0.328 ± 0.014	13.82	+
May	R	9.15	127.0	0.732 ± 0.007	0.377 ± 0.003	2.39	–
May	R	7.61	80.2	0.726 ± 0.01	0.389 ± 0.010	8.39	+
May	R	11.9	170.0	0.673 ± 0.008	0.353 ± 0.002	1.65	–
July	**C**	**3.61**	**33.9**	**0.788 ± 0.002**	**0.291 ± 0.004**	**4.63**	+
July	**C**	**3.2**	**13.6**	**0.799 ± 0.001**	**0.248 ± 0.004**	**5.35**	+
July	**C**	**4.29**	**32.2**	**0.768 ± 0.001**	**0.235 ± 0.004**	**5.82**	+
July	**C**	**8.43**	**47.4**	**0.8 ± 0.004**	**0.296 ± 0.005**	**4.84**	+
July	**C**	**4.03**	**17.8**	**0.829 ± 0.002**	**0.29 ± 0.004**	**4.10**	+
July	**C**	**5.18**	**24.0**	**0.777 ± 0.012**	**0.320 ± 0.006**	**6.16**	+
July	**CF**	**3.89**	**27.3**	**0.804 ± 0.009**	**0.239 ± 0.006**	**8.33**	+
July	**CF**	**2.95**	**21.2**	**0.777 ± 0.008**	**0.187 ± 0.006**	**9.26**	+
July	**CF**	**2.74**	**17.3**	**0.771 ± 0.003**	**0.231 ± 0.007**	**9.18**	+
July	**CF**	**3.13**	**20.6**	**0.777 ± 0.017**	**0.21 ± 0.008**	**11.53**	+
July	**CF**	**2.13**	**15.8**	**0.742 ± 0.003**	**0.178 ± 0.005**	**8.22**	+
July	R	13.8	115.0	0.778 ± 0.003	0.452 ± 0.003	2.06	–
July	R	5.05	59.2	0.677 ± 0.01	0.341 ± 0.009	8.14	+
July	R	10.4	83.9	0.754 ± 0.002	0.264 ± 0.004	4.94	–
July	R	8.69	71.3	0.735 ± 0.002	0.305 ± 0.003	3.47	–
July	**R**	**4.61**	**38.6**	**0.738 ± 0.003**	**0.256 ± 0.006**	**7.15**	+
July	R	4.57	52.8	0.782 ± 0.001	0.259 ± 0.007	8.56	+

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Conflict of interest

None declared.

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Susceptibility to *Melampsora* Leaf Rust of Poplar Clones From Diverse Genetic Backgrounds: Effects on Photochemistry and Water Relations

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Abstract

The selection of resistant genotypes is the most appropriate approach in the prevention of the reduction of biomass and mortality caused by rust infection in poplar plantations. Thus, it is pertinent that we improve our understanding of the consequences that this fungal disease has on leaf physiology. Here, we studied the susceptibility to *Melampsora* rust in three different poplar clones of commercial interest: Lux clone – *Populus deltoides* Batr. (cottonwood) and Luisa Avanzo and Adige clones – both *Populus* × *canadensis* Mönch. The most susceptible clone to the infection was L. Avanzo whereas Lux and especially Adige were only slightly affected. The propagation of the disease was very rapid in L. Avanzo; their leaves showed a high incidence and severity of the disease in early and advanced stages of infection as was clearly evidenced by the degree of infection. Infected leaves of L. Avanzo were shown to have drought impaired water relations during summer as reflected by the marked decline in the relative water content (RWC). Chlorophyll fluorescence imaging revealed heterogeneity of the effect of the pathogen in the leaves, and areas with pustules showed low maximum quantum yield (Fv/Fm) and PSII quantum yield (Φ_{PSII}) values, indicative of strong photoinhibition. In L. Avanzo, with a greater pustule density, rust provoked a decline in whole leaf photochemistry as indicated by Fv/Fm and photochemical reflectance index (PRI) results. Leaf structural parameters were not affected by the disease but results in L. Avanzo and Lux showed higher leaf mass per area (LMA) and higher leaf density (D) indicating an adaptation to increasing summer drought. In all clones, the effect of the pathogen was reflected in lower leaf chlorophyll content.

Keywords: *Populus* spp., Chlorophyll fluorescence, Chlorophyll content, Radiometric index

1. Introduction

Rust infection caused by species of the genus *Melampsora* Cast. (Basidiomycota, Uredinales, Melampsoraceae) is one of the most widespread and devastating diseases in poplars, willows and pines (Pinon & Frey, 2005; Royle & Hubbes, 1992; Vialle et al., 2011).

