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EFECTOS DEL RIGOR INVERNAL SOBRE LA MORFOLOGÍA Y FISIOLOGÍA FOLIAR DE TRES ESPECIES ARBÓREAS PERENNIFOLIAS

PATRICIA GONZÁLEZ ZURDO

~ TESIS DOCTORAL ~

SALAMANCA, 2016

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FACULTAD DE BIOLOGÍA

DEPARTAMENTO DE BIOLOGÍA ANIMAL, PARASITOLOGÍA, ECOLOGÍA, EDAFOLOGÍA Y QUÍMICA AGRÍCOLA

Efectos del rigor invernal sobre la morfología y fisiología foliar de tres especies arbóreas perennifolias.

Memoria presentada por la Licenciada en Biología Patricia González Zurdo para optar al Título de Doctor en Biología por la Universidad de Salamanca bajo la dirección de la Dra. Sonia Mediavilla Gregorio y el Dr. Alfonso Escudero Berián.

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Esta Tesis Doctoral está formada por un compendio de cuatro artículos, de los cuales tres están publicados y otro ha sido remitido y se encuentra en fase de revisión en el momento de depósito del documento, todos ellos en revistas internacionales especializadas incluidas en el SCI. A continuación se detalla toda la información sobre cada uno de ellos:

CAPÍTULO I **Cost of leaf reinforcement in response to winter cold in evergreen species.**

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N resorption efficiency and proficiency in response to winter cold in three evergreen species.

Patricia González-Zurdo, Alfonso Escudero, Sonia Mediavilla

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Losses of leaf area owing to herbivory and early senescence in three tree species along a winter temperature gradient.

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CAPÍTULO IV

Responses of photosynthetic parameters to differences in winter temperatures throughout a temperature gradient in two evergreen tree species.

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La Dra. Sonia Mediavilla Gregorio y el Dr. Alfonso Escudero Berián, Profesora Titular y Catedrático, respectivamente, del Departamento de Biología Animal, Parasitología, Ecología, Edafología y Química Agrícola de la Facultad de Biología de la Universidad de Salamanca

Certifican que:

La Tesis Doctoral titulada *Efectos del rigor invernal sobre la morfología y fisiología foliar de tres especies arbóreas perennifolias* ha sido realizada bajo su dirección por la Licenciada en Biología, Patricia González Zurdo, en el Área de Ecología de la Facultad de Biología de la Universidad de Salamanca, y reuniendo todos los requisitos científicos y formales para ser defendida y optar al Título de Doctora en Biología por la Universidad de Salamanca, autorizan que sea presentada en el formato de compendio de artículos.

En Salamanca, a de Mayo de 2016

Fdo. Sonia Mediavilla Gregorio Fdo. Alfonso Escudero Berián

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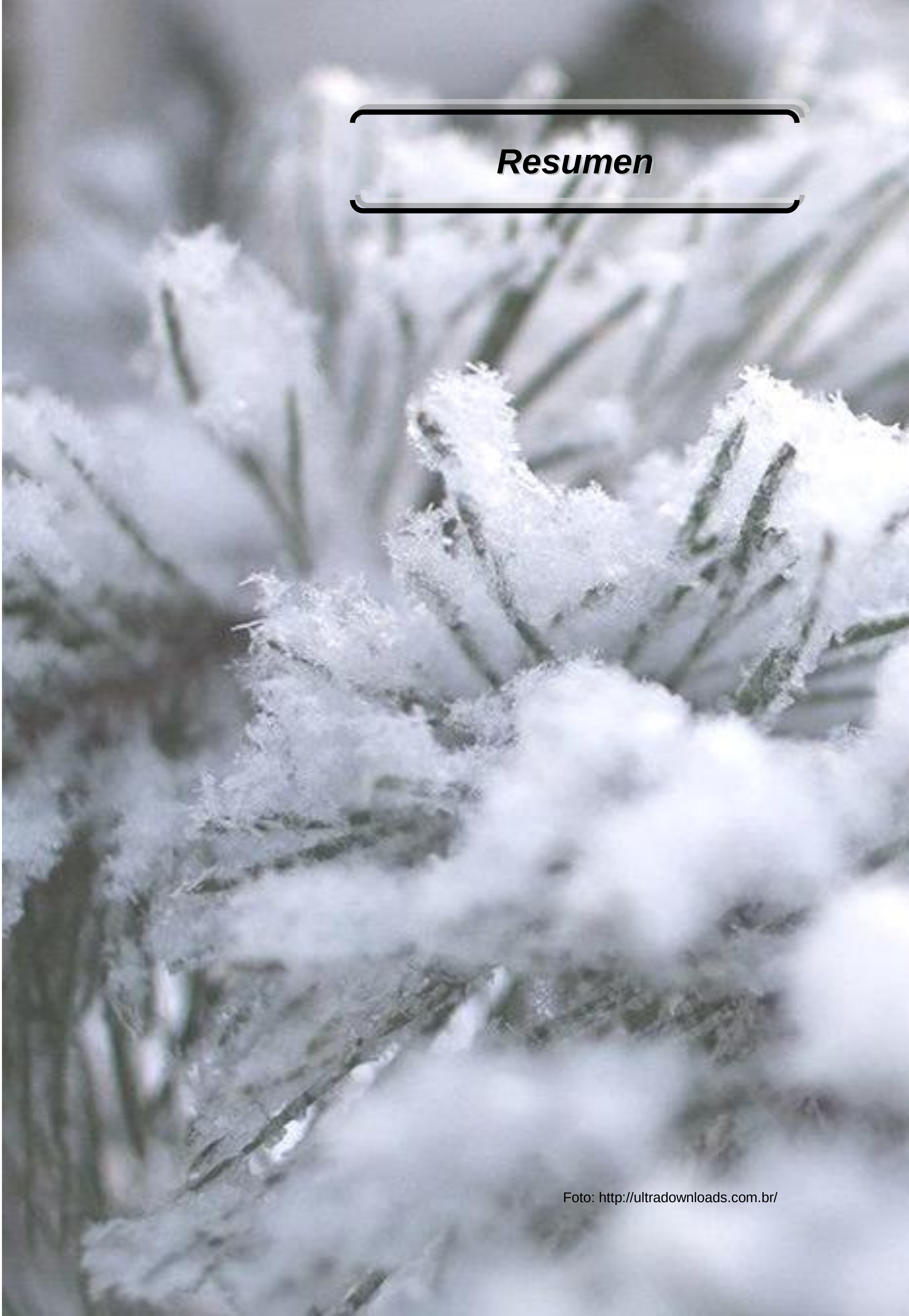

Resumen

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El estudio de las estrategias foliares desplegadas por las diferentes especies arbóreas ha acaparado desde siempre una gran atención, por el simple hecho de que las características de las hojas condicionan numerosos aspectos que finalmente determinan el funcionamiento de los ecosistemas. En los últimos años, no obstante, las preocupantes proyecciones contenidas en los sucesivos informes del Panel Intergubernamental de Expertos en el Cambio Climático han dado aún más relevancia a este tema de investigación, a causa de la fuerte influencia de la productividad vegetal sobre el ciclo del carbono y sobre el control de la concentración atmosférica de CO_2. Una de las consecuencias ecológicas importantes del cambio climático puede ser la sustitución de las especies dominantes actuales por otras mejor adaptadas a las nuevas condiciones. O, lo que es más importante aún, la sustitución de especies caducifolias por perennifolias o a la inversa, que podría tener lugar si los cambios estacionales alteran el balance de C de uno u otro de estos hábitos foliares, lo que, debido a las implicaciones de los rasgos foliares sobre los intercambios gaseosos, obviamente afectaría a la respuesta global al cambio climático.

En las regiones de clima mediterráneo, cabe esperar que los cambios más trascendentales para los equilibrios caducifolias-perennifolias tengan lugar durante el invierno porque las posibles alteraciones climáticas durante las estaciones más cálidas afectarían a ambos hábitos, ya que los dos mantienen hojas activas en estas épocas. En el caso de los climas templados, está ampliamente aceptado además que el funcionamiento de las plantas y en general de los ecosistemas depende en gran medida de los procesos ecológicos durante el invierno. Y son precisamente estas condiciones climáticas en invierno las que están sujetas a un cambio climático más intenso y rápido, un patrón que se espera que sea incluso más acusado en el futuro.

Teniendo en cuenta estas consideraciones, en el presente trabajo **nos propusimos analizar** los efectos de las diferencias en la intensidad del rigor invernal sobre la morfología, composición química y distintos aspectos de la fisiología foliar de tres especies perennifolias (*Quercus suber*, *Q. ilex* y *Pinus pinaster*). Las tres figuran entre las especies arbóreas más representadas en la Península y muestran amplias áreas de distribución, lo que nos permite encontrar un número de enclaves suficientes con condiciones climáticas contrastadas en los periodos más fríos del año. Además, exhiben un rango de longevidad foliar suficientemente amplio como para permitir el análisis de posibles diferencias en las respuestas en función de la duración y características foliares asociadas a esta distinta duración. Entre estos rasgos asociados a la longevidad, el peso por unidad de superficie (LMA) ha despertado particular interés, porque figura entre los atributos que experimentan un cambio más acusado entre especies y lleva asociado un enorme número de implicaciones para la productividad y la fisiología foliar. Así, especies con bajo LMA tienden a tener mayores concentraciones de proteínas y minerales y menor concentración de fibras, lo que tiende a estar asociado con altas tasas de asimilación, pero incrementa la vulnerabilidad frente a la herbivoría y a otros factores de estrés.

Pero, aunque LMA varía ampliamente entre especies, también se ha comprobado que lo hace a nivel de una misma especie, en respuesta a diferencias en el rigor del hábitat, como son, por ejemplo, la intensidad del estrés hídrico o nutricio. Por tanto, en primer lugar nos planteamos comprobar si la intensidad del rigor invernal puede añadirse a la serie de factores que determinan diferencias en el nivel de reforzamiento exigido a las hojas dentro de una misma especie, y si el mayor LMA que supuestamente deberían presentar las hojas en los sitios más fríos para soportar las condiciones más adversas se acompaña de cambios también en otros rasgos morfológicos o en composición química (**Capítulo I**). Nuestros resultados revelan que, efectivamente, la mayor parte de los rasgos analizados muestran diferencias significativas entre localizaciones, que parecen estar relacionadas con diferencias en la intensidad del rigor invernal, con una respuesta similar en las tres especies. El mayor LMA de las hojas en los ambientes con inviernos más fríos y heladas más intensas y continuadas sugiere que también soportar estas condiciones invernales más duras exige un mayor reforzamiento, que parece conseguirse únicamente a través de un mayor espesor y cantidad de pared celular, como se desprende del contenido de carbohidratos estructurales más alto que muestran las hojas en estos sitios más fríos. Aunque no observamos ninguna tendencia en el contenido de nitrógeno foliar asociada a las diferencias en temperatura invernal, la concentración de clorofila, Rubisco y proteínas solubles es siempre más baja en los sitios más fríos, lo que sugiere que el incremento en el reparto de N a las paredes celulares en estos ambientes ocurre a expensas de reducir la asignación al aparato fotosintético. Contrariamente, la concentración de fósforo sí se incrementa entre sitios con la intensidad del rigor invernal, lo que podría ayudar a compensar los efectos desfavorables de las bajas temperaturas sobre la asimilación de carbono.

Una vez comprobados estos cambios en morfología y composición química, en los siguientes capítulos nos propusimos analizar sus implicaciones sobre distintos aspectos del funcionamiento de las hojas de una misma especie en los distintos ambientes. En concreto, en el **Capítulo II** nuestro objetivo fue estimar los efectos de las temperaturas invernales sobre la eficiencia de retranslocación del nitrógeno y proficiencia a nivel de las hojas de cada especie. Observamos que las tres especies muestran menor potencial para la reabsorción de nitrógeno en los enclaves con temperaturas invernales más bajas, donde, por tanto, es mayor la dependencia del nitrógeno edáfico. Según nuestro estudio, este resultado parece deberse a las mayores cantidades de N inmovilizadas en la mayor cantidad de pared celular necesaria para hacer frente a las condiciones más adversas de los sitios más fríos, como apuntan las cantidades más altas de N encontradas en las hojas desprendidas en estos sitios. Este es el primer estudio, que nosotros conozcamos, en el que se pone de manifiesto la importancia de las diferencias en composición química asociadas con el distinto reforzamiento en respuesta a las temperaturas invernales sobre las diferencias en la eficiencia de reabsorción a nivel de una misma especie ocupando distintos ambientes.

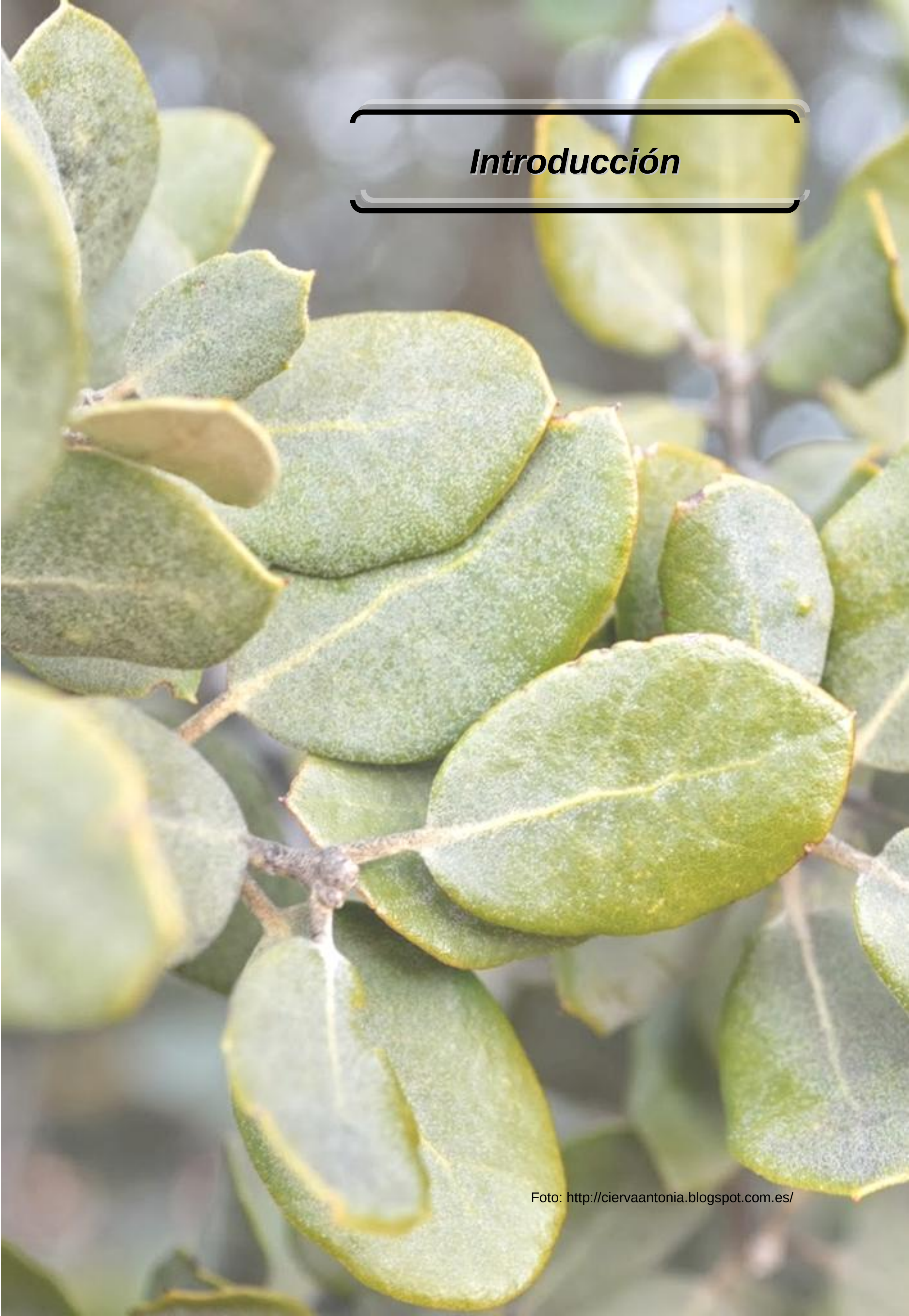
Otro de los aspectos que abordamos en nuestro estudio fue el análisis de las implicaciones de las diferencias en las características de las hojas a lo largo del gradiente de temperaturas sobre la intensidad de los daños sufridos en la superficie fotosintética, tanto por el consumo de herbívoros, como por el efecto de factores abióticos (**Capítulo III**). En las dos especies para las que disponíamos de información sobre las pérdidas por herbivoría, encontramos que los niveles de consumo (tanto el porcentaje de hojas atacadas, como la fracción de área media consumida por hoja) resultan siempre inferiores en las parcelas más frías, debido al mayor reforzamiento que ya hemos visto muestran las hojas en estos sitios. Sin embargo, a pesar de este mayor reforzamiento, las condiciones climáticas más severas se traducen también en mayores daños por factores abióticos, con más hojas dañadas por el efecto de las bajas temperaturas y más fracción de área pérdida en las parcelas más frías, en las tres especies de estudio. Y, en principio, este incremento en la intensidad de los daños abióticos en los sitios más fríos respecto a los más cálidos supera claramente al descenso registrado en la intensidad de la herbivoría. Los resultados de nuestro estudio, el primero donde se aportan datos sobre las diferencias en los daños en hojas de una misma especie entre distintos ambientes climáticos, sugieren, por tanto, que también respecto a las pérdidas anticipadas de área foliar, ocupar sitios más fríos implicaría una clara desventaja para nuestras especies perennifolias.