Melampsora spp. depends on the host tissues for their development, proliferation and reproduction. The fungus invades host cells, depleting their sugars and nutrients, thereby producing a decrease in stem growth, premature defoliation and predisposition to attack by insects and secondary pathogens, such as *Cytospora* or *Dothichiza*, and in some cases, it may cause plant death (Newcombe et al., 2001; Wang & van der Kamp, 1992). The life cycle of some *Melampsora* species is completed by infection of poplars and coniferous hosts (Feau et al., 2007). During the summer, urediniospores are produced in yellow-orange pustules (uredinia) on the underside of *Populus* spp. leaves. These spores serve as the inoculum to spread the disease across *Populus* spp. Teliospores are produced in late summer and overwinter on the underside of dead poplar leaves on the ground (Torres, 1998). The intensive cultivation of poplars due to their high growth potential, their use in renewable energy systems (Scholz & Ellerbrock, 2002), their capacity to capture atmospheric CO_2 (Isebrand & Karnosky, 2001) and to remediate metal pollution in soils and water (Fernàndez et al., 2012) has made them susceptible to many pathogens (Gérard et al., 2006). Moreover, stands are even-aged and monoclonal and the number of cultivars planted in a given region is limited. Simplistic monoclonal and intensive breeding, combined with the obviously high adaptive potential of *Melampsora* spp., have led to successive resistance breakdowns. As they do not have a secondary line of defence, most cultivars have been exposed to the epidemic spread of newly emerged virulent strains of the fungus (Frey et al., 2005).

In order to prevent the harmful effects on plant productivity brought about by infection by *Melampsora* spp., the cultivation of hybrids for their resistance to pathogens and for their wood quality highly regarded by growers is a standard practice (Newcombe et al., 1996). Lux clone – *Populus deltoides* Batr. (cottonwood) and Luisa Avanzo and Adige clones – both *Populus* × *canadensis* Mönch. were selected on the basis of their relevance as crops and because they respond differently to biotic and abiotic constraints. L. Avanzo and Adige are highly resistant to mosaic virus infections, whereas Lux is sensitive; the latter is tolerant to infection by the fungus *Marsonnina brunnea*, whereas Adige has a low tolerance and L. Avanzo is sensitive. L. Avanzo can withstand windy conditions, while Adige is less capable of doing so and Lux is sensitive to such conditions (Facciotto & Frison, 1999).

The functional characteristics of leaves can be modified by environmental and biotic stresses (Niinemets, 2001). Direct measurements of photosynthetic parameters and other aspects of primary metabolism have not yet been reported for poplars with pathogen infections (Major et al., 2010). Foliar pathogens can reduce net CO_2 assimilation (Hajji et al., 2009) by affecting stomatal conductance (Pinkard & Mohammed, 2006) and chlorophyll decay (Holloway et al., 1992). Furthermore, the effects of foliar pathogens on plant water relations and the consequences for the crop water-use efficiency (WUE) have recently been reviewed (Grimmer et al., 2012).

The aim of this study was to determine the susceptibility to *Melampsora* infection in the selected poplar clones. The analysis of rust incidence (number of infected leaves per tree and number of infected trees per genotype) and severity (expressed as the degree of infection in an individual in function of the percentage of affected leaf area) allowed for the observation of the propagation of the disease in the different clones.

Another aim was to characterize the effect of the disease on physiological parameters related to photosynthetic processes and to leaf water relations in early and advanced stages of the infection during the summer. The use of chlorophyll fluorescence imaging and leaf reflectance indices allowed for a non-destructive evaluation. Chlorophyll fluorescence imaging has been used during biotic stress (Pineda et al., 2011) and provides information on electron transport characteristics both in whole leaves and in specific leaf areas affected by fungal infection.

Determination of the susceptibility to leaf rust and its effect on physiology can help to identify the most resistant clones to this infection and the most suitable ones for multiple ecological services.

2. Materials and Methods

2.1 Experimental Site and Plant Material

Poplar clones were selected from a collection of fifty clones established since 2001 in the IBAF-Institute experimental field near Rome in the Tevere valley. This area has an alluvial soil type. The climate is Mediterranean with cold winters, cool wet springs and autumns, and hot dry summers. The area registers a mean annual temperature of 13-14 ºC, and an annual rainfall of 500-700 mm. Three poplar clones from different genetic backgrounds were used: Lux clone – *Populus deltoides* Batr. (cottonwood), which was successfully introduced into Europe from North America; and Luisa Avanzo and Adige clones – both *Populus* × *canadensis* Mönch., a hybrid from *Populus deltoides* and *Populus nigra*. *Populus nigra*, is native European taxon. All clones were female. Each clone was represented by 10-30 individuals in single plots.

2.2 Sampling

Sampling for the purpose of physiological measurements was performed in mid-July (a period when the *Melampsora* spp. uredinia are produced on the *Populus* leaves) and mid-September (after the disease has been able to develop over the summer). Climatic parameters during the study are shown in Table 1. June was relatively humid with elevated temperatures with respect to the period of sampling in July which was in contrast dry with even higher temperatures. Conditions in August were similar to those of July. In September, just before sampling, the temperatures decreased while precipitation increased markedly. All measurements were recorded on 3 randomly selected 8-years old individuals per clone in healthy leaves (control) and leaves that showed *Melampsora* sp. pustules (infected leaves). In both cases, leaves were fully expanded, South-oriented and collected at 2 to 4 m height from the base of the tree. Taking into account the different degrees of infection in each leaf throughout the whole plant, measurements on the infected leaves were recorded on those showing the average degree of severity of the measured clone. Total chlorophyll content and radiometric measurements were obtained at the IBAF-Institute experimental fields at 13.30 – 16.30 h local time. Leaf structural, hydric and imaging fluorescence measurements were analysed in the IBAF laboratories.