En el **Capítulo IV** nos propusimos por último explorar las implicaciones que en términos de capacidad fotosintética (variabilidad en los parámetros fotosintéticos) conlleva para una misma especie ocupar ambientes con diferente intensidad del rigor invernal. Puesto que la estimación fidedigna de los parámetros fotosintéticos requiere medir un gran número de curvas de respuesta de la asimilación a cambios en la concentración de CO_2, en este caso limitamos el estudio únicamente a las dos especies quercíneas. Nuestros resultados muestran que, debido al mayor reforzamiento de las hojas necesario en los sitios más fríos, es menor la proporción de nitrógeno asignada a la maquinaria fotosintética, lo que contribuye a reducir la máxima tasa de carboxilación (V_{cmax}) y de transporte de electrones (J_{max}), y por tanto la capacidad fotosintética y eficiencia en el uso del N respecto a los sitios

más cálidos. La menor limitación estomática que también muestran nuestras especies en estos ambientes no es suficiente para compensar la menor actividad de la maquinaria fotosintética, lo que resulta finalmente en menores tasas de fotosíntesis a concentración ambiente de CO_2. Según nuestro estudio entonces, serían más bien las diferencias en la asignación de nitrógeno a distintas funciones, y no tanto en la cantidad absoluta de N en las hojas, las principales responsables de la variabilidad en la capacidad fotosintética a nivel de las hojas de una misma especie.

En resumen, este trabajo revela que ocupar ambientes con inviernos más fríos exige un mayor reforzamiento foliar, que a su vez conlleva una menor eficiencia de retranslocación de N (y por tanto mayor dependencia del N edáfico), menor capacidad fotosintética y mayores pérdidas prematuras de área foliar. Todos nuestros resultados sugieren entonces que el carácter perennifolio llevaría asociado mayores costes en estos ambientes, lo que necesariamente supone alguna desventaja añadida respecto a las caducifolias que los ocupan. Es obvio que se necesitan nuevos estudios que permitan abordar otros aspectos del funcionamiento de las hojas para comprobar si existen ventajas que aún no hemos identificado o para tratar de predecir si el cambio climático previsto puede permitir o no mitigar en alguna medida estas desventajas detectadas. En cualquier caso, está claro que el efecto de los cambios en temperatura durante el invierno debe ser tenido muy en cuenta en los modelos predictivos de la posible composición de especies de nuestros bosques en el futuro.

Introducción



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La investigación de las adaptaciones de las plantas terrestres a los factores de estrés ha atraído desde siempre la atención de numerosos científicos (Balaguer et al. 2001, Gratani et al. 2003, Gimeno et al. 2008, Kamiyama et al. 2014) por la obvia razón de que la producción vegetal es la base de la energía que fluye por la biosfera y, a través de las plantas, las condiciones climáticas y edáficas determinan el funcionamiento de los ecosistemas. La alteración artificial de la química atmosférica da más relevancia aún a este tema de investigación, a causa de la fuerte influencia de la productividad vegetal sobre el ciclo del carbono y sobre el control de la concentración atmosférica de dióxido de carbono (Melillo et al. 1993, Dewar et al. 2009, Shevliakova et al. 2013). De especial importancia es averiguar si los ecosistemas actúan como sumideros o fuentes de CO_2, y, particularmente, analizar los cambios previsibles en el papel de los ecosistemas en el ciclo del carbono, porque, evidentemente, pueden actuar como freno o, alternativamente, pueden acelerar los procesos antropogénicos de alteración de la química atmosférica.

En este sentido, las distintas estrategias foliares desplegadas por las diferentes especies arbóreas deben ser investigadas con especial atención, porque las diferencias en las características foliares afectan fuertemente a los patrones de intercambios gaseosos (Reich et al. 1997, Mediavilla et al. 2001, Niinemets and Sack 2006, Reich 2014) y a las propiedades de la materia orgánica en el suelo, una vez que las hojas mueren y se desprenden (Facelli and Pickett 1991, Aerts 1995, Rubbo and Kiesecker 2004), de modo que los rasgos foliares específicos determinan el balance de carbono de los ecosistemas, tanto cuando las hojas viven como incluso después de muertas. Todos los autores coinciden en que la comparación perennifolias-caducifolias es especialmente relevante, porque, al menos en zonas de climas templados, las diferencias en longevidad foliar y fenología entre especies caducifolias y perennifolias están asociadas a diferencias en numerosos rasgos foliares (Warren and Adams 2000, Mediavilla et al. 2001, Takashima et al. 2004, Villar et al. 2006) que, a su vez, determinan los patrones de intercambios gaseosos, la composición química de las hojas, la dinámica interna de los nutrientes, la descomposición de los detritos vegetales en el suelo y muchos otros fenómenos que trascienden a las propias funciones de las plantas y terminan teniendo efectos sobre las propiedades de los ecosistemas e, incluso, efectos globales sobre el funcionamiento de la biosfera.

Una de las consecuencias ecológicas importantes del cambio climático puede ser el cambio de la distribución de las especies arbóreas, a causa de la sustitución de las especies dominantes actuales por otras mejor adaptadas a las nuevas condiciones (Walther et al. 2009, Sheldon et al. 2011, Wardle et al. 2011, Mori et al. 2014). Más importante, en realidad, que la simple sustitución de unas especies por otras será la sustitución de hábitos foliares. Entre ellos, la sustitución de especies caducifolias por perennifolias o a la inversa, que podría tener lugar si los cambios estacionales alteran el balance de carbono de uno u otro de estos hábitos foliares. Los cambios en las estrategias foliares pueden tener profundas implicaciones sobre la fenología, los intercambios gaseosos, las propiedades de los suelos, el paisaje, etc. Y, lo que es más importante, por las razones explicadas más arriba, la sustitución de unas especies por otras con distintos hábitos foliares puede afectar al ciclo del carbono y a la respuesta global al cambio climático.

En nuestras regiones de clima mediterráneo, cabe esperar que los cambios más trascendentales para los equilibrios caducifolias-perennifolias tengan lugar durante el invierno. Durante los meses más cálidos y secos del año, ambos grupos de especies mantienen hojas activas, de modo que las posibles alteraciones climáticas operadas durante las estaciones más cálidas afectarían tanto a hojas caducas como perennes, aunque evidentemente sus respuestas pueden diferir. Serían más bien entonces las condiciones invernales las que deberían determinar las diferencias principales entre hojas caducas y perennes, por lo que parece especialmente importante analizar las respuestas a los factores de estrés típicos de la estación invernal. En todo caso, cualquier alteración de los factores de estrés en cualquier época del año a causa del cambio climático debería tener especial efecto sobre las características de las hojas de las especies perennifolias, porque las hojas de vida más corta son funcionales sobre todo durante épocas favorables para la fotosíntesis, y podrían responder a cualquier cambio climático con una modificación de su fenología (Norby et al. 2003, Chen et al. 2005, Gordo and Sanz 2010), que mantuviera ajustada la duración de la hoja a la estación favorable en las nuevas condiciones, ahorrando cambios morfológicos que perjudiquen la fotosíntesis. Por el contrario, las hojas de duración superior a un año deben mantenerse vivas durante todas las estaciones, y la única manera de conseguirlo es adoptar una morfología y composición química que les permita soportar las condiciones de estrés de cualquier época del año y, eventualmente, responder con adaptaciones morfológicas a cualquier agravamiento de dichas condiciones.

Por otra parte, aunque el cambio climático más esperado sea el aumento de la temperatura, si hemos de explotar mejor sus oportunidades y minimizar los daños, es imprescindible, paradójicamente, entender las respuestas de las plantas a las bajas temperaturas (Sage and Kubien 2007, Kreyling 2010). Es ampliamente aceptado que el funcionamiento de las plantas y en general de los ecosistemas depende en gran medida de los procesos ecológicos durante el invierno en el caso de los climas templados (Makoto et al. 2014, Mori et al. 2014). Y son estas condiciones climáticas en invierno las que están sujetas a un cambio climático más acusado y rápido. En la reunión Intergubernamental sobre el Cambio Climático de 2007 ya se puso de manifiesto que el calentamiento observado en los últimos treinta años ha sido especialmente intenso en climas templados o fríos durante el invierno. Y este patrón se espera que sea incluso más acusado en el futuro. La moderación de las temperaturas invernales en climas templados o fríos permitirá alargar la estación de crecimiento en invierno y primavera temprana, lo que favorecerá a las especies adaptadas a fotosintetizar a menores temperaturas. A pesar de estos cambios ya constatados, el número de estudios sobre las implicaciones del cambio climático durante el invierno son marcadamente reducidos comparados a los estudios llevados a cabo en relación al impacto del cambio climático sobre las plantas durante la estación de crecimiento (Sturm et al. 2005, Wipf and Rixen 2010, Cornelissen and Makoto 2014). Y, entre estos escasos estudios, los llevados a cabo en regiones templadas en particular son prácticamente inexistentes (Kreyling 2010, Kreyling et al. 2011).

Por todos estos motivos, en esta Tesis nos propusimos analizar los efectos de las diferencias en la intensidad del rigor invernal sobre las características de las hojas perennes de tres especies arbóreas mediterráneas que se encuentran entre las más ampliamente distribuidas en la Península (*Quercus suber, Q. ilex* y *Pinus pinaster*) y comprobar las implicaciones de dichas diferencias sobre diversos aspectos del funcionamiento de estas especies. Tanto el género *Pinus*, como particularmente el género *Quercus*, constituyen dos grupos de gran importancia para comprender cómo los factores ambientales determinan la variación en morfología y características foliares, debido a sus particularmente altos niveles de variación no sólo entre especies, sino incluso entre poblaciones, entre árboles y también dentro de un mismo individuo (Bruschi et al. 2003, Tovar Sánchez and Oyama 2004, Sacks et al. 2006). Dada la aparente capacidad de aclimatación en los rasgos foliares (Andivia et al. 2012, Klein et al. 2013, Matesanz and Valladares 2014), cabe esperar que dentro de una misma especie distribuida por regiones de temperaturas diferentes se den cambios en los rasgos foliares, lo que proporciona una buena oportunidad para prever las respuestas al cambio climático mediante el estudio comparativo de los atributos de las hojas de una misma especie en zonas con diferentes características climáticas.

Entre los rasgos foliares que han despertado un mayor interés se encuentra el peso por unidad de superficie (LMA), que a su vez depende del espesor y/o la densidad foliar (Witkowski and Lamont 1991, Niinemets 1999). Este rasgo, que determina el nivel de reforzamiento estructural, y, por tanto, está íntimamente ligado a la duración de las hojas (Turner 1994, Niinemets 2001, Onoda et al. 2011, Kikuzawa et al. 2013), figura entre los que experimentan un cambio más acusado entre especies y lleva asociado un enorme número de implicaciones para la productividad y la fisiología foliar. Así, especies con bajo

LMA tienden a tener mayores concentraciones de proteínas y minerales y menor concentración de fibras, lo que tiende a estar asociado con altas tasas de asimilación, pero incrementa la vulnerabilidad frente a la herbivoría y a otros factores de estrés (Wright and Cannon 2001, Wright et al. 2005, Shipley et al. 2006, Mediavilla et al. 2008). La generalidad de estas relaciones observadas por numerosos autores ha dado lugar al concepto "leaf economics spectrum" (Wright et al. 2004, Donovan et al. 2010, Heberling and Fridley 2012), que ha acabado constituyendo una guía de la investigación de las adaptaciones de las plantas y de la productividad vegetal durante los últimos años.

Pero, aunque LMA es uno de los rasgos que más ampliamente varían entre especies, también lo hace a nivel de una misma especie en respuesta a diferencias en el rigor del hábitat (He et al. 2006, Milla et al. 2008, Messier et al. 2010). Tradicionalmente, estos cambios en LMA se han analizado sobre todo en relación al estrés hídrico o nutricio (Wright et al. 2002, Wright et al. 2004), pero también se ha constatado que las bajas temperaturas contribuyen a incrementar LMA y que este rasgo cambia en las hojas producidas en distintas condiciones de temperatura (Ogaya and Peñuelas 2007, Atkin et al. 2008). Si es cierto entonces que alargar la vida de la hoja exige un refuerzo estructural que permita superar los rigores climáticos, en principio podríamos esperar que cuanto más duras y estresantes sean las condiciones climáticas, en nuestro caso el rigor de la estación invernal, tanto mayor debería ser el refuerzo estructural, lo que, teniendo en cuenta las connotaciones que llevan asociadas las diferencias en LMA, debe traducirse en importantes diferencias en distintos aspectos del funcionamiento de las hojas de una misma especie en los distintos ambientes. En concreto en la presente memoria nos proponemos:

- Determinar los efectos del rigor invernal sobre la morfología (tamaño, peso por unidad de superficie, espesor y densidad) y composición química (contenido de nitrógeno, fósforo, fibras, clorofila, proteínas solubles y Rubisco) en nuestras especies de estudio.

- Estimar los efectos de las temperaturas invernales sobre la eficiencia y proficiencia de retranslocación del nitrógeno a nivel de las hojas de cada especie.

- Cuantificar los daños en la superficie fotosintética causados por distintos factores de estrés en enclaves caracterizados por distinta intensidad del rigor invernal y tratar de identificar las causas de las posibles diferencias en la intensidad de los daños a lo largo del gradiente de temperaturas.

- Analizar las implicaciones que en términos de capacidad fotosintética (variabilidad en los parámetros fotosintéticos) conlleva para nuestras especies de estudio ocupar ambientes con diferente intensidad del rigor invernal.

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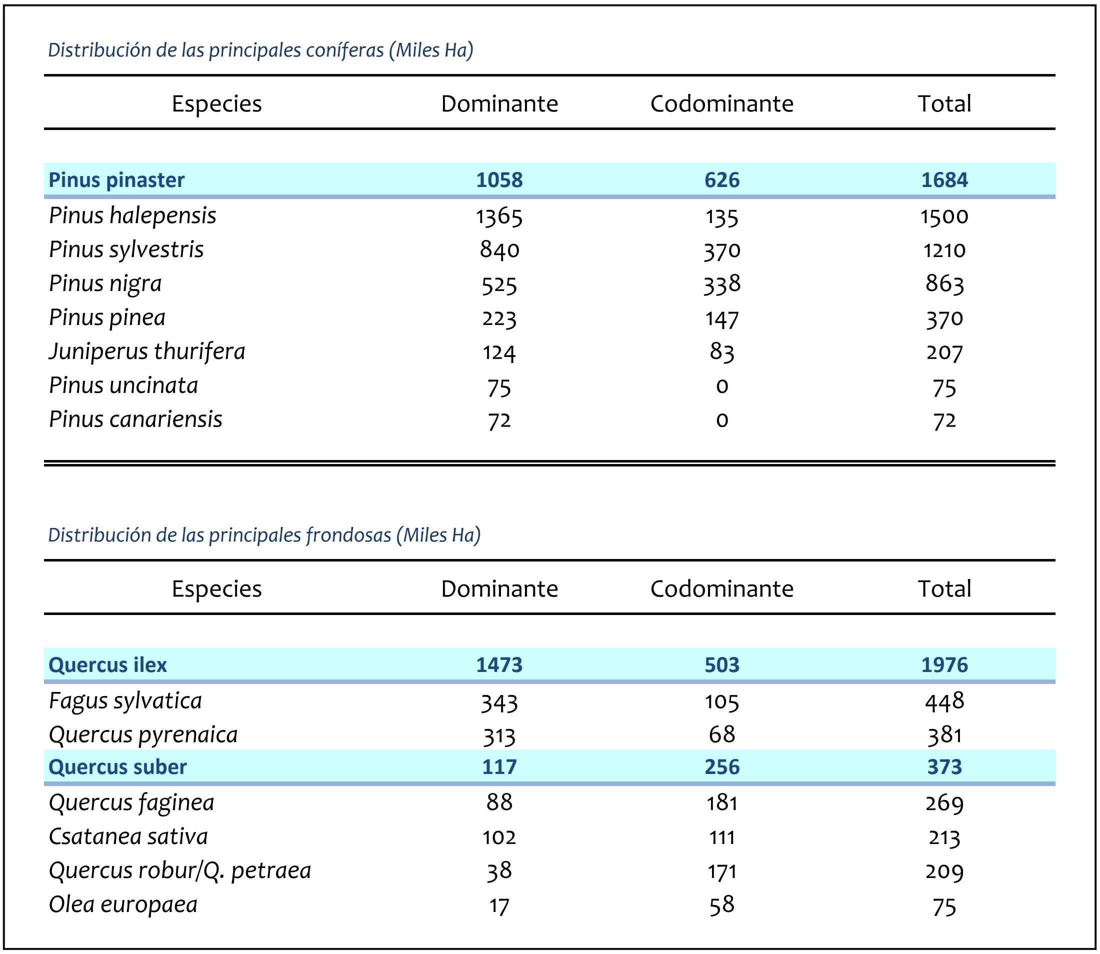
Especies y zonas de estudio

Tres especies arbóreas perennifolias fueron seleccionadas para el presente estudio: el alcornoque (*Quercus sube*r L.), la encina (*Quercus ilex* L. subsp. *ballota* (Desf.) Samp. (= *Q. rotundifolia* Lam.) y el pino marítimo (*Pinus pinaster* Aiton). Su selección se debe, en primer lugar, al hecho de que las tres se encuentran entre las especies arbóreas más representadas en la Península (Tabla 1) y muestran amplias áreas de distribución (Figura 1), lo que nos proporciona la posibilidad de encontrar un número de enclaves suficiente como para obtener un contraste adecuado en las condiciones climáticas en los periodos más fríos del año.