Table 1. Climatological data from the meteorological station closest to the site of the study. The first sampling corresponds to July 11th and the second sampling to the Sepember 11th

	June 1st	**June 11th**	**June 21st**	**July 1st**	**July 11th**	**July 21st**	**August 1st**	**August 11th**	**August 21st**	**September 1st**	**September 11th**
Precipitation (mm)	41.2	8.8	**12.2**	**10.4**	**0.2**	**0**	**0**	**2**	**12.2**	**0.4**	**65.8**
Radiation ($kJ \cdot m^{-2}$)	25365	23795	25501	27367	**27160**	30858	27138	27463	24989	20765	**15330**
T max (ºC)	28.7	29.7	30	34.6	**36.4**	35.6	33.5	36.5	35.9	31	**27.3**
T min (ºC)	12.4	14.3	12.8	17.5	**19**	15.8	16.4	17.8	18	17.1	**14.6**

2.3 Incidence and Severity of Melampsora rust

We evaluated the incidence of the pathogen first on the basis of the percentage of individuals of each clone showing visual symptoms of the disease. Then the incidence in each individual was evaluated as a percentage of the number of infected leaves (presence of pustules). Fifteen leaves were selected at random in an individual and in total 3 individuals per clone were evaluated. Severity was considered as the degree of the infection in an individual. This parameter was determined in 3 plants per clone. For each individual, we examined a total of 24 leaves from different orientations and plant height to make sampling as representative as possible. Three leaves each were taken from 8 different points on the tree: facing North, South, East and West, and from the base of the tree (2 m from the soil surface) and from the top of the tree. The degree of severity in each leaf was established from the percentage of leaf area covered by *Melampsora* rust, as shown in Table 2. For each clone, different severity degrees were established by calculating the percentage of leaves that exhibited each degree of severity in each individual.

Table 2. Degrees of severity of the foliar infection caused by *Melampsora* sp.

Degree of severity	% Affected leaf area	Infection level
1	0	Absent
1.5	<1	Traces
2	1-5	Mild
3	6-25	Moderate
4	26-50	Severe
5	>50	Very severe

The different percentages of affected area correspond to a degree of severity.

2.4 Chlorophyll Fluorescence Imaging

Chlorophyll fluorescence was recorded with the pulse-amplitude-modulated chlorophyll fluorometer Imaging-PAM (MICRO-version (Walz, Effeltrich, Germany)) operated using the Imaging Win v.2.21d (Heinz Walz) software. Chlorophyll parameters were obtained for one squared image area of 26 x 34 mm^2 per leaf. After 40 minutes dark-adaptation, minimum (F_o) and maximum fluorescence (Fm), and maximum quantum yield of PSII photochemistry (F_v/F_m) (equivalent to (F_m-F_o)/F_m) were obtained. Subsequently, the light-adapted components of chlorophyll fluorescence (minimum fluorescence yield (F'_o), maximum fluorescence yield (F'_m) and quantum yield of photosystem II (Φ_{PSII}; equivalent to (F'_m-F)/F'_m)) (Genty et al., 1989) were obtained after five minutes of light adaptation with an incident actinic light of 300 µmol m^{-2} s^{-1} (Pietrini et al., 2010). Measurements were performed in 3 controls and in 3 infected leaves for 3 individuals of each clone. They were also performed on pustules and in non-affected areas of infected leaves.

2.5 Relative Water Content and Leaf Structural Parameters

For all analyses, relative water content (RWC) was measured at midday on 3 infected and 3 control leaves of the 3 selected plants per clone. RWC was calculated as $[(M_f\text{-}M_d)/(M_{fs}\text{-}M_d)\cdot 100]$, with M_f being plant fresh mass; M_{fs}, plant fresh saturated mass (after rehydrating samples for 24h in the dark at 4 ºC); and M_d, plant dry mass (after oven-drying samples at 65 ºC until a constant weight was achieved). Leaf area (LA) was determined with a CI 2003 Laser Leaf Area Meter (CI-203) (CID, Inc., Camas, WA 98607, USA). Leaf mass per area (LMA) was calculated as M_d/LA and its components Thickness and Density were calculated as M_f/LA and $[(M_d/M_f)\cdot 100]$ respectively (Dijkstra, 1989).

2.6 Leaf reflectance Indices and Total Chlorophyll Content

Leaf reflectance was measured at midday *in situ* on 3 infected and 3 control South-oriented leaves for 3 individuals of each clone (the same plants for all analyses) with a portable spectral analysis system with artificial light (USB4000, Oceanoptics), operated with the Spectrasuite (Oceanoptics) software. One measurement was calculated as an integration of ten scans (integration time 50 ms). The photochemical reflectance index (PRI) and the water index (WI) were derived from the spectra. PRI was calculated as $[(R_{531}\text{-}R_{570})/(R_{531}+R_{570})]$, where R_n is the reflectance at n nm (Peñuelas et al., 1995). WI was calculated as (R_{900}/R_{970}), where reflectance at 970 nm is associated with water absorption, and 900 nm is a reference wavelength (Peñuelas et al., 1993). For methodological reasons PRI and WI results obtained in July or September could not be compared (Peñuelas et al., 1995). Total chlorophyll content was measured with a leaf chlorophyll meter (SPAD, Minolta, Osaka, Japan) on 8 infected and 8 control leaves of 3 plants per clone.

2.7 Statistical Analysis

All statistical procedures were performed using Statgraphics for Windows (Statgraphics v. 15.2.14, Statpoint Inc., Virginia, USA). Analysis of variance (ANOVA) was used to test the main effects against appropriate error terms between treatments (leaf types: control, infected), clones and time (July, September) on the measured parameters. A multiple comparison test of the means was carried out using the Tukey HSD post-hoc test. Statistical significance was set at $p \leq 0.05$.

3. Results

3.1 Incidence and Severity of the Infection by Melampsora sp.

In L. Avanzo the incidence of the infection originated by *Melampsora* sp. expressed as a percentage of individuals showing symptoms of infection was approximately twice as much as in Lux and Adige individuals during July (Table 3). In September, all individuals of L. Avanzo and Lux were infected while only 20% of the individuals of Adige showed symptoms. In July, the incidence of infection expressed as percentage of affected leaves was more than double in L. Avanzo that of Lux and Adige. In September, all leaves of L. Avanzo were infected; in Lux most leaves showed signs of the disease and in Adige only around a 16% of leaves showed symptoms. In July, 61% of L. Avanzo leaves were infected and presented a degree of severity between 2 and 3 while in September, 86% of leaves were infected and showed a degree of severity between 4 and 5 (Table 4). In July, 83% of Lux leaves were infected, which decreased to 68% in September. The degree of severity ranged from between 1 and 1.5. Most Adige leaves were infected during the study period with a degree of severity of 1.

Table 3. Incidence of *Melampsora* infection in July and September

	Incidence of infection			
	% individuals		**% leaves**	
	July	**September**	**July**	**September**
L. Avanzo	91.7	100	53.3±7.7	100.0±0.0
Lux	41.2	100	17.8±2.2	73.3±26.7
Adige	50	20	17.8±5.9	15.6±2.2

The incidence is expressed as a percentage of infected individuals and as a percentage of the number of infected leaves of 45 randomly selected leaves of 3 infected individuals (15 per individual) per clone expressed as mean ± SE.

Table 4. Percentage of infected leaves showing the different degrees of severity in L. Avanzo, Lux and Adige clones

Degree of Rust Severity	July			September		
	Percentage of infected leaves					
	L. Avanzo	Lux	Adige	L. Avanzo	Lux	Adige
1	0	36.1±9.1	63.9±7.7	0	23.6±17.7	88.9±3.7
1.5	16.7±8.7	47.2±6.1	30.6±6.9	0	44.4±2.8	11.1±3.7
2	23.6±11.4	16.7±9.6	5.6±1.4	0	15.3±7.7	0
3	37.5±9.6	0	0	19.4±2.8	13.9±7.4	0
4	18.1±12.3	0	0	45.8±7.2	2.8±2.8	0
5	4.2±2.4	0	0	34.7±8.5	0	0

The degree of severity was determined for each clone in 3 plants and in 24 leaves (three leaves each facing North, South, East and West, and at two different heights: one at the base (2m from the soil surface) and the other at the top of the individuals). Values are mean ± SE.

3.2 Relative Water Content (RWC) and Water Index (WI)

No differences in RWC between the control and infected leaves were observed throughout the study except in the infected leaves of Lux, which showed a significant decrease in July (Figure 1 a, b). In September, infected leaves of L. Avanzo and both kinds of leaf of Adige showed the lowest RWC values. The WI was highest for L. Avanzo in July especially in infected leaves (Figure 1 c). In September, Adige and Lux showed the lowest values (Figure 1 d). Methodological reasons did not permit the comparison between the WI results obtained in July and September.