Tabla 1. Distribución nacional de las principales especies de coníferas y frondosas (Plan Forestal Nacional 2002).



Distribución de las principales coníferas (Miles Ha)

Especies	Dominante	Codominante	Total
Pinus pinaster	**1058**	**626**	**1684**
Pinus halepensis	1365	135	1500
Pinus sylvestris	840	370	1210
Pinus nigra	525	338	863
Pinus pinea	223	147	370
Juniperus thurifera	124	83	207
Pinus uncinata	75	0	75
Pinus canariensis	72	0	72

Distribución de las principales frondosas (Miles Ha)

Especies	Dominante	Codominante	Total
Quercus ilex	**1473**	**503**	**1976**
Fagus sylvatica	343	105	448
Quercus pyrenaica	313	68	381
Quercus suber	**117**	**256**	**373**
Quercus faginea	88	181	269
Csatanea sativa	102	111	213
Quercus robur/Q. petraea	38	171	209
Olea europaea	17	58	75

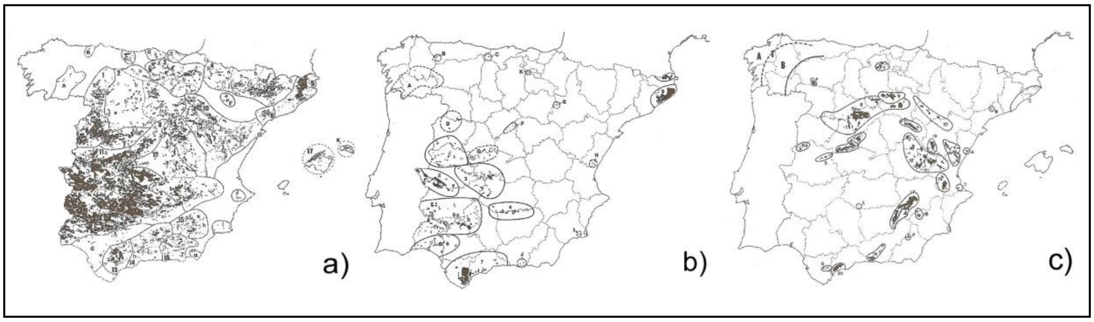


Figura 1. Mapas de distribución de *Q. ilex* (a), *Q. suber* (b) y *P. pinaster* (C).

Las tres especies exhiben además un amplio rango de longevidad foliar (entre los 15 meses de media para el caso del alcornoque, pasando por los casi 24 de la encina, hasta una duración media de en torno a 52 meses para las hojas del pino), suficientemente grande como para permitir el análisis de las diferencias en las respuestas de las distintas especies en función de la duración de sus hojas.

Describimos a continuación, a rasgos generales, las **principales características** de estas especies de estudio, siguiendo principalmente la “Guía de los árboles y arbustos de la Península Ibérica y Baleares” de Ginés López (2007), la “Flora Ibérica” (1986-2012) y el “Atlas forestal de Castilla y León” (2007).

Quercus suber L. (Alcornoque). Es un árbol de tamaño medio, que no suele sobrepasar los 20 ó 25 m de altura, con una corteza muy gruesa, esponjosa y ligera. Su copa suele ser muy amplia, casi hemisférica e irregular. Sus hojas son parecidas a las de la encina, aunque más triangulares, con el margen entero o dentado (con dientes poco profundos), alternas y de color verde lustroso en el haz y blanquecino en el envés, debido a la capa de pelos que presentan en esta zona. Estas hojas tienen, además, los nervios muy bien marcados en el envés. Florecen en marzo, abril o mayo aunque a veces la floración se puede alargar hasta el verano o el otoño, y sus frutos (bellotas parecidas a las de la encina, aunque de sabor menos dulce) maduran de septiembre a febrero las primeras y en primavera o verano las de floración otoñal. Al igual que en el caso de la encina, las flores son de pequeño tamaño, unisexuales, las masculinas con envuelta amarillenta agrupadas en amentos finos y las femeninas solitarias o en grupos reducidos.

Se trata de una especie localizada preferentemente en terrenos silíceos, con suelos sueltos y permeables (sobre todo arenosos), en zonas húmedas y sin fuertes heladas. A pesar de formar las mayores masas en las zonas silíceas, pueden presentarse pequeños grupos o ejemplares aislados en suelos calizos descalcificados. Es poco resistente al frío, por lo que prefiere inviernos suaves y algo húmedos, además de veranos calurosos. Rara vez aparece en lugares en los que no se superan los 500 mm de precipitación anual, encontrándose su óptimo en los 600-1000 mm. Se sitúa entre el nivel del mar y los 1200 m de altitud, pero su óptimo está entre los 300 y los 600 m. Se asocia frecuentemente con la encina, pero requiere más humedad y es más termófila que ella, por lo que el área de distribución del alcornoque es más restringida que la de la encina, apareciendo especialmente representada en la mitad oeste de la Península, así como en el nordeste de Cataluña (Figura 1).

Quercus ilex subsp. ***ballota*** (Desf.) Samp. (Encina). Ésta es una especie de copa amplia y redondeada, que raramente sobrepasa los 15 ó 20 m, cuyo tronco es fuerte y está algo torcido y que tiene la corteza cenicienta y resquebrajada en grietas poco profundas. Las hojas son simples y alternas, coriáceas, gruesas, con el margen entero o provisto de dientes, de color verde intenso por el haz y blanquecinas por el envés, debido a la presencia de una corta pubescencia en esta zona. Florece en primavera, en marzo, abril o mayo, y sus frutos, bellotas de forma ovoide con escamas reducidas y soldadas formando una cúpula, maduran y se diseminan en otoño, de octubre a noviembre. Las flores son de tamaño reducido, unisexuales, las masculinas sobre ejes flexibles y ramificados y las femeninas solitarias o en pequeños grupos.

La encina se encuentra en todo tipo de sustratos, incluso en los más pobres, creciendo tanto sobre suelos silíceos como sobre calizos. Además está adaptada tanto al clima mediterráneo y al continental, resistiendo bien temperaturas extremas y las fuertes sequías estivales. Debido a esta gran resistencia y plasticidad, esta especie ocupa ambientes muy diversos, tanto desde el punto de vista climático como edáfico. A pesar de ser indiferente en cuanto al tipo de suelo (crece tanto sobre suelos silíceos como calizos), tolera mal los terrenos húmedos o encharcados y tampoco le resultan favorables los suelos arcillosos, margosos y excesivamente compactos. Se puede encontrar desde el nivel del mar hasta unos 1400 m de altitud, pero algunos ejemplares aislados se pueden encontrar hasta cerca de los 2000 m. Se trata del árbol dominante en gran parte de las regiones de clima mediterráneo seco, así como en las de clima continental, por lo que su distribución por la Península Ibérica comprende casi su totalidad, abundando especialmente en la zona centro-oeste de la misma (Figura 1).

Pinus pinaster Aiton (Pino resinero). Se trata de un árbol con porte piramidal, en el caso de juveniles, y copa redondeada, en los adultos. Es de talla mediana, de entre 20 y 30 m de altura. Su tronco es derecho y con la corteza áspera y profundamente resquebrajada, de color pardo-rojizo. Las hojas son aciculares, largas y rígidas, de color verde oscuro, que se agrupan por parejas envueltas en una vaina membranosa que las recubre por la base. Florece en primavera, entre abril y mayo. Posee flores masculinas y femeninas. Las masculinas, formadas por estambres afilados, con abundante polen amarillo, el cual desprenden en primavera. Las femeninas se agrupan en conos que cuando maduran forman piñas de forma cónica alargada, de color pardo y con escamas poco prominentes, madurando a finales del verano o en el otoño del siguiente año, pero los piñones no se diseminan hasta el tercer año.

Esta especie se puede encontrar desde el nivel del mar hasta unos 1500 m de altitud, principalmente en terrenos silíceos, prefiriendo suelos sueltos y arenosos, en los que sí soporta la caliza. Tiene carácter termófilo y gran exigencia de luz, resistiendo bien tanto la sequía estival como las heladas. Se trata de una especie propia de regiones mediterráneas, aunque se encuentra en casi toda la Península Ibérica, teniendo gran parte de sus poblaciones origen en las reforestaciones de amplias zonas ocupadas por matorral (sobre todo jarales y brezales) y monte bajo de frondosas, aunque se ha extendido en numerosas ocasiones de forma artificial a expensas de robledales, alcornocales y encinares (Figura 1). Puede aparecer tanto en masas monoespecíficas como en bosques mixtos, junto a otras coníferas (especialmente *P. sylvestris* L. y *P. pinea* L.), o bien acompañado de encinas, quejigos y melojos.

Las tres especies fueron seleccionadas en un total de once enclaves distribuidos por las provincias de Soria, Guadalajara, Zamora, Salamanca y Cáceres (Figura 2).

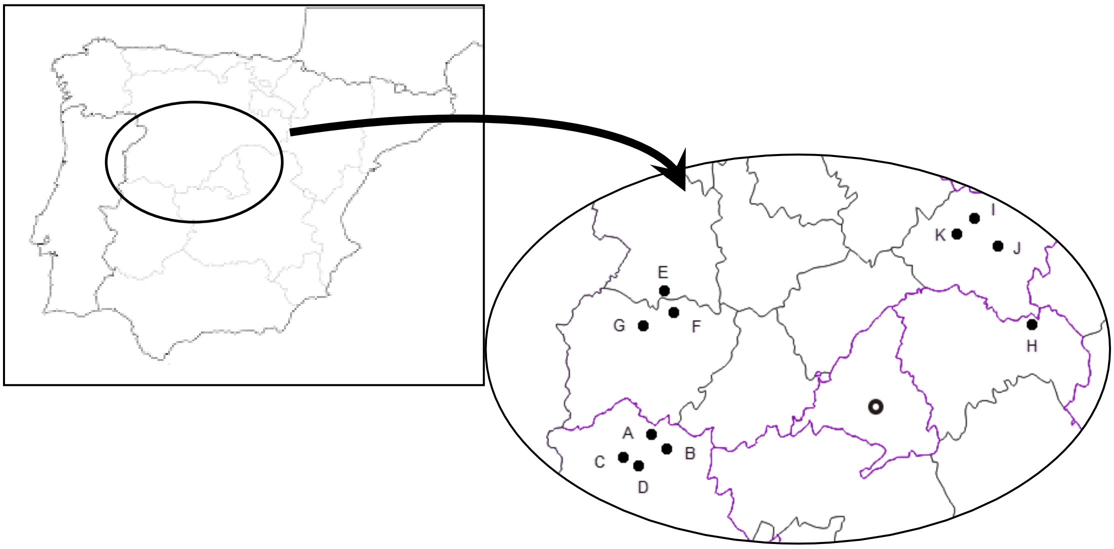


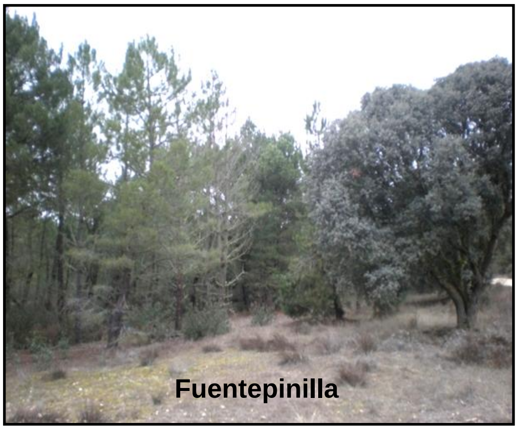
Figura 2. Mapa de distribución de las diferentes parcelas experimentales.

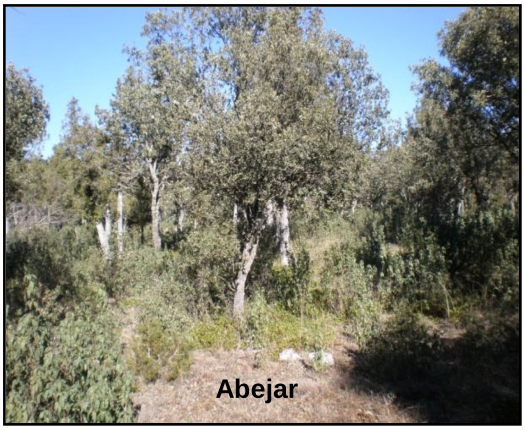
Como ya hemos comentado en la introducción, nuestro objetivo principal consiste en comparar diferentes poblaciones de cada una de dichas especies en condiciones contrastantes de rigor invernal, pero procurando que las restantes variables climáticas se mantengan lo más parecidas posible entre las diferentes poblaciones. De este modo, los enclaves incluidos han sido aquellos que, presentando el mayor número posible de especies distintas, entre las tres objeto de esta Memoria, ofreciesen un rango lo suficientemente amplio de temperaturas durante los periodos más fríos del año, pero sin presentar diferencias acusadas en otras variables ambientales. Basándonos en estos criterios y teniendo en cuenta los patrones de distribución de cada especie, siete fueron, de entre las 11 parcelas consideradas en nuestro estudio, las seleccionadas en el caso del alcornoque, ocupando las posiciones más occidentales: una en la provincia de Zamora (Teso Santo, E), dos en Salamanca (Valdelosa y Garcirrey, F y G respectivamente) y cuatro más en la provincia de Cáceres (Segura de Toro, Jaraíz, Cañaveral y Casatejada, parcelas A, B, C y D respectivamente). Debido a sus amplios patrones de distribución, la encina pudo ser seleccionada en un total de diez, de entre las once parcelas de estudio, con la única excepción de Jaraíz, en la provincia de Cáceres. Finalmente, los ejemplares de *P. pinaster* fueron seleccionados en tres enclaves en la provincia de Soria, en la que se distribuye formando amplias masas (Abejar, Fuentepinilla y Muriel, parcelas I, J y K respectivamente), un enclave en la provincia de Zamora (Teso Santo, E), otro en Salamanca (Valdelosa, F) y dos más en Cáceres (Cañaveral y Casatejada, C y D).

Describimos a continuación las principales características correspondientes a cada uno de los enclaves de estudio que integran el gradiente seleccionado para cada especie. Las coordenadas geográficas, altitud, precipitación y datos edáficos se presentan en la sección de “Material y Métodos” de los sucesivos capítulos de esta Memoria. Los datos climáticos (temperatura media mensual característica de cada una de las parcelas, obtenida como promedio de los datos registrados en los últimos 10 años, y precipitación total anual para el periodo concreto a lo largo del cual se extiende nuestro estudio) fueron proporcionados por el Instituto Nacional de Meteorología a partir de la información recogida en las estaciones más próximas a cada parcela. En el caso de la temperatura, puesto que es ésta la variable que condiciona las diferencias en la intensidad del rigor invernal, recurrimos a una estimación más exhaustiva mediante la colocación bajo la copa de un ejemplar seleccionado al azar en cada una de las localizaciones de un registrador serie Hobo para intemperie (Hobo Pendant temperature/light datalogger, Part UA-002-XX). Los sensores, que están programados para obtener estimaciones a intervalos de tan sólo 10 minutos, fueron mantenidos en el campo a lo largo de 4 años (octubre de 2008 − octubre 2012). Tanto su programación (fijación de la hora de inicio, modo para guardar valores y cuota de medición), como la lectura de los valores de medición, eran llevadas a cabo en el campo en cada una de las diferentes fechas de muestreo a través de un ordenador portátil con un software específico destinado a tal fin.

En cuanto a la caracterización edafológica de cada una de las parcelas, ésta se obtuvo tomando muestras de suelo a distintas profundidades y en diversos puntos de la misma zona para aumentar la representatividad. Las muestras fueron sometidas a determinaciones granulométricas, de pH y de las concentraciones de los principales nutrientes minerales, y se efectuaron en el Laboratorio de Suelos del Instituto de Recursos Naturales y Agrobiología (CSIC) de Salamanca, con arreglo a los métodos descritos en Bremner (1960), Chapmann and Pratt (1973) y Walkley and Black (1934).