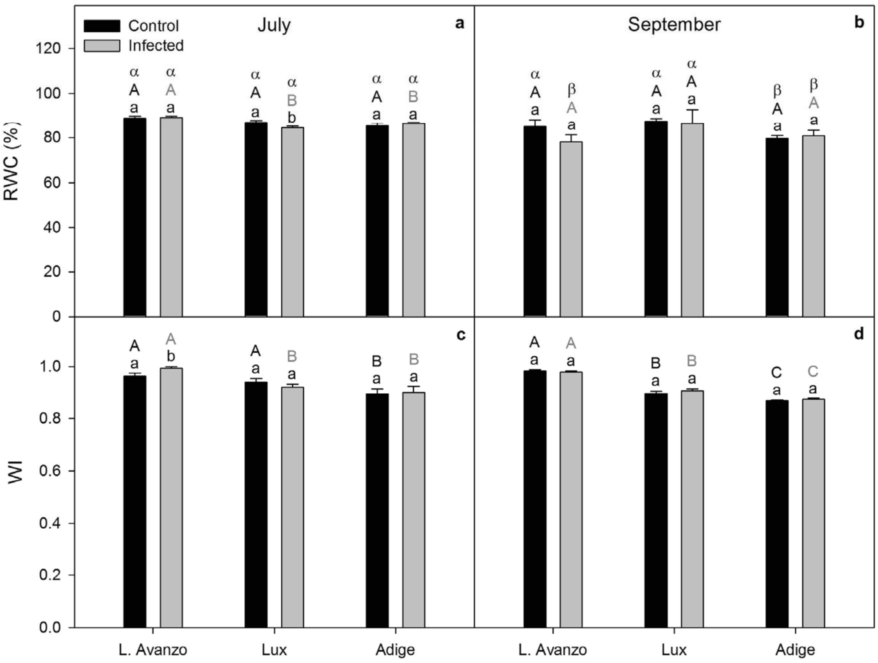

Figure 1 a, b) Relative water content (RWC) and c,d) the radiometric index WI during the study in infected and control leaves of the different *Populus* spp. clones. The number of replicates was 3 leaves per types of leaf, clone and time. Significant differences ($p < 0.05$) between infected and control leaves for each clone are expressed by different lower–case letters (a, b). Differences between controls or between infected leaves of the different clones are expressed by different upper-case letters (A, B). Differences between times are expressed by different Greek letters (α, β). Values are mean ± SE

3.3 Leaf Structural Parameters

Leaf area (LA) in Lux was higher than that of the other clones (Table 5) and no differences between the control and infected leaves were observed in any of the measurements. L. Avanzo showed the greatest reduction in LA throughout the summer. Control leaves of Lux showed the highest leaf mass per area (LMA) in July, and control leaves of L. Avanzo showed the lowest LMA. For each clone, no differences in LMA were observed between the control and infected leaves. Control leaves of Lux showed the highest leaf thickness (T) in July, followed by the control leaves of Adige and then by those of L. Avanzo. Infected leaves of Lux had a lower T than did the control leaves. In September, similar values were observed in the control leaves of all three clones, whereas infected leaves in Lux and Adige showed a higher T than those of L. Avanzo. In July, leaf density (D) was highest in Lux and lowest in L. Avanzo. For each clone, the control and infected leaves showed similar D values. Comparing September with July, increases in D were observed in both kinds of leaf except for infected leaves in Adige.

Table 5. Variations in structural parameters during the study in infected and control leaves of the different *Populus* spp. clones: leaf area (LA, cm^{-2}), leaf mass per area (LMA, $gM_d \cdot m^{-2}$), leaf thickness (T, $g \cdot m^{-2}$) and leaf density (D, %)

		L. Avanzo	**L. Avanzo**	**Lux**	**Lux**	**Adige**	**Adige**
		Control	**Infected**	**Control**	**Infected**	**Control**	**Infected**
July	**LA**	$45.3 \pm 4.6^{aA\alpha}$	$52.8 \pm 4.8^{aA\alpha}$	$76.6 \pm 5.1^{aB\alpha}$	$85.3 \pm 10.2^{aB\alpha}$	$49.0 \pm 4.1^{aA\alpha}$	$56.9 \pm 5.0^{aA\alpha}$
	LMA	$66.3 \pm 4.1^{aA\alpha}$	$69.9 \pm 3.7^{aA\alpha}$	$89.8 \pm 3.0^{aB\alpha}$	$81.3 \pm 4.2^{aA\alpha}$	$77.5 \pm 2.7^{aC\alpha}$	$79.7 \pm 2.0^{aA\alpha}$
	T	$195.9 \pm 6.3^{aA\alpha}$	$210 \pm 8.1^{aA\alpha}$	$240.1 \pm 6.1^{aB\alpha}$	$218.4 \pm 8.1^{bA\alpha}$	$220.9 \pm 5.0^{aC\alpha}$	$225.6 \pm 4.2^{aA\alpha}$
	D	$33.6 \pm 1.1^{aA\alpha}$	$33.2 \pm 0.8^{aA\alpha}$	$37.4 \pm 0.7^{aB\alpha}$	$37.1 \pm 0.7^{aB\alpha}$	$35.0 \pm 0.5^{aAB\alpha}$	$35.3 \pm 0.4^{aB\alpha}$
September	**LA**	$17.7 \pm 0.4^{aA\beta}$	$32.1 \pm 3.7^{aA\beta}$	$74.9 \pm 8.6^{aB\alpha}$	$81.6 \pm 6.0^{aB\alpha}$	$69.2 \pm 5.4^{aB\alpha}$	$47.4 \pm 5.0^{aC\alpha}$
	LMA	$98.0 \pm 9.8^{aA\beta}$	$83.7 \pm 2.2^{aA\beta}$	$98.3 \pm 8.4^{aA\alpha}$	$92.8 \pm 1.3^{aA\alpha}$	$94.1 \pm 10.7^{aA\beta}$	$89.5 \pm 1.0^{aA\alpha}$
	T	$221.3 \pm 7.8^{aA\alpha}$	$211.7 \pm 6.9^{aA\alpha}$	$217.3 \pm 7.0^{aA\alpha}$	$235.3 \pm 11.4^{aAB\alpha}$	$223.9 \pm 19.2^{aA\alpha}$	$239.1 \pm 8.0^{aB\alpha}$
	D	$44.1 \pm 3.2^{aA\beta}$	$40.0 \pm 2.2^{aA\beta}$	$45.1 \pm 2.4^{aA\beta}$	$39.5 \pm 1.3^{aA\beta}$	$41. 8 \pm 1.3^{aA\beta}$	$37.2 \pm 1.0^{aA\alpha}$