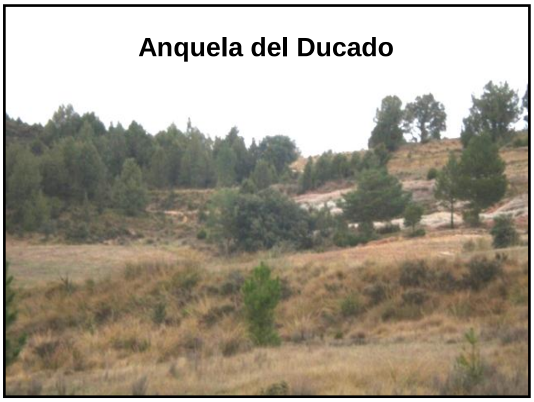
En la provincia de Soria seleccionamos tres de las once parcelas de estudio, **Muriel de la Fuente**, **Fuentepinilla** y **Abejar**, localizadas cada una en los municipios de los que toman su nombre, y con marcadas similitudes climatológicas. El clima de esta zona varía desde el Mediterráneo templado al frío, con temperaturas medias anuales que oscilan entre los 6 y los 12 ºC, siendo la temperatura media del mes más frío de -2 a 2 ºC, y la temperatura media del mes más cálido de 14 a 20 °C. La precipitación media anual oscila entre los 450 y 600 mm. La topografía de la zona es fundamentalmente llana. La superficie arbolada está constituida por sabinas (*Juniperus thurifera* L.) y pequeñas masas de pino silvestre (*Pinus sylvestris* L.) que forman mosaico con el pino resinero (*P. pinaster* Aiton). Esta última es la especie





forestal más difundida. Mezcladas con los pinos encontramos otras especies, de las cuales la encina (*Quercus ilex* L.) es la que presenta mayor superficie en masas puras, mientras que es el melojo (*Q. pyrenaica* Willd.) el que aparece asociado en mayor superficie con *P. pinaster*. Los suelos son profundos, muy mineralizados y con buena estructura. El subsuelo suele estar constituido por gravas.

La parcela situada en el municipio de **Anquela del Ducado** está localizada al noroeste de la provincia de Guadalajara. Su clima es Mediterráneo templado o templado-fresco, con una temperatura media anual entre los 10 y 12 °C. La temperatura media del mes más frío es de 2 a 4 ºC, y la del mes más cálido de 20 a 24 ºC. La precipitación media anual se sitúa entre los 400-500 mm. La vegetación natural es de bosquetes esclerófilos perennifolios presididos por la encina (*Quercus ilex* L.) y asociados en algunos casos con el quejigo (*Q. faginea* Lam.). En ocasiones se encuentran pies de enebro (*Juniperus communis* L.) en estas superficies arboladas y a veces se observan pequeñas masas de sabina albar (*J. thurifera* L.). La zona presenta un relieve accidentado. Los materiales predominantes de los suelos son areniscas, conglomerados, calizas y margas, de texturas francas tendiendo a gruesas



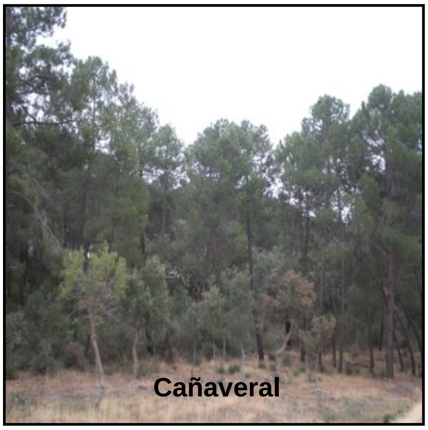
(arenosas). Se caracterizan por presentar poca materia orgánica y, en general, poco espesor.

Al suroeste de la provincia de Zamora se encuentra la parcela de **Teso Santo**, localizada en el municipio de Peñausende. Su clima es Mediterráneo continental, presentando una temperatura media anual de 11-12 °C. La temperatura media del mes más cálido está comprendida entre los 18 y los 28 °C, y la temperatura media del mes más frío se encuentra entre los 0 y los 9 °C. Las precipitaciones oscilan en torno a los 350-450 mm de media anual. Su entorno ha sido repoblado con pino albar (*Pinus sylvestris* L.), pero en la ladera sur se conserva el ecosistema de bosque mixto de alcornoque (*Quercus suber* L.) y roble (*Q. pyrenaica* Willd.), con la presencia de monte bajo de jara (*Cistus ladanifer* L.) y numerosos ejemplares de encina (*Q. ilex* L.) y pino resinero (*P. pinaster* Aiton). Es un cerro de tierra roja arcillosa, que destaca del paisaje predominante de penillanura granítica. Los suelos son ácidos, de textura arenosa-franca y con mucha porosidad, ya que presentan un alto contenido de arena gruesa.

En Salamanca se encuentran las parcelas de **Garcirrey** y **Valdelosa**, ambas localizadas en los municipios con el mismo nombre. La primera se halla más hacia el centro de la provincia, mientras que la segunda está más al norte, cerca de la frontera con Zamora. El clima dominante es Mediterráneo frío, con una temperatura media anual que varía entre los 11 y los 13 °C. La temperatura media del mes

más frío está comprendida entre 0 y 6 °C, mientras que la temperatura media del mes más cálido está entre los 20 y los 24 °C. La precipitación anual se encuentra entre los 350 y los 450 mm. Las lluvias son particularmente escasas durante los meses de julio y agosto, en los que también son más altas las temperaturas, especialmente en el primero. La vegetación está constituida por monte y pastizal arbolado, con las características propias de la dehesa. Se trata de un ecosistema derivado de la actividad humana a partir del bosque de encinas (*Quercus ilex* L.) y robles (*Q*. *pyrenaica* Willd.). La orografía es la de una penillanura típica, con suaves ondulaciones. Los suelos son ácidos, de textura ligera franco-arenosa y presentan buena porosidad.

En la provincia de Cáceres tenemos cuatro parcelas, **Segura de Toro**, **Jaraíz de la Vera**, **Cañaveral** y **Casatejada**, ubicadas en las inmediaciones de las localidades de las que toman su nombre. El enclave de **Segura de Toro** se encuentra al norte de Cáceres, colindando con la provincia de Salamanca. Su clima varía entre el Mediterráneo templado y subtropical, con una temperatura media anual entre 10 y 16 °C, encontrándose la temperatura media del mes más frío entre 2 y 8 °C y la del mes más cálido entre 20 y 28 °C. La precipitación anual está en torno a 700-850 mm. La vegetación que encontramos es de encinas (*Quercus ilex* L.), entre las que se aprecian a menudo manchas o franjas de alcornoques (*Q. suber* L.), muchas veces formando superficies de pastizal con arbolado, y también varios núcleos de melojo (*Q*. *pyrenaica* Willd.), alternando a veces con castaños (*Castanea sativa* Mill.). De forma natural encontraríamos robledales y hayedos, con inclusión por invasión secundaria de pinares. Los suelos tienen textura ligera, con débiles estructuras migajosas o grumosas en superficie y subangulares en profundidad. Presentan buena porosidad, bajo contenido en agua útil, permeabilidad rápida y un contenido en materia orgánica bajo.



Las parcelas de **Jaraíz de la Vera**, **Cañaveral** y **Casatejada** presentan unas condiciones más similares. Su clima es Mediterráneo subtropical, con una temperatura media anual de entre 8 y 16 °C, oscilando la temperatura media del mes más frío entre 2 y 7 °C y la del mes más cálido de 20 a 26 °C. Las mayores diferencias entre las tres parcelas se dan a nivel de su precipitación anual, la cual se sitúa en torno a los 900-1000 mm en Jaraíz, pero alcanza valores inferiores en los otros dos enclaves (600-800 mm). La vegetación natural estaría integrada por bosques y bosquetes esclerófilos perennifolios, más o menos presididos por la encina (*Quercus ilex* L.). Sin embargo, se trata de una zona fuertemente intervenida, con zonas dedicadas a cultivos, frutales de regadío y muchas praderas naturales. De este modo encontramos una vegetación predominante tipo pastizal-matorral, con arbolado en algunos puntos de encina (*Q. ilex* L.), alcornoque (*Q. suber* L.) y melojo (*Q. pyrenaica* Willd.) y algunas masas de pino resinero (*Pinus pinaster* Aiton). El relieve es fundamentalmente llano, con pendientes inferiores al 5%. Dominan los suelos de textura ligera de tipo franco-arenosa y franco-arcillo-arenosa, con un contenido alto en materia orgánica, bastante ácidos y duros en seco.

A través de esta investigación pretendemos, como ya hemos comentado repetidamente, analizar los efectos de las diferencias en el rigor climático durante las estaciones más frías del año sobre la morfología, composición química y fisiología de las hojas de nuestras especies de estudio. Por lo tanto, además de analizar las características generales de cada una de las parcelas, resulta indispensable la **caracterización climática** de mismas, para comprobar que, efectivamente, incluimos un rango de temperaturas invernales lo suficientemente amplio como para poder satisfacer nuestros objetivos. Para ello, recurrimos a los datos de temperatura recogidos por los sensores colocados en cada enclave, a partir de los cuales obtuvimos la temperatura media mensual (media de las medias del conjunto de días de cada mes), temperatura media mínima y máxima mensual (media de las mínimas y máximas del conjunto de días de cada mes) y temperatura mínima y máxima absoluta (los valores extremos de temperatura registrados en cada mes) de cada uno de los meses a lo largo de los cuales se mantuvieron en el campo (octubre de 2008 hasta octubre de 2012). Para completar nuestras estimas del rigor climático en relación al frío, incluimos además el número total de días de heladas registrado en cada periodo mensual. Teniendo en cuenta el volumen de datos (11 parcelas, 48 meses, 6 estimas de temperatura), para facilitar la comparación entre parcelas hemos optado en el presente apartado por reflejar los datos promediados por estaciones, tal y como se muestran en las Tablas 2 a 7.

Analizando las parcelas en conjunto podemos distinguir, de forma más o menos clara, tres grupos en función de su rigor invernal. Las condiciones climáticas más duras, tanto en otoño como en invierno, las encontramos en las parcelas del nordeste, las de las provincias de Soria y Guadalajara, que son las que reciben menor influencia atlántica. En el extremo opuesto, las que presentan unas condiciones más suaves son las que se localizan más al suroeste, las pertenecientes a la provincia de Cáceres. Por último, en una posición

Tabla 2. Valor medio del número total de días de **helada** por año (S.E. entre paréntesis), y del periodo a lo largo del cual se extienden las heladas**,** junto con la fecha en la que se registran las últimas heladas, para los cuatro años de estudio en cada una de las parcelas experimentales.

	S Toro	Jaraíz	Cañaveral	Casatejada	T. Santo	Valdelosa	Garcirrey	Anquela	Abejar	Fuentepinilla	Muriel
Nº total días de helada/año	7 (1)	15 (2)	5 (2)	20 (3)	57 (3)	61 (9)	59 (9)	98 (9)	105 (12)	105 (2)	112 (8)
Duración media periodo de heladas (días)	24 (5)	68 (14)	55 (3)	90 (12)	127 (7)	154 (12)	100 (11)	193 (9)	222 (12)	202 (22)	214 (9)
Fecha media última helada	Febrero (1ª quincena)	Febrero (2ª quincena)	Enero (2ª quincena)	Marzo (1ª quincena)	Abril (1ª quincena)	Abril (2ª quincena)	Marzo (2ª quincena)	Mayo (1ª quincena)	Mayo (2º quincena)	Mayo (1ª quincena)	Mayo (2º quincena)

Tabla 3. Valor medio (promedio de los cuatro años de estudio, S.E. entre paréntesis) de la **temperatura media mínima mensual** (ºC) registrada en las distintas estaciones del año en cada una de las parcelas de estudio.

Estación	S. Toro	Jaraíz	Cañaveral	Casatejada	T. Santo	Valdelosa	Garcirrey	Anquela	Abejar	Fuentepinilla	Muriel
Otoño	9.54 (0.63)	7.57 (0.44)	8.97 (1.03)	6.47 (0.58)	3.76 (0.73)	4.05 (0.84)	3.79 (1.22)	1.68 (0.75)	1.55 (0.51)	0.95 (0.88)	0.95 (0.52)
Invierno	6.09 (0.44)	4.44 (0.39)	5.86 (0.52)	4.40 (0.51)	0.97 (0.27)	0.36 (0.35)	0.75 (0.13)	-1.24 (0.27)	-1.91 (0.49)	-1.59 (0.50)	-2.00 (0.60)
Primavera	13.6 (0.54)	12.4 (0.48)	13.3 (0.99)	11.8 (0.28)	8.25 (0.45)	7.92 (0.52)	8.87 (0.48)	5.79 (0.30)	5.50 (0.31)	7.21 (1.24)	5.50 (0.57)
Verano	20.1 (0.43)	18.3 (0.38)	18.9 (0.47)	16.4 (0.56)	12.6 (0.16)	12.7 (0.01)	13.9 (0.42)	11.8 (0.01)	10.5 (0.94)	9.11 (0.75)	9.61 (0.18)

Tabla 4. Temperatura mínima absoluta (ºC) registrada en cada una de las estaciones del año a lo largo del periodo completo de estudio en cada parcela experimental.

Estación	S. Toro	Jaraíz	Cañaveral	Casatejada	T. Santo	Valdelosa	Garcirrey	Anquela	Abejar	Fuentepinilla	Muriel
Otoño	-1.68	-3.43	-3.50	-4.76	-7.27	-8.58	-6.74	-11.3	-11.3	-13.6	-13.0
Invierno	-2.15	-4.15	-3.55	-5.50	-8.18	-9.82	-7.50	-13.1	-13.7	-15.0	-16.9
Primavera	3.26	3.68	3.47	1.33	-2.73	-3.15	-2.50	-3.55	-3.55	-5.87	-4.03
Verano	10.9	10.9	9.60	10.4	5.24	3.66	5.55	0.67	-0.21	-2.73	-1.57

Tabla 5. Valor medio (promedio de los cuatro años de estudio, S.E. entre paréntesis) de la **temperatura media máxima mensual** (ºC) registrada en las distintas estaciones del año en cada una de las parcelas de estudio.

Estación	S. Toro	Jaraíz	Cañaveral	Casatejada	T. Santo	Valdelosa	Garcirrey	Anquela	Abejar	Fuentepinilla	Muriel
Otoño	17.9 (0.40)	22.3 (0.55)	17.4 (1.48)	17.9 (0.40)	16.8 (1.47)	15.0 (1.17)	15.5 (1.32)	11.7 (0.94)	11.4 (1.13)	13.5 (1.67)	13.7 (0.93)
Invierno	15.7 (1.17)	19.3 (1.62)	14.4 (0.97)	15.7 (1.76)	14.0 (1.49)	11.4 (0.63)	12.5 (1.28)	9.21 (0.88)	9.25 (1.17)	11.7 (1.69)	11.0 (0.83)
Primavera	24.7 (0.72)	30.3 (0.46)	25.7 (1.43)	28.3 (2.71)	23.5 (0.59)	22.5 (0.82)	25.7 (1.04)	19.1 (0.58)	22.4 (1.27)	22.5 (1.08)	20.8 (0.83)
Verano	32.7 (0.42)	39.2 (0.56)	32.8 (0.79)	36.7 (2.76)	30.3 (0.07)	30.4 (0.02)	35.3 (0.07)	27.0 (0.18)	29.2 (0.77)	30.0 (0.69)	28.4 (0.20)

Tabla 6. Temperatura máxima absoluta (ºC) registrada en cada una de las estaciones del año a lo largo del periodo completo de estudio en cada parcela experimental.

Estación	S. Toro	Jaraíz	Cañaveral	Casatejada	T. Santo	Valdelosa	Garcirrey	Anquela	Abejar	Fuentepinilla	Muriel
Otoño	35.0	40.2	36.2	34.3	36.6	33.1	41.6	25.7	30.1	33.9	33.3
Invierno	33.7	35.0	32.5	32.2	30.0	24.8	36.0	22.5	28.1	29.9	27.1
Primavera	38.2	46.5	41.5	43.7	37.3	37.5	42.6	34.0	36.6	37.4	34.7
Verano	40.4	43.6	43.0	44.1	39.6	38.9	43.9	39.2	41.1	39.5	40.4

Tabla 7. Valor medio (promedio de los cuatro años de estudio, S.E. entre paréntesis) de la **temperatura media mensual** (ºC) registrada en las distintas estaciones del año en cada una de las parcelas de estudio.

Estación	S. Toro	Jaraíz	Cañaveral	Casatejada	T. Santo	Valdelosa	Garcirrey	Anquela	Abejar	Fuentepinilla	Muriel
Otoño	12.6 (0.65)	12.2 (0.48)	13.2 (1.22)	11.5 (0.54)	7.84 (0.96)	9.11 (1.01)	8.32 (0.39)	6.09 (0.74)	6.03 (0.59)	6.51 (0.84)	6.35 (0.61)
Invierno	9.55 (0.60)	9.07 (0.39)	9.81 (0.70)	9.20 (0.70)	5.55 (0.37)	5.30 (0.20)	5.86 (0.36)	3.04 (0.32)	3.14 (0.39)	3.99 (0.40)	3.53 (0.22)
Primavera	18.4 (0.61)	19.3 (0.32)	19.0 (1.23)	19.1 (1.05)	15.0 (0.49)	14.9 (0.59)	15.7 (0.54)	12.0 (0.36)	13.0 (0.64)	14.5 (1.05)	13.2 (0.61)
Verano	25.6 (0.38)	25.8 (0.40)	25.6 (0.64)	26.3 (1.13)	20.8 (0.05)	21.4 (0.17)	22.3 (0.27)	19.4 (0.08)	19.1 (0.18)	19.0 (0.48)	19.1 (0.16)

intermedia, con un clima suavizado por la influencia del Océano Atlántico, se encontrarían las parcelas distribuidas por la zona noroeste de nuestro estudio, las pertenecientes a las provincias de Zamora y Salamanca. Dentro de cada grupo se pueden apreciar ligeras variaciones en la ubicación de las parcelas a lo largo de un gradiente dependiendo de cuál sea la estima de rigor invernal empleada en cada caso. Sin embargo, los tres grupos quedan claramente definidos cuando todas las estimas disponibles (temperatura mínima media mensual, mínima absoluta y número y duración de las heladas) son consideradas conjuntamente.

En las parcelas más frías, el número total de días de heladas por año está en torno a 100 en todos los casos, distribuidos a lo largo de más de la mitad del año, apareciendo incluso en la segunda mitad del mes de mayo (Tabla 2), muy avanzada ya la primavera. En las parcelas consideradas como intermedias, la duración del periodo de heladas es menor que en el grupo anterior, extendiéndose, por lo general, sólo hasta las fases iniciales o mediados de la primavera. El número total de heladas en este caso se reduce a tan solo en torno a la mitad del total registrados en las parcelas más frías. Un número de heladas inferior a los 20 días, que se extienden a lo largo de tan sólo en torno a entre uno y tres meses, caracteriza a las parcelas más cálidas (Tabla 2).

Estas diferencias entre parcelas se confirman al observar las temperaturas mínimas, tanto las medias mensuales como las absolutas, con diferencias en algunos casos de hasta 10 °C entre las parcelas con inviernos más rigurosos y las de inviernos más suaves. Así, las parcelas definidas como más frías no superan los valores positivos de temperatura media mínima mensual en ningún caso durante el invierno (Tabla 3). Además, alcanzan valores mínimos absolutos que tampoco superan, en ningún caso, los -13 °C en la estación más fría y que incluso en otoño permanecen por debajo de -11 °C (Tabla 4). Las parcelas catalogadas como intermedias, las de Zamora y Salamanca, presentan valores de temperatura media mínima mensual positivos en invierno, aunque nunca superan 1 °C (Tabla 3). Sus valores mínimos absolutos, aunque muy bajos también, oscilan entre los -10 y los -7 °C a lo largo de todo el periodo frío (otoño e invierno) (Tabla 4). Las parcelas más cálidas presentan temperaturas medias mínimas que superan los 4 °C en invierno (Tabla 3). En cuanto a las mínimas absolutas, aunque negativas tanto en otoño como en invierno, se sitúan siempre entre -5 y -2 °C (Tabla 4).

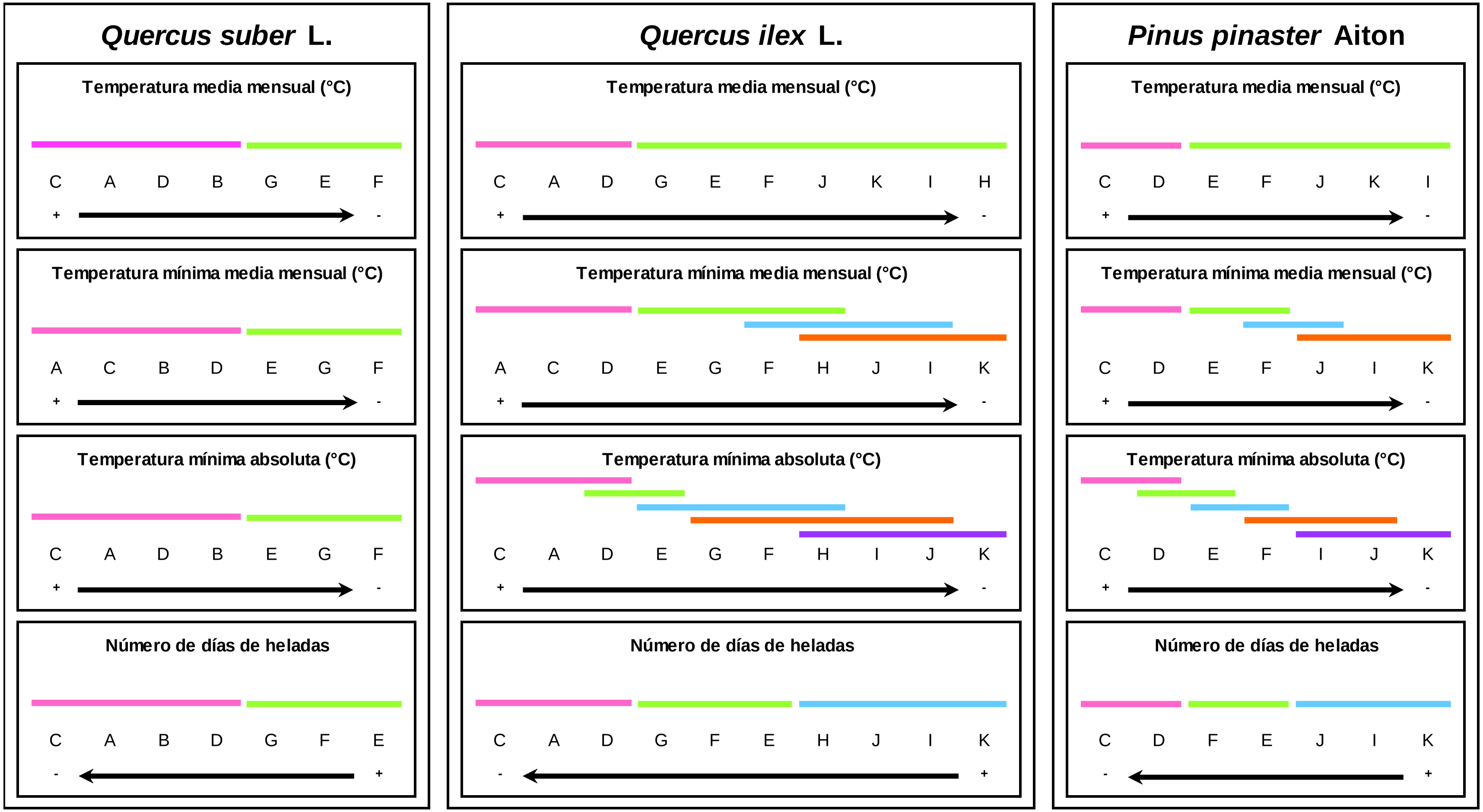
Las temperaturas máximas (Tablas 5 y 6) corroboran la clasificación que acabamos de exponer de nuestras parcelas, entre cuyos tres grupos (frías, intermedias y las más cálidas) se aprecian, sin embargo, diferencias menos acusadas que las que hemos visto para valores mínimos de temperatura. Estas tendencias a nivel de las temperaturas tanto máximas como mínimas se traducen lógicamente en un gradiente prácticamente similar a la hora de calificar las parcelas por su temperatura media mensual (Tabla 7). Abejar, Anquela, Fuentepinilla y Muriel oscilan entre los 3 y 7 ºC de media para el periodo de otoño e invierno, mientras que las parcelas más cálidas no bajan de 9 ºC de temperatura media en esta misma época del año en ningún caso, ascendiendo incluso hasta los 13 ºC. Finalmente, y como cabría esperar, las parcelas localizadas en la zona noroeste de nuestro estudio alcanzan unas temperaturas medias que se encuentran entre los dos grupos descritos, no superando en ningún caso los 10 °C, ni descendiendo por debajo de los 5 °C, durante el otoño y el invierno respectivamente.

Para comprobar la significación de estas diferencias y asegurarnos de que para cada especie hemos incluido un rango de temperaturas lo suficientemente amplio como para que pueda reflejarse en diferencias en los rasgos foliares entre parcelas, recurrimos a un análisis de la varianza utilizando los datos obtenidos mes a mes en los distintos años de estudio y para las diferentes estimas de temperatura. Puesto que nuestro interés se encuentra en los periodos más fríos del año, centramos nuestras comparaciones en las estaciones de otoño y fundamentalmente invierno. En este caso, sin embargo, las comparaciones se efectuaron separadamente para cada especie, puesto que, como ya hemos comentado, los patrones de distribución no son los mismos en todos los casos y ninguna de las tres especies se encuentra representada en la totalidad de las parcelas. Así, por ejemplo, *Q. suber* es una especie que, por su carácter más termófilo, se distribuye a lo largo de un rango de temperaturas más reducido, no encontrándose en las provincias más frías de Soria y Guadalajara. De este modo, parcelas que desde el punto de vista del rigor invernal podrían ser consideradas intermedias en el caso de la encina (como es el caso de Valdelosa, Teso Santo o Garcirrey), pasarían a ocupar la categoría de frías en el caso del alcornoque.

Para ninguna de las tres especies se obtuvieron diferencias significativas en las temperaturas máximas (ni absolutas, ni máximas medias mensuales) entre las parcelas en las que aparecían representadas, ni en otoño, ni tampoco en invierno (datos no mostrados). Para el resto de las estimas de temperatura, las diferencias eran siempre más reducidas, o incluso desaparecían, en otoño en comparación al invierno. Así, por ejemplo, entre los enclaves en los que aparece representado el alcornoque, únicamente se obtenían diferencias significativas en otoño a nivel del número de días de heladas (datos no mostrados), el mismo parámetro que, junto a las temperaturas mínimas absolutas, reflejaba diferencia entre las parcelas seleccionadas para *P. pinaster* en esta estación del año (datos no mostrados). En la encina las diferencias en otoño se extendían también a las temperaturas mínimas medias mensuales (datos no mostrados), aunque eran menos acentuadas que en invierno. En esta última estación, sin embargo, los ANOVAs revelaban en todos los casos diferencias significativas entre parcelas para todas las estimas de temperatura indicativas del rigor invernal (Figura 3). Estos resultados ponen de manifiesto, que, efectivamente, es la intensidad del rigor invernal el principal factor ambiental en función del cual se diferencian nuestras parcelas y que, por tanto, su selección se adecua bien a los objetivos de nuestro estudio.

En resumen, por tanto, en el caso del alcornoque, podrían diferenciarse dos grupos de parcelas, el de las más frías, compuesto por Teso Santo, Garcirrey y Valdelosa, siendo esta última la más extrema, y el de las más cálidas, constituido por el resto. Entre las parcelas ocupadas por la encina, al ser más numerosas, se aprecia una gradación más marcada.

Figura 3. Representación gráfica de los resultados de los ANOVAs efectuados para comprobar diferencias entre parcelas (con respecto a los datos de temperatura de invierno) en las tres especies estudiadas.



Abejar, Anquela, Fuentepinilla y Muriel integran el grupo de las parcelas con un invierno más riguroso, significativamente diferente al resto, tanto en número de días de helada como en temperatura mínima media mensual y absoluta. Por su parte, Teso Santo, Garcirrey y Valdelosa ocuparían posiciones intermedias, mientras que Segura de Toro, Cañaveral y Casatejada serían las más cálidas, pero con diferencias menos marcadas. En el caso del pino, Muriel, Fuentepinilla y Abejar conforman el grupo de las parcelas más frías, sin diferencias significativas entre ellas. Teso Santo y Valdelosa componen el grupo intermedio. Por último, Cañaveral y Casatejada formarían el grupo de parcelas más cálidas.

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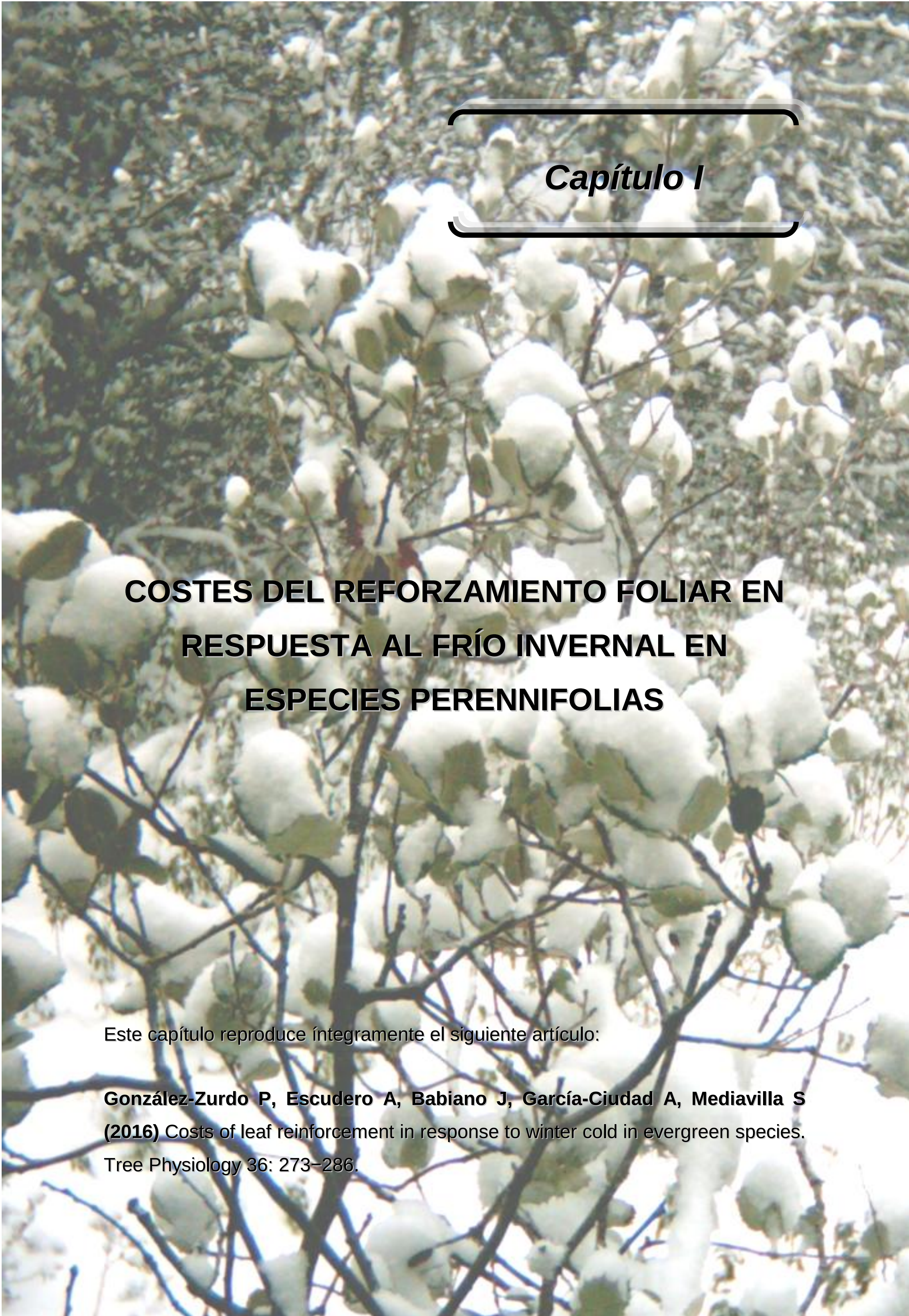

Capítulo I

COSTES DEL REFORZAMIENTO FOLIAR EN RESPUESTA AL FRÍO INVERNAL EN ESPECIES PERENNIFOLIAS

Este capítulo reproduce íntegramente el siguiente artículo:

González-Zurdo P, Escudero A, Babiano J, García-Ciudad A, Mediavilla S (2016) Costs of leaf reinforcement in response to winter cold in evergreen species. Tree Physiology 36: 273−286.

RESUMEN

El equilibrio competitivo entre las especies caducifolias y perennifolias depende en gran medida de la intensidad de la reducción en la ganancia de carbono sufrida por las hojas perennes, asociada a los rasgos foliares que confieren resistencia frente a las condiciones de estrés durante el periodo desfavorable del año. Este estudio examina los efectos del rigor invernal sobre los rasgos de resistencia de las hojas perennes. Para ello, se determinó el peso por unidad de área foliar (LMA), el espesor de las hojas y las concentraciones de fibra, nitrógeno (N), fósforo (P), proteínas solubles, clorofila y ribulosa 1,5-bisfosfato carboxilasa/oxigenasa (Rubisco) en tres especies perennifolias y en dos especies caducifolias distribuidas a lo largo de un gradiente de temperatura invernal. En las especies perennifolias, LMA, el espesor y el P y las concentraciones de carbohidratos estructurales aumentaban con la disminución de las temperaturas invernales. Las concentraciones de nitrógeno y de lignina no mostraban patrones definidos en este sentido. La clorofila, las proteínas solubles y Rubisco disminuían con el incremento del rigor invernal. Nuestros resultados sugieren que un incremento en LMA y en la concentración de carbohidratos estructurales sería un requisito para que las hojas pudieran hacer frente a las bajas temperaturas invernales. El hábito perennifolio estaría asociado, por tanto, con mayores costes en los lugares más fríos, ya que los rasgos de resistencia al frío implican costes adicionales de mantenimiento y una reducción de la asignación de N a la maquinaria fotosintética, asociados con el reforzamiento estructural en los lugares más fríos.