The number of replicates was 3 leaves per types of leaf, clone and time. Significant differences ($p < 0.05$) between infected and control leaves for each clone are expressed by different lower–case letters (a,b). Differences between the controls or between infected leaves of the different clones are expressed by different upper-case letters (A, B). Differences between times are expressed by different Greek letters (α, β). Values are mean ± SE.

3.4 Chlorophyll Fluorescence, Photochemical Reflectance Index (PRI) and Total Chlorophyll Content

In all studied clones, the chlorophyll fluorescence images obtained for Fv/Fm and Φ PSII (Figure 2) revealed lower values on the areas containing fungal pustules than in areas free of the pathogen fructification. Values obtained for Fv/Fm for each clone were between 0.75-0.85 (Figure 3 a, b). In L. Avanzo, infected leaves showed a significant decline in Fv/Fm values from July to September with lower values than the control leaves, while Lux showed low values in September both in control and infected leaves. L. Avanzo showed the lowest PRI values in both July and September compared to the other clones, especially in the infected leaves (Figure 3c, d). Lux and Adige did not show differences between the control and infected leaves but showed lower values in September. In July, the total chlorophyll content of the infected leaves was lower than in the control leaves in L. Avanzo and Adige (Figure 3 e, f). L. Avanzo showed lower values than Lux and Adige. In September, Lux and L. Avanzo showed a decline in total chlorophyll content in infected leaves compared to the control leaves. In Adige, values were higher in comparison to those of L. Avanzo and Lux.

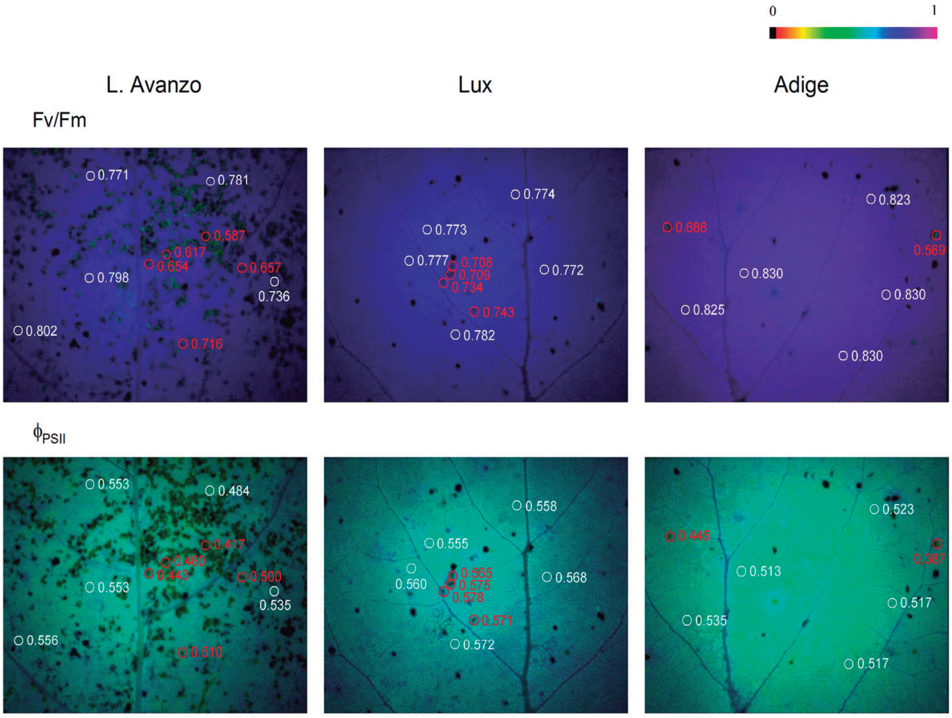

Figure 2. Chlorophyll fluorescence images of maximum quantum yield (Fv/Fm) in a dark-adapted state and quantum yield of photosystem II photochemistry (Φ_{PSII}), at steady-state with actinic illumination of 300 µE·m^{-2}·s^{-1}. Measurements were recorded in pustules (red circles) or not-affected areas (white circles) of infected leaves of the studied clones. The false colour code displayed at the top of the image ranges from 0 (black) to 1 (pink)

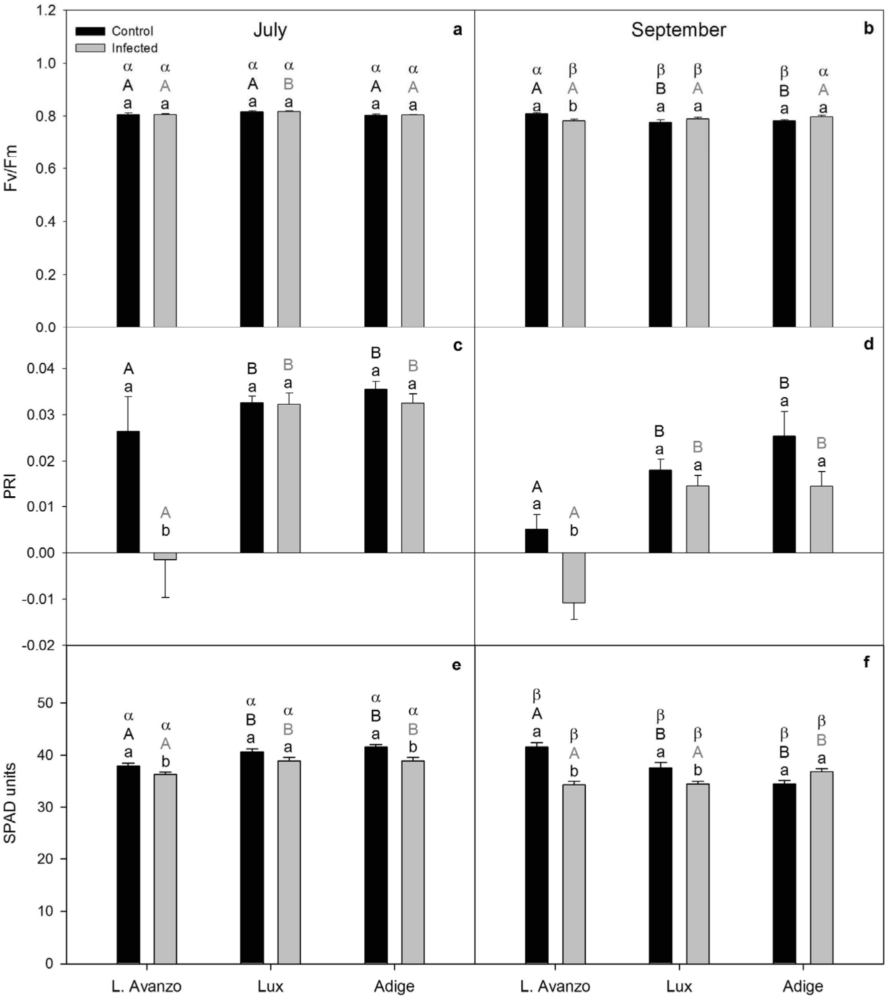

Figure 3 a, b) Maximum quantum yield (Fv/Fm), c,d) the Radiometric index PRI and e,f) Total Chlorophyll content during the study in infected and control leaves of the different *Populus* spp. clones. The number of replicates was 3 leaves for Fv/Fm and PRI and 8 leaves for chlorophyll content per types of leaf, clone and time. Significant differences ($p < 0.05$) between infected and control leaves for each clone are expressed by different lower–case letters (a, b). Differences between the controls or between infected leaves of the different clones are expressed by different upper-case letters (A, B). Differences between times are expressed by different Greek letters (α, β). Values are mean ± SE

4. Discussion

The evaluation of the effects of leaf rust on the physiological responses of poplar clones such as monitoring changes in the photochemistry processes and water relations affecting whole leaf photosynthesis can help to determine their potential in productivity programs (Widin & Schipper, 1980).

In our study, we detected significant differences in the susceptibility to rust infection and its effect on the physiology of the selected clones during the development of their leaves.

Plants did not show signs of infection until July, thereafter L. Avanzo showed the highest level of incidence of *Melampsora* rust. In July, most of their individuals were infected and the percentage of affected leaves was more than double with respect to Lux and Adige. In July, the degree of severity in L. Avanzo was mild to moderate, while in September, this clone was severely affected. Other studies reported differences in the susceptibility to rust of L. Avanzo. Artificially inoculated leaf discs of two strains of *M. larici-populina* showed that this clone was highly resistant to the E1 strain and highly susceptible to E3 (Giorcelli et al., 1996). Frey and Pinon (1997) also observed a wide range of reactions in L. Avanzo, ranging from susceptibility to resistance, depending on the isolate. Lux, a clone considered to be resistant to the E1 and E3 strains of *M. larici-populina* (Giorcelli et al.,

1996) showed moderate susceptibility in this study. In July, 18% of leaves presented traces of the infection or were not infected. In September, the percentage of infection had increased to 73%, while the severity remained at the same degree. In contrast to Facciotto and Frison (1999), Adige was the most resistant clone with a low incidence and degree of severity in July, and registering decreases in both parameters in September.