Palabras clave: contenido foliar de fibras, peso por unidad de superficie foliar, espesor foliar, contenido de nutrientes, Rubisco, gradiente de temperatura invernal.

COSTS OF LEAF REINFORCEMENT IN RESPONSE TO WINTER COLD IN EVERGREEN SPECIES

ABSTRACT

The competitive equilibrium between deciduous and evergreen plant species to a large extent depends on the intensity of the reduction in carbon gain undergone by evergreen leaves, associated with the leaf traits that confer resistance to stressful conditions during the unfavourable part of the year. This study explores the effects of winter harshness on the resistance traits of evergreen leaves. Leaf mass per unit area (LMA), leaf thickness and the concentrations of fibre, nitrogen (N), phosphorus (P), soluble protein, chlorophyll and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) were determined in three evergreen and two deciduous species along a winter temperature gradient. In the evergreen species, LMA, thickness, and P and structural carbohydrate concentrations increased with the decrease in winter temperatures. Nitrogen and lignin concentrations did not show definite patterns in this regard. Chlorophyll, soluble proteins and Rubisco decreased with the increase in winter harshness. Our results suggest that an increase in LMA and in the concentration of structural carbohydrates would be a requirement for the leaves to cope with low winter temperatures. The evergreen habit would be associated with higher costs at cooler sites, because the cold resistance traits imply additional maintenance costs and reduced N allocation to the photosynthetic machinery, associated with structural reinforcement at colder sites.

Key-words: leaf fibre content, leaf mass per unit area, leaf thickness, nutrient content, Rubisco, winter temperature gradient.

INTRODUCTION

For many years, the study of leaf traits has been an important focus of research in ecology because leaves govern the patterns of gas exchange and many other processes that affect the properties of ecosystems. The fact that leaves are plastic organs with marked variability in their responses to environmental conditions has led the study of leaf traits to acquire great relevance in the investigation of climate change (Taub 2010, Guerin et al. 2012). In many tree species, significant correlations have been observed between intraspecific variations in leaf traits and different environmental factors (Valladares et al. 2002, Klein et al. 2013). Accordingly, study of phenotypic variation among populations within a given species has become a useful tool for predicting variations in the distribution and composition of forest species in a changing climatic scenario (Nicotra et al. 2010, Matesanz and Valladares 2014). However, even for the most frequently studied species, the number of observations available in the existing databases is limited, especially with respect to the coverage of the geographic range of a species (Niinemets 2015). Thus, more data are needed to gain insight into within-species variation in leaf traits.

Among the different leaf traits, leaf mass per unit area (LMA) has attracted the most attention, mainly because changes in LMA are accompanied by changes in other characteristics, such as leaf lifespan and fibre and nutrient contents (Wright et al. 2004), which leads to important trade-offs between productivity and persistence (Reich 2014). It has been reported that LMA varies widely at the single-species level in response to differences in the harshness of the habitat (He et al. 2006, Messier et al. 2010). Traditionally, these changes in LMA have been interpreted mainly as responses to changes in water or nutrient stress (Niinemets 2001, Wright et al. 2004, Poorter et al. 2009). Less attention has been focused on changes in LMA in response to low winter temperatures (Ogaya and Peñuelas 2007, Mediavilla et al. 2012). However, if lengthening leaf lifespan demands a structural reinforcement that will allow leaves to overcome climate harshness (Kikuzawa et al. 2013), we also could expect that the harsher the climate conditions in winter, the greater the demand for structural reinforcement in the leaves that must survive during winter. This means that keeping leaves alive during winter (the evergreen habit) should be associated with larger investments in structural tissues at colder sites. Accordingly, low winter temperatures may also be involved as an additional factor in the trade-offs between productivity and persistence.

The competitive equilibrium between deciduous and evergreen tree species depends strongly on leaf productivity along the different seasons of the year and on the morphological and chemical adaptations necessary for leaf survival during the different seasons. Prolonging leaf lifespan over more than one growth season is only advantageous if the lengthening of productive life compensates for both additional maintenance costs and reduced carbon gain, which are associated with tolerance to unfavourable circumstances (van Ommen Kloeke et al. 2012). A greater LMA requires more plant material to achieve a given leaf area for light interception and, hence, implies higher construction costs per unit leaf area. However, the costs associated with a greater LMA derive mainly from its negative relationship with instantaneous carbon assimilation (Reich et al. 1997, Niinemets and Sack 2006). The greater allocation of biomass and nitrogen (N) to structural components versus photosynthetic components has been proposed to be one of the factors responsible for the lower carbon assimilation rate in leaves with a larger LMA (Vitousek et al. 1990, Niinemets 1999). At the interspecific level, some authors have reported a reduction in the proportion of N allocated to ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) with an increase in LMA, suggesting that this would reflect the need for greater N investment in structural components (Ellsworth et al. 2004, Onoda et al. 2004, Takashima et al. 2004). For example, it is known that cell walls accumulate a significant amount of N compounds: up to 10% of the cell content (Reiter 1998). A greater LMA in the leaves produced at colder sites would be explained by their greater thickness (Mediavilla et al. 2012), which would be achieved via a thickening of the cell walls, a characteristic of leaves growing in cold climates (Kubacka-Zębalska and Kacperska 1999, Stefanowska et al. 1999). Accordingly, it can be expected that a greater amount of available N would be allocated to cell walls in environments with harsher winters, leading to a reduction in the amount available for allocation to chlorophyll

(CF) or photosynthetic proteins (PTs), which would negatively affect CO_2 assimilation rates. In addition, an increased thickness of cell walls contributes to increasing the photosynthetic limitations due to internal diffusion (Niinemets et al. 2011), which should exacerbate the disadvantages of the structural reinforcement under unfavourable climatic conditions.

In the present work, we analyse the effects of the differences in climate harshness during winter on the morphology (LMA and thickness) and chemical composition (content of N, P, fibres, CF, soluble PTs and Rubisco) of the leaves of three evergreen (*Quercus ilex* ssp*. ballota* (Desf.) Samp, *Quercus suber* L. and *Pinus pinaster* Aiton) and two deciduous (*Quercus pyrenaica* Willd. and *Quercus faginea* Lam.) species. These species are widely distributed across the Iberian Peninsula. Although some authors have addressed possible changes in LMA in response to decreases in temperature along altitudinal gradients, their results are so disparate that it is not possible to draw reliable conclusions. Thus, whereas an increase in LMA with altitude has been observed in several species (Vitousek et al. 1990, Bresson et al. 2011, Körner 2012), it has also been found that it remains constant (Birmann and Körner 2009, Vitasse et al. 2014) or even decreases in some cases (Schoettle and Rochelle 2000, Wright et al. 2004). A similar situation is seen in the case of nutrients. The levels of N and phosphorus (P) in plant tissues have been positively correlated with altitude and hence with decreases in temperature in several works (Weih and Karlsson 2001, Reich and Oleksyn 2004, Jian et al. 2009). In contrast, other authors have found a marked decrease in the N content per unit leaf area (Zhang et al. 2005, Li et al. 2006) or no trends with altitude (Hultine and Marshall 2000, Premoli and Brewer 2007) or with temperature (Niinemets 2015). With respect to the remaining leaf components included in the present study, as far as we are aware, no other authors have analysed the changes at the single-species level in response to gradients of winter harshness. Differences in the contents of CF, PTs and Rubisco in response to differences in light intensity (Miyazawa et al. 2004), drought stress (Haldimann et al. 2008), CO_2 concentrations (Blaschke et al. 2001) and growth temperatures (Campbell et al. 2007) have been analysed, but only based on short-term responses in controlled environments. Possible differences in the photosynthetic machinery associated with a greater or lesser structural reinforcement of the leaves in different environments have not been addressed. Our general aim here is to check whether winter climate harshness (in particular, the intensity and frequency of frosts) contributes to intensifying the leaf traits that confer persistence, and hence to reducing the amount of N associated with photosynthetic components and photosynthetic capacity. We surmise that in temperate climates, the evergreen habit would involve costs of adaptation to freezing that are largely unknown (van Ommen Kloeke et al. 2012) and that must logically be stronger in colder environments. Our hypothesis is that the most important cost would be an unfavourable distribution of N, associated with a greater concentration of fibre in cold climates. Thus, our aim is to clarify the implications of cold for leaf traits and productivity with a view to unravelling the possible repercussions of climate change on the distribution of woody species.

MATERIALS AND METHODS

Study species and area

The set of species studied included three evergreens with leaf lifespans of more than 1 year, and accordingly with leaves that survive during at least one winter (*P. pinaster* Aiton, *Q. suber* L. and *Q. ilex* ssp*. ballota* (Desf.) Samp), and two deciduous species (*Q. pyrenaica* Willd. and *Q. faginea* Lam.) with leaf lifespans of about 5-7 months (data taken from Mediavilla and Escudero 2003).

The species were distributed on 11 sites located in the regions of Castilla-Leon and Extremadura (central-western Spain) between latitudes 41º 45' N and 40º 01' N and between longitudes 6º 22' W and 2º 08' W (Figure 1). Owing to differences in altitude and to the effects of continentality, there were strong between-sites differences in temperature that were especially pronounced for the minimum winter temperatures and the number of frosts

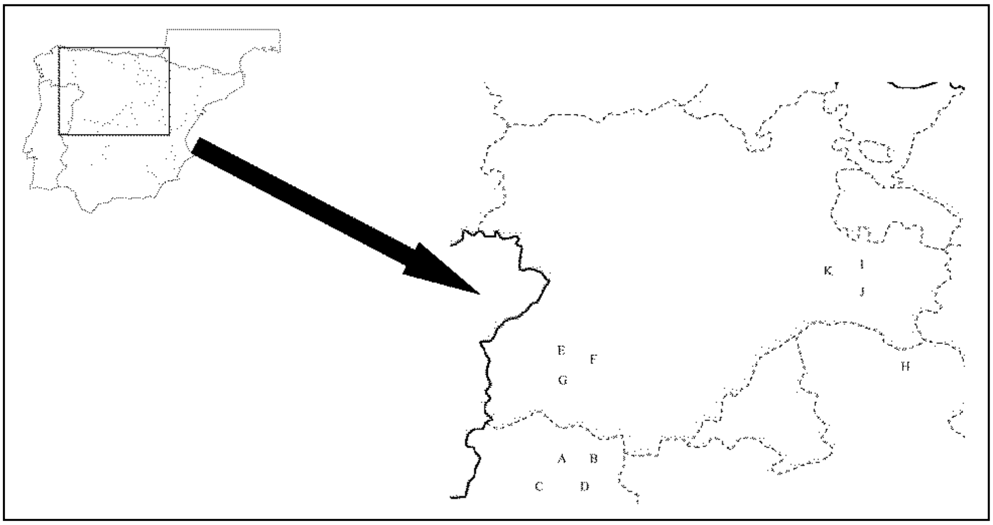

Figure 1. Distribution of the sample locations.

per year (Table 1). In contrast, the differences in summer temperatures were less intense. Accordingly, the annual temperature range was higher in colder sites, especially because of the effects of continentality (Ninyerola et al. 2000). The sites consisted of flat areas with sparse populations (between 50 and 100 specimens ha^{-1}) of mature (> 100 years old) individuals. Trunk diameter at 1.3 m height ranged from 20 to 60 cm and mean heights were 4–10 m. Each site was selected so as to include as many study species as possible and to cover a wide gradient in winter temperatures, although taking care that the rest of climate characteristics would be as homogeneous as possible. Nevertheless, there is a tendency for rainfall levels to be higher in the hottest and southernmost sites, which helps to reduce the differences in the intensity of drought stress between cold and hot sites (Table 1). Owing to its lower tolerance to low temperatures, *Q. suber* was absent at the coldest sites. Rainfall and solar irradiance data were obtained from the digital climatic atlas of the Iberian Peninsula (Ninyerola et al. 2005): a set of digital climatic maps of mean air temperature, precipitation and solar radiation elaborated with 200 m resolution using data from climate stations and a combination of geographical variables (altitude, latitude, continentality, solar radiation and terrain curvature). Temperature data were obtained for each site by means of data loggers (Hobo Pendant temperature/light datalogger, Part UA-002-08, Onset Computer

Table 1. Site characteristics. Pp, *Pinus pinaster*; Qi, *Quercus ilex;* Qs, *Quercus suber;* Qp, *Quercus pyrenaica*; Qf, *Quercus faginea*.

	Stands										
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**
Longitude (W)	5º 57´	5º 48´	6º 22'	5º 02´	5º 52´	5º 47´	6º 07´	2º 08´	2º 37´	2º 43´	2º 52´
Latitude (N)	40º 13´	40º 01´	39º 49'	40º11´	41º 14´	41º 08´	40º 55´	40º 59´	41º 45´	41º 32´	41º 43´
Altitude (m above sea level)	619	466	600	449	985	834	832	1246	1189	982	1045
Mean temperature (ºC)											
Annual	16.5	16.6	16.8	16.4	12.3	12.7	13.0	10.1	10.3	11.0	10.6
Absolute maximum temperature	40.4	43.6	43	44.1	39.6	38.9	43.9	39.2	41.1	39.5	40.4
Absolute minimum temperature	-2.15	-4.15	-3.55	-5.50	-8.18	-9.82	-7.50	-13.1	-13.7	-15.0	-16.9
Days with frost per year	7	15	5	20	57	61	59	98	105	105	112
Annual rainfall (mm)	1109	986	672	1001	495	460	554	605	567	535	646
Emberger's index	72	105	60	60	52	46	48	58	79	69	78
Daily mean radiation (W m^{-2})	184	185	184	186	186	186	183	182	177	179	177
Soil											
Sand content (%)	81.1	77.2	73.9	75.7	84.6	74.3	67.9	57.8	75.1	84.8	84.0
Clay content (%)	8.5	11.7	12.3	10.6	6.4	12.2	16.2	22.8	14.4	8.1	8.3
Silt content (%)	10.4	11.1	13.8	13.7	9.00	13.5	15.9	19.4	10.5	7.10	7.7
Soil N content (%)	0.082	0.143	0.104	0.126	0.072	0.021	0.078	0.112	0.115	0.040	0.048
Available P (p.p.m.)	31	38	5	6	10	4	20	2	7	9	6
Organic matter (%)	1.66	4.47	4.10	2.78	2.6	0.37	1.66	1.96	5.12	0.69	1.92
pH	5.7	4.5	4.7	5.1	4.6	4.5	4.4	7.6	4.8	7.0	6.1
Species	Qi, Qs, Qp, Qf	Qs, Qp	Pp, Qi, Qs	Pp, Qi, Qs	Pp, Qi, Qs, Qp	Pp, Qi, Qs, Qp, Qf	Qi, Qs, Qp, Qf	Qi, Qf	Pp, Qi, Qp, Qf	Pp, Qi, Qp	Pp, Qi

Corporation, Pocasset, MA, USA). The data loggers were programmed to obtain temperature data every 10 min and they were kept at each site for 4 years (October 2008 – October 2012).

We used the Emberger's pluviothermic index (Emberger 1930) to analyse the effects of water stress on leaf traits:

$$Q = \frac{1000 \times P}{(273 + \frac{T_X + T_N}{2}) \times (T_X - T_N)}$$

where P is the annual precipitation (mm), T_X is the average temperature of the hottest month (ºC) and T_N is the average temperature of the coldest month (°C). This index is commonly used in Mediterranean climates (Kunstler et al. 2007).

Soil samples were taken up to a depth of 20 cm (excluding the forest floor) from each stand. Determinations of soil granulometry, pH and N and P concentrations were carried out at the Soils Laboratory of the Institute of Natural Resources and Agrobiology in Salamanca according to the methods described by Chapmann and Pratt (1973) (Olsen analysis for available P) and Walkley and Black (1934).

Measurements of leaf morphology and chemistry

At each site, four or five mature specimens of each species were selected randomly during each sampling session. A composite sampling of sun-exposed branches with leaves from different crown positions in each canopy was undertaken for each individual selected. Samples were collected during three different periods of the year (autumn, winter and end of spring-beginning of summer) from October 2008 to October 2012, providing 4-year data for each sampling date. Some additional samplings during the same years were made to obtain estimates of leaf lifespan.

The samples were taken immediately to the laboratory and the branches were separated into annual segments (shoots) of different age classes. Only one flush of leaf growth was observed in all species. Accordingly, all the leaves sprouting in a given year were considered to belong to the same age class. All shoots bearing leaves of a given age were identified as belonging to the same age class. In the evergreen species, the number of leaves or needles per shoot was counted for each age class and the data were used to construct static life tables, which made it possible to estimate the mean leaf lifespan for each species according to standard methods.