Productivity in fast growing species like poplars depends on water availability (Tschaplinski et al., 1994). Relative water content (RWC) and water index (WI) determinations provided information about poplar water status. In L. Avanzo, the higher RWC and WI of infected leaves in July with respect to the infected leaves of the other clones would favour the proliferation of *Melampsora* sp. infection in its early stages since high humidity promotes rust spore germination (Agrios, 2005), as reported for poplars (Coyle et al., 2006). Most leaves, both healthy and infected, showed elevated RWC indicating a sustained capacity to retain water during the summer. However, in L. Avanzo, RWC declined below 80% in infected leaves at the end of the summer which could be related to a possible increase in respiration and a higher limitation in photosynthetic activity (González & González-Vilar, 2001). In Lux, infected leaves also showed lower RWC than control leaves in July but values did not fall markedly during summer drought. Foliar disease has been reported to impair stomatal closure in the dark and stomatal opening in the light thereby affecting plant CO_2 assimilation and the ability to conserve water (Grimmer et al., 2012). In Adige, results for RWC and WI showed that both in July and September there was a higher susceptibility to water stress with respect to the other clones, however, no direct effects on these parameters as a result of infection were observed.

Leaf structural characteristics such as higher total and individual leaf area are related to higher productivity rates in several *Populus* clones (Marron, 2005). The leaf mass per area (LMA) and their two components – density (D) and thickness (T) – have been used to indicate poplar response to water stress and photosynthetic capacity. An increase in D is associated with thicker cell walls while an increase in T accounts for additional mesophyll layers (Niinemets, 2001). In spite of disease infection, no differences in D, LMA and LA were observed between control and infected clones. In September, increased sclerophylly (increases in D and LMA) and reductions in leaf transpiratory area (LA) (mainly in L. Avanzo) indicate that structural adaptations occur during summer drought.

Rust infection was not observed as having an effect on morphological parameters which contrasts with studies on defence mechanisms during pathogen infection in *Populus* spp. These report the strengthening of cell walls through lignin deposition (Duplessis et al., 2009) which would imply an increase in cell density (D). We only observed a decline in T in the early stages of *Melampsora* rust in Lux.

Chlorophyll fluorescence imaging provides a sensitive method for determining the impact of fungal pathogens on the photosynthetic metabolism of their hosts (Scholes & Rolfe, 2009). Our results showed differences between pustule (rust-affected) and nearby non-affected areas of infected leaves for each clone. The leaf zones where the fungus developed fructification had lower potential efficiency of PSII photochemistry (Fv/Fm) and PSII quantum yield (Φ_{PSII}). In fact, Fv/Fm values lower than 0.75 indicated a strong photoinhibition (Demmig-Adams & Adams, 1993). The distribution of the mycelium of this pathogen is heterogeneous in leaves and as demonstrated by Alves et al. (2011) in eucalyptus infected by the rust fungus *Puccinia psidii*, photosynthesis was only reduced in diseased leaf area. The effect of infection on whole leaves depends on the ratio between infected and non-infected leaf areas. Highest pustule density was observed for L. Avanzo, followed by Lux and Adige. By September, when the disease had progressed strongly in L. Avanzo (all individuals and 100% of leaves were infected mostly with a high-severity degree of infection where leaf global Fv/Fm values were significantly lower than those of the other clones. Lux showed lower global Fv/Fm values in September but no effects from the infection. However, whole-leaf Fv/Fm values (between 0.75 and 0.85) in all cases indicate that the clones were not subjected to a strong photoinhibition as a result of infection. In fact, although it can limit carbon assimilation, this disease is seldom responsible for tree death (Feau et al., 2007). The effect of the pathogen on L. Avanzo photochemistry was also apparent at high incident light intensities in the field (about 1100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with the lowest PRI values in both July and September compared to the other clones, PRI is related to PSII efficiency (Peñuelas et al., 1995). In general, a decline in total chlorophyll content was observed in September, especially in infected leaves of L. Avanzo and Lux in accordance with Bertamini et al. (2005). In Adige, the effect of the pathogen was not detected at the end of the summer which is in accordance with the observed reduction in the proliferation of the fungus.

Determination of susceptibility to *Melampsora* rust infection can help to identify the most resistant *Populus* clones. In this study, L. Avanzo was the most susceptible clone to rust showing a high incidence and increased severity during the summer, affecting photochemical processes, chlorophyll content and water relations. Lux and especially Adige were more tolerant to the pathogen and showed only traces of infection and slight physiological alterations.

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