For morphological analyses, 25 individual leaf samples for each species and leaf age class were finally selected at each site and sampling date. Leaf thickness was measured with a digital micrometer (Digimatic Micrometer, Mitutoyo, Japan) as a mean of three measurements taken at random positions on each leaf or needle, avoiding the main ribs on flat leaves. The total projected leaf and needle areas were determined by an image analysis system (Delta-T Devices Ltd, Cambridge, UK). In the case of *Pinus*, we also measured needle length with the digital micrometer. The leaf volume of flat leaves was calculated as the product of mean leaf thickness x leaf area. The transverse cross-sectional area of needles was measured with amplified scanning images, and needle volume was estimated as cross-sectional area x needle length. The samples were then oven-dried at 70ºC to constant weight and the total dry mass was determined. From the data thus obtained, we calculated the leaf dry mass per area (LMA) and leaf tissue density (dry mass/volume). Once all the data had been collected, a value for each species and age class at each site and sampling date was calculated as the average of 25 leaves taken in each case.

Once dried, the 25 individual leaves taken from each species and leaf age class selected at each site and sampling date were ground together to obtain a sufficient amount of sample for the chemical analyses. Leaf N concentrations were determined with a CE-Instruments NA-2100 autoanalyser (ThermoQuest, Milan, Italy). Phosphorus concentrations were measured colorimetrically as molybdo-vanado-phosphoric acid (Duque-Macías 1970).

After the N and P analyses, the remaining material was used to analyse the fibre content (hemicellulose, cellulose and lignin) with an Ankom Analyser (A220, ANKOM, Macedon, NY, USA), following the method of Goering and Van Soest (1970). The nutrient and fibre contents of leaves were expressed per unit dry mass (as milligrams of nutrient or fibre per gram of leaf dry mass), and nutrients also as per unit of leaf area, obtained as the nutrient concentration per unit dry mass multiplied by LMA.

Owing to the high cost in time and money involved in determining the Rubisco content, in this case, and for the CF and soluble PT content, we limited our analyses to current-year leaves of the two oak species (*Q. ilex* and *Q. suber*) and six sites (the two warmest, two intermediate and the two coldest in order to obtain replications of each of the temperature ranges). The leaf samples were taken from five mature specimens of each species at each site during winter and at the end of spring-beginning of summer of 2012. At the laboratory, the plant material was weighed and immediately plunged into liquid N and kept at -80ºC until analysis. For PT extraction and CF and PT determinations, we used the method of Agrisera (Sweden). Chlorophyll was measured according to Whatley and Arnon (1963) and total soluble PT was measured according to Bradford (1976). The dry mass and LMA of the leaves used for the analyses were also determined, and the CF and PT contents of leaves were expressed per unit dry mass and per unit leaf area. For western blotting and Rubisco analysis, we used the method of Agrisera, with minor modifications (see Vicente et al. 2011). The relative amount of the Rubisco large subunit was calculated by densitometric scanning of polyvinylidene difluoride membranes by image analysis using the Scion ImagePC software (Scion, MD, USA) and expressed in arbitrary units.

Data analysis

The relationships between leaf traits and the different temperature measurements were described by means of linear regression analysis. To better explore site effects on leaf traits, for each site, we obtained a single value for the absolute annual maximum and minimum temperatures, the number of days with frost per year and the total annual rainfall.

The effects of climatic variables on leaf traits were initially explored by simple regression analyses. However, several environmental variables were correlated with each other. Accordingly, the data were also explored using multiple regression models with stepwise selection of variables based on Akaike's information criterion to determine the best model for leaf traits with environmental variables.

Temperature is known to exert a marked effect on the time of leaf emergence. Accordingly, the leaf traits data corresponding to the spring period for the current-year leaf cohort were excluded in order to avoid possible differences among the sites due to the different state of development of the recently emerged leaves. We did not include the leaves of the second age class of *Q. suber* either, since its maximum leaf lifespan is slightly longer than 1 year and we did not find old leaves in sufficient numbers to be able to perform the different chemical analyses. The test for significantly different slopes in analysis of covariance (ANCOVA) was used to determine whether the slopes of the change in each leaf trait with temperature change were significantly different for the different species. Between-sites differences in CF, soluble PT and Rubisco concentrations were explored using one-way analysis of variance. We performed the statistical tests using R ver. 3.0.3 software (R Development Core Team 2007).

RESULTS

Within-species variability of leaf morphology related to temperature gradients

Leaves of evergreen species showed a definite trend to decreasing LMA and thickness with the increase in mean annual temperatures across the different sites (Figure 2). This trend was not apparent in the deciduous species, which also exhibited smaller between-sites differences in LMA and leaf thickness (Figure 2, upper panels). Both leaf traits presented

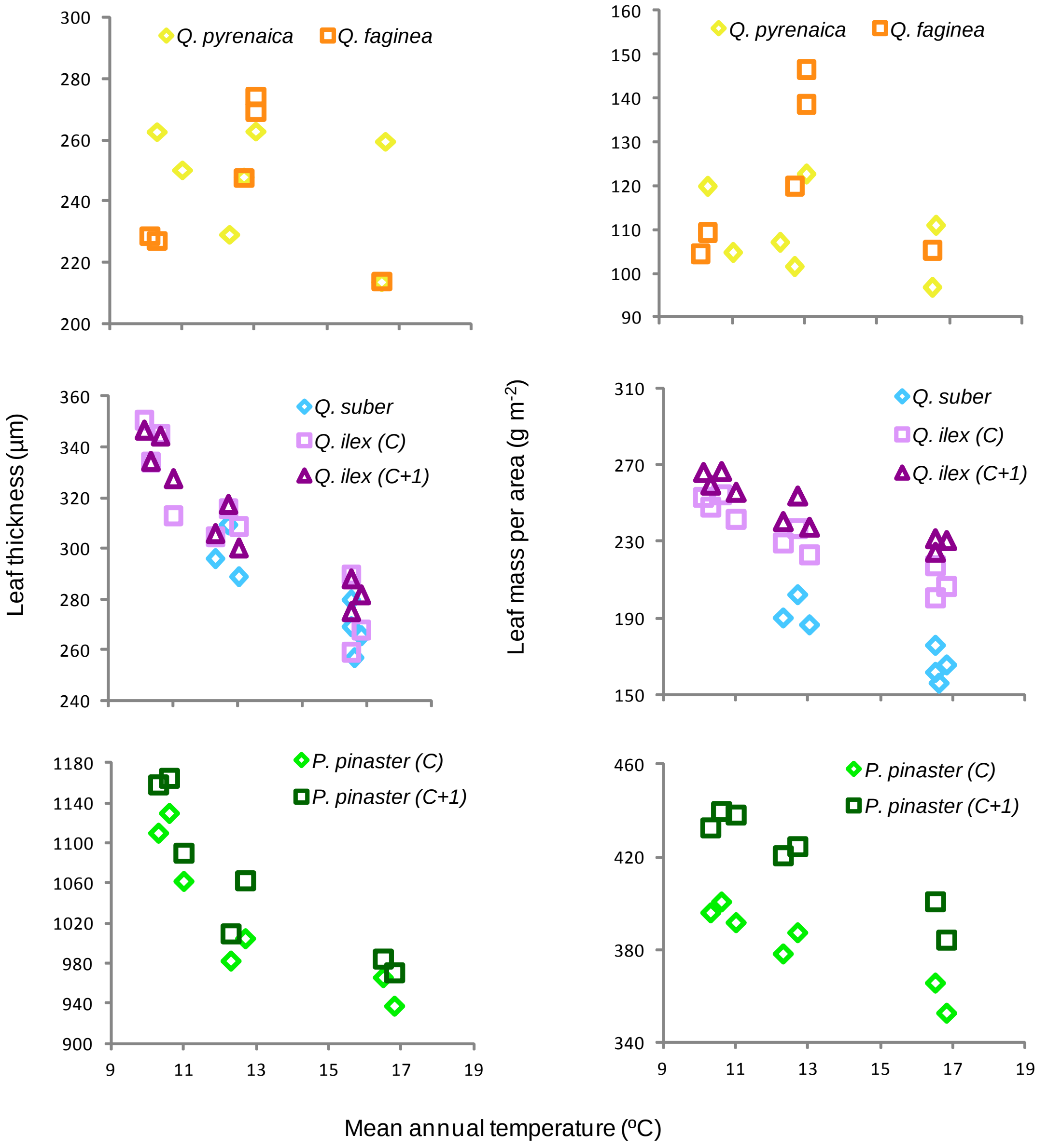

Figure 2. Leaf thickness and LMA as a function of mean annual temperature at the different sites. C, current-year leaves; C + 1, 1-year-old leaves.

higher values in the species with longer leaf lifespan, and the interspecific differences tended to be stronger at colder sites. Among the evergreens, leaf lifespan was independent from mean annual temperature for *Q. suber* and *Q. ilex* (Figure 3). However, *P. pinaster* needles tended to maintain longer duration in the coldest sites.

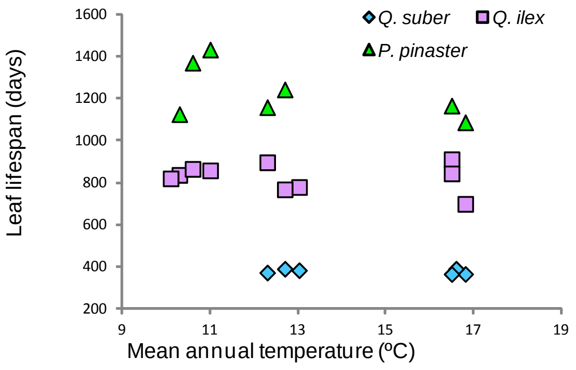

Figure 3. Leaf lifespan of evergreen species as a function of mean annual temperature at the different sites.

Leaf responses of the evergreen species to temperature gradients were more pronounced when calculated only for winter conditions. In all leaf age classes, LMA and leaf thickness showed a pronounced response to the harshening of winter climatic variables, since the minimum temperatures and the number of days with frost were the two variables that best accounted for the highest percentage of variation observed among sites in thickness and LMA (Table 2). In contrast, in many cases, the relationships with the maximum temperatures disappeared or, if present, always reached a much lower significance level. Leaf tissue density was the only trait that did not show any definite trend along the thermal gradient for any type of leaf (data not shown). Therefore, the increase in LMA as the harshening of winter conditions progresses seems to occur only through leaf thickening, with no associated changes in density. Leaf lifespan of *P. pinaster* was marginally (P=0.054) significantly correlated with absolute minimum temperatures. The two deciduous species showed no responses to any of the environmental factors studied. In some cases, the differences in leaf traits of evergreen species were also correlated with other climatic and soil variables, although always with relatively low percentages of variance explained. Since the warmest sites tended to have higher rainfall and solar radiation levels (Table 1), we also explored the combined effects of minimum winter temperatures and other factors by means of multiple regression analysis. In all cases, the minimum annual temperature was selected as the primary predictor for LMA and leaf thickness at each site. In a few cases, the best models included other independent variables, but always with lower significance levels than minimum temperatures (Table 3).

Table 2. Linear regression parameters for different morphological leaf traits (LT, leaf thickness; LMA, leaf mass per unit area) depending on different climatic and soil variables (C, current-year leaves; C + 1, 1-year-old leaves; C + 2, 2-year-old leaves). Only significant ($P < 0.05$) relationships are shown. *R* , total annual rainfall; *I* , Emberger's pluviothermic index; SR, solar global radiation; min *T* , minimum winter temperature; max *T* maximum summer temperature; *F* , number of days with frost per year.

Independent variables	R^2	*F*	Intercept	Slope	*P*
LT (µm)					
Quercus suber C (*n* = 49)					
R (mm)	0.49	25.3	319	-0.051	<0.0001
I	0.35	13.8	330	-0.601	<0.0001
SR (W m^{-2})	0.15	4.7	-702	5.32	0.0390
F	0.69	59.0	261	0.636	<0.0001
Min *T* (ºC)	0.73	71.4	245	-6.08	<0.0001
Max *T* (ºC)	0.31	11.8	489	-4.96	0.0020
Soil N (%)	0.63	44.4	316	-390	<0.0001
Soil P (ppm)	0.32	12.1	294	-0.808	0.0018
Q. ilex C (*n* = 75)					
R (mm)	0.27	13.7	366	-0.086	<0.0001
SR (W m^{-2})	0.25	12.6	1165	-4.70	0.0011
F	0.64	66.8	266	0.679	<0.0001
Min *T* (ºC)	0.62	59.7	257	-5.47	<0.0001
Max *T* (ºC)	0.12	5.1	571	-6.40	0.0295
Soil P (ppm)	0.25	12.3	329	-1.95	0.0012
Q. ilex C+1 (*n* = 107)					
R (mm)	0.32	26.1	361	-0.073	<0.0001
SR (W m^{-2})	0.42	39.8	1176	-4.74	<0.0001
F	0.83	266	275	0.599	<0.0001
Min *T* (ºC)	0.83	265	265	-4.94	<0.0001
Max *T* (ºC)	0.27	20.8	616	-7.40	<0.0001
Soil P (ppm)	0.30	23.9	330	-1.68	<0.0001
Pinus pinaster C (*n* = 56)					
I	0.16	5.0	808	2.74	0.0341
SR (W m^{-2})	0.39	16.5	3655	-14.4	<0.0001
F	0.45	20.9	919	1.63	<0.0001
Min *T* (ºC)	0.45	21.6	881	-14.1	<0.0001
P. pinaster C+1 (*n* = 78)					
R (mm)	0.10	4.2	1172	-0.172	0.0481
I	0.14	6.2	868	2.44	0.0168
SR (W m^{-2})	0.45	31.9	3791	-15.0	<0.0001
F	0.55	47.1	950	1.72	<0.0001
Min *T* (ºC)	0.55	46.9	910	-14.8	<0.0001
Max *T* (ºC)	0.16	7.2	1905	-20.5	0.0106
P. pinaster C+2 (*n* = 78)					
SR (W m^{-2})	0.26	13.0	3512	-13.3	<0.0001
F	0.36	21.4	974	1.70	<0.0001
Min *T* (ºC)	0.36	21.1	936	-14.5	<0.0001
Max *T* (ºC)	0.13	5.8	2013	-22.6	0.0213

Independent variables	R^2	*F*	Intercept	Slope	*P*
LMA (g m^{-2})					
Quercus suber C (*n* = 49)					
R (mm)	0.51	27.0	215	-0.051	<0.0001
I	0.39	16.5	228	-0.623	<0.0001
F	0.64	45.9	158	0.597	<0.0001
Min *T* (ºC)	0.69	57.9	143	-5.78	<0.0001
Max *T* (ºC)	0.21	6.7	342	-3.93	0.0155
Soil N (%)	0.51	26.8	207	-343	<0.0001
Soil P (ppm)	0.32	12.3	190	-0.797	0.0017
Q. ilex C (*n* = 75)					
R (mm)	0.31	16.7	268	-0.056	<0.0001
SR (W m^{-2})	0.33	18.0	816	-3.21	<0.0001
F	0.75	114	203	0.443	<0.0001
Min *T* (ºC)	0.76	115	196	-3.65	<0.0001
Max *T* (ºC)	0.21	9.8	438	-5.05	0.0034
Soil P (ppm)	0.31	16.5	245	-1.31	<0.0001
Q. ilex C+1 (*n* = 107)					
R (mm)	0.25	19.1	276	-0.045	<0.0001
SR (W m^{-2})	0.30	24.6	755	-2.79	<0.0001
F	0.60	85.3	224	0.352	<0.0001
Min *T* (ºC)	0.62	92.1	218	-2.95	<0.0001
Max *T* (ºC)	0.23	16.3	437	-4.65	<0.0001
soil P (ppm)	0.26	19.7	258	-1.07	<0.0001
Pinus pinaster C (*n* = 56)					
SR (W m^{-2})	0.25	8.6	844	-2.53	0.0069
F	0.53	29.2	356	0.390	<0.0001
Min *T* (ºC)	0.52	27.8	347	-3.31	<0.0001
Max *T* (ºC)	0.27	9.7	635	-6.19	0.0045
R (mm)	0.11	4.6	457	-0.059	0.0382
P. pinaster C+1 (*n* = 78)					
SR (W m^{-2})	0.15	7.0	944	-2.88	0.0118
F	0.36	22.2	389	0.464	<0.0001
Min *T* (ºC)	0.35	21.2	379	-3.93	<0.0001
Max *T* (ºC)	0.22	11.0	750	-8.05	0.0020
R (mm)	0.13	5.5	499	-0.072	0.0239
P. pinaster C+2 (*n* = 78)					
I	0.12	5.1	386	0.836	0.0298
SR (W m^{-2})	0.45	31.0	1452	-5.49	<0.0001
F	0.64	66.5	406	0.701	<0.0001
Min *T* (ºC)	0.62	62.2	391	-5.94	<0.0001
Max *T* (ºC)	0.21	10.2	817	-8.88	0.0028

Table 3. Multiple regression for different morphological leaf traits against minimum annual temperature and other climatic and soil factors. Model selection was conducted based on Akaike's information criteria. Abbreviations as in Table 2.

Independent variables	R^2	*F*	Intercept	Slope	*P*	Independent variables	R^2	*F*	Intercept	Slope	*P*
LT (μm)						LMA (g m^{-2})					
Quercus suber C (*n* = 49)											
Min *T* (ºC)	0.82	58	274	-4.18	<0.0001	Min *T* (ºC)	0.62	62	391	-5.94	<0.0001
Soil N (%)				-198	0.00142						
Q. ilex C (*n* = 75)											
Min *T* (ºC)	0.62	60	257	-5.47	<0.0001	Min *T* (ºC)	0.80	46	-146	-4.77	<0.0001
						SR (W m^{-2})				1.78	0.02
						Soil N (%)				84	0.05
Q. ilex C + 1 (*n* = 107)											
Min *T* (ºC)	0.85	100	-31	-5.93	<0.0001	Min *T* (ºC)	0.62	92	218	-2.95	<0.0001
Soil N (%)				92	0.02						
SR (W m^{-2})				1.520	0.03						
Pinus pinaster C (*n* = 56)											
Min *T* (ºC)	0.45	22	881	-14.13	<0.0001	Min *T* (ºC)	0.52	28	347	-3.31	<0.0001
P. pinaster C + 1 (*n* = 78)											
Min *T* (ºC)	0.57	25	950	-15.68	<0.0001	Min *T* (ºC)	0.35	21	379	-3.93	<0.0001
Soil P (ppm)				-7.44	0.18						
P. pinaster C + 2 (*n* = 78)											
Min *T* (ºC)	0.36	21	936	-14.50	<0.0001	Min *T* (ºC)	0.62	62	391	-5.94	<0.0001

Patterns of leaf chemical composition with respect to winter temperatures

In all evergreen species, the structural carbohydrates concentration (cellulose + hemicellulose) increased between sites with the intensity of their winter harshness (mean number of days with frost per year along the study period) (Figure 4). The maximum temperatures either had no influence or the effect was less significant than that of winter harshness (not shown). Again, among the deciduous species, no trends with temperature were observed in fibre concentration (Figure 4, upper panels). No trend was observed also for the lignin concentration in response to any of the different temperature estimates in any of the species (Figure 4). Fibre concentration tended to be higher in the species with longer leaf lifespan. In addition, the ANCOVA results revealed that the slopes of the regression lines of cellulose + hemicellulose concentration against number of frosts did not differ for the

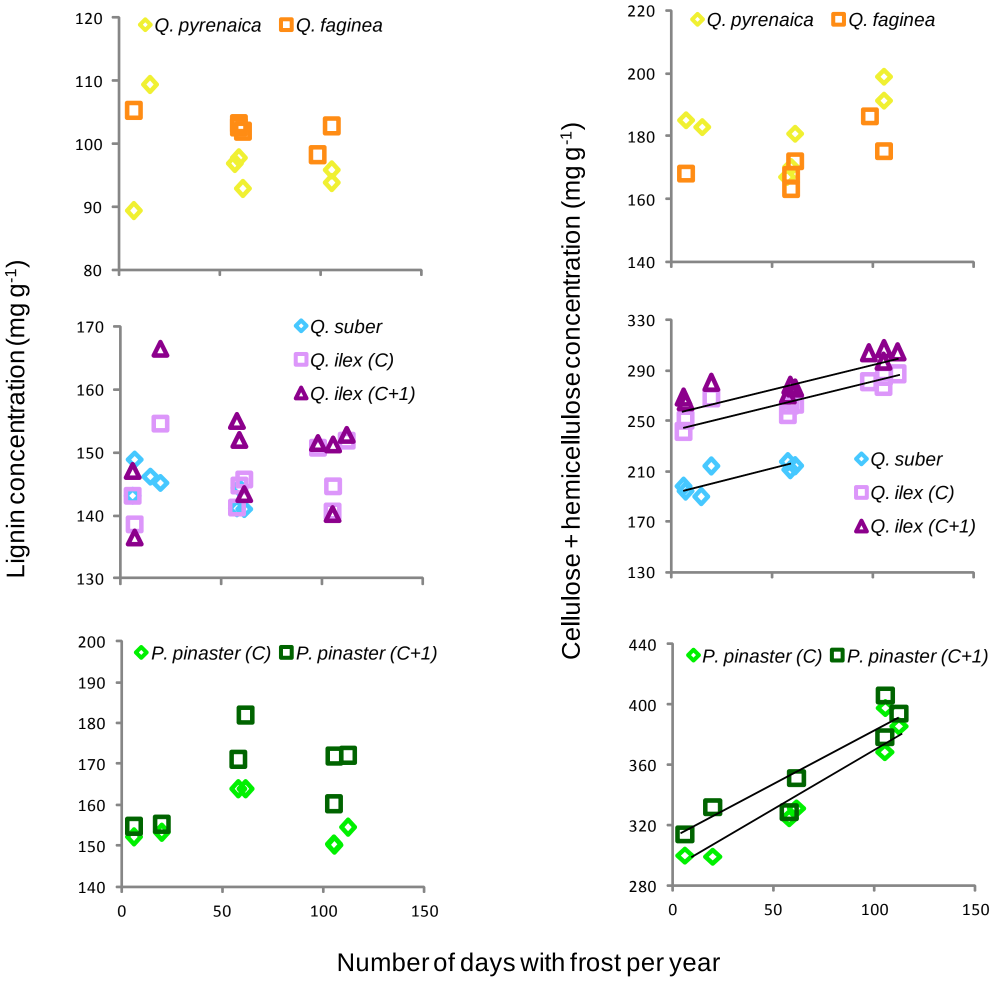

Figure 4. Relationships between the number of days with frost per year at each site and fibre concentration in the different leaf types. Each point is an average of eight sampling dates for current-year (C) leaves and 12 for 1-year-old (C + 1) leaves. Fitted equations for cellulose + hemicellulose concentrations: *Q. suber* ($y = 0.34x + 195$, $R^2 = 0.61$, $P = 0.04$), *Q. ilex* C ($y = 0.33x + 247$, $R^2 = 0.78$, $P = 0.0008$), *Q. ilex* C + 1 ($y = 0.35x + 264$, $R^2 = 0.79$, $P = 0.0005$), *P. pinaster* C ($y = 0.90x + 285$, $R^2 = 0.92$, $P = 0.0007$) and *P. pinaster* C + 1 ($y = 0.77x + 308$, $R^2 = 0.86$, $P = 0.0025$). No significant relationships were observed for lignin concentration. *Quercus pyrenaica* and *Q. faginea* produced no significant relationships for any variable.

two evergreen *Quercus* species, but were significantly higher in *Pinus* than in both *Quercus* species. This resulted in stronger between-species differences in structural carbohydrates concentration for cold sites (Figure 4).

No significant relationships were observed between the leaf N concentration and the number of days with frost per year (Figure 5), despite a slight trend of the colder sites to exhibit lower concentrations. Owing to this non-significant trend, N contents per unit leaf area were also uncorrelated with winter temperatures, despite the increase in LMA observed in colder sites. Leaf N concentrations in the two deciduous species were also independent of temperature (Figure 5, upper panel). The differences in winter harshness were accompanied, however, by differences in the leaf P content in the evergreen species.

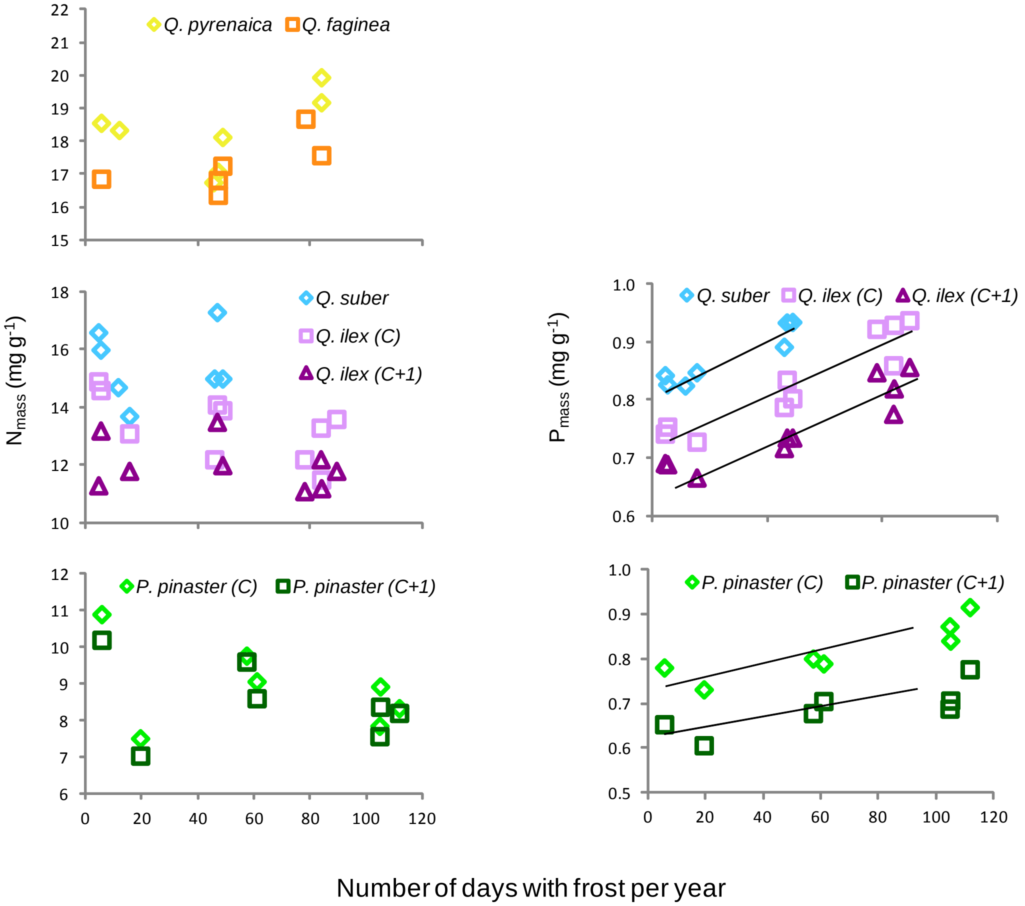

Figure 5. Relationships between the number of days with frost per year in each site and nitrogen (N_{mass}) and phosphorus (P_{mass}) concentrations in the different leaf types. Each point is an average of eight sampling dates for current-year (C) leaves and 12 for 1-year-old (C + 1) leaves. Fitted equations for P concentrations: *Q. suber* ($y = 0.0018x + 0.815$, $R^2 = 0.89$, $P = 0.0015$), *Q. ilex* C ($y = 0.0018x + 0.716$, $R^2 = 0.88$, $P < 0.0001$), *Q. ilex* C + 1 ($y = 0.0015x + 0.658$, $R^2 = 0.84$, $P < 0.0001$), *P. pinaster* C ($y = 0.0013x + 0.733$, $R^2 = 0.78$, $P = 0.0087$) and *P. pinaster* C + 1 ($y = 0.0010x + 0.622$, $R^2 = 0.63$, $P = 0.0334$). Phosphorus concentrations were not available for *Q. pyrenaica* and *Q. faginea* .

The P concentrations per unit leaf mass responded to the changes in temperature, increasing among sites with the number of frosts per year along the study period (Figure 5). This was particularly patent when the P amounts were expressed per unit leaf area (not shown), because the increase in P concentration per unit mass was accompanied by the increase in LMA associated with the decrease in winter temperatures. Summer temperatures again either had no influence on P concentrations or the effect was less significant than that of winter harshness (data not shown). The same trends between sites were repeated in all the leaf age classes. In this case, the ANCOVA results revealed that there were no significant interspecific differences in the response slopes of P concentrations to the number of frosts. No relationship was observed between the concentration of N and P in the leaves at each site and the levels of both nutrients recorded in the soils of the same sites (see Table 1).

There were significant differences in the concentration of CF and soluble PT between sites with different temperatures. The data concerning the two sites selected for each climatic category as a function of the intensity of their winter harshness (two warmer sites, two intermediate sites and two sites with the coldest winters) were pooled after checking that there were no significant differences in the mean values obtained for each of them in any of the cases (data not shown). Expressed per unit leaf mass, the concentrations of CF and soluble PTs fell with the reduction in winter temperatures, with significantly higher values in the leaves produced in warmer environments with respect to the colder sites (Figure 6). The sites with intermediate conditions were not always significantly differentiated from the warmer or colder sites, although they consistently showed intermediate values between both. The same differences between environments persisted for the CF content but disappeared for that of soluble PTs when both were expressed per unit leaf area (not shown). In both species, it was also possible to note a decrease in the relative amounts of Rubisco per unit leaf mass with the increase in the intensity of winter harshness (Figure 6).

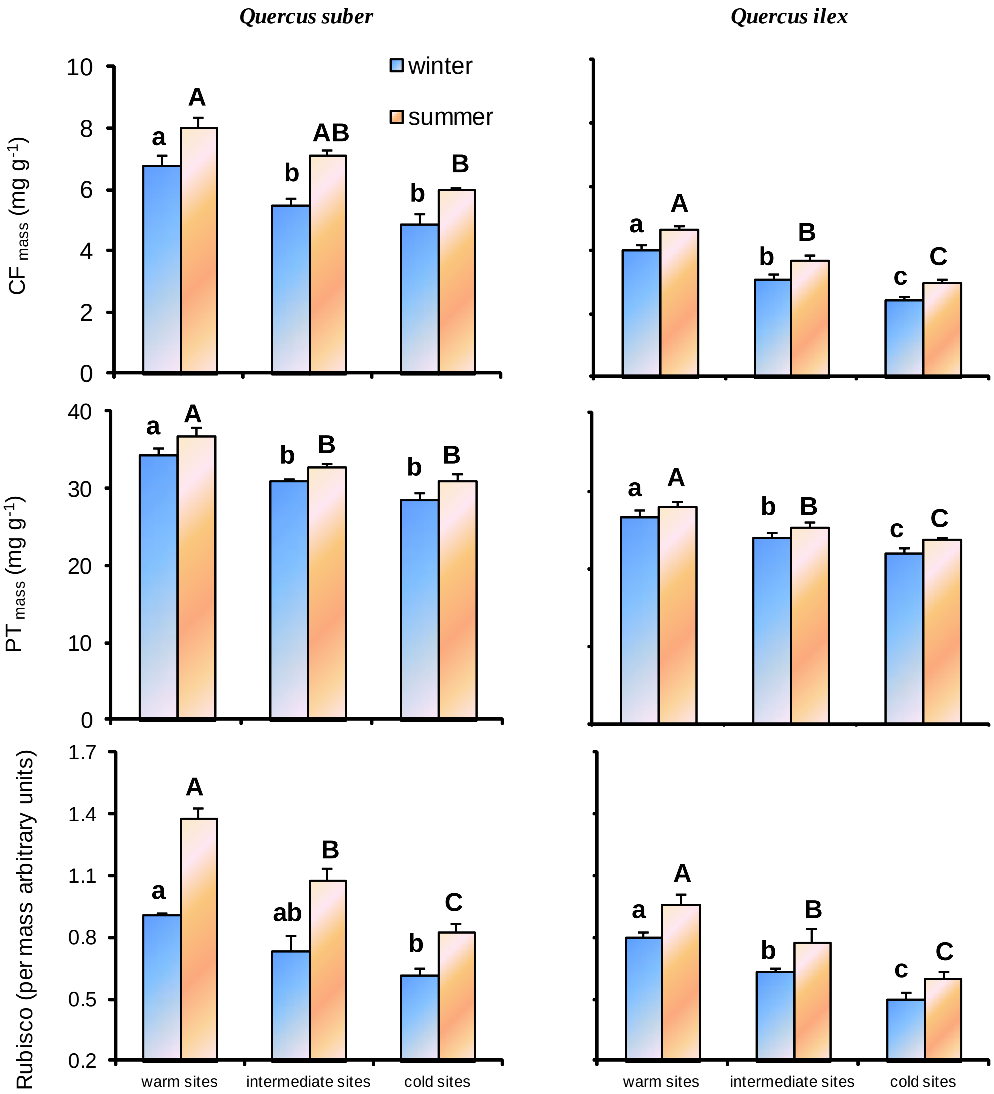

Figure 6. Mean (±SE, *n* = 10) concentration of chlorophyll (CF_{mass}), soluble protein (PT_{mass}) and Rubisco for the different species. The significant differences among sites are marked with different letters (Fisher LSD test, $P < 0.05$).

DISCUSSION

Most of the leaf traits analysed here showed significant differences between sites and such differences seemed to be related to differences in the intensity of winter harshness, with a similar response in all three evergreen species studied. Thus, the leaves from one species developed in environments with cooler winters had a greater LMA and a higher concentration of structural carbohydrates and P, but lower concentration of CF, soluble PTs and Rubisco than those produced at the sites with milder winters. A trend to decreasing Rubisco contents at high altitudes has also been reported for beech by Rajsnerová et al. (2015).

These temperature responses were not observed in the two deciduous species studied, which suggests that the between-sites differences in environmental conditions during the growth season were not responsible for the trends observed in evergreen leaves. Although other soil and climatic factors also exerted significant effects on leaf traits, these effects were mainly due to the existence of correlations among the different environmental variables. In multiple regression analysis, in most cases, only winter temperatures showed significant relationships with leaf traits, which suggests a direct effect of winter harshness on leaf characteristics. Differences in solar radiation between our sites were small. Possibly for this reason, despite the known effects of irradiance on LMA (Poorter et al. 2009), in the present study, we did not detect independent effects of this factor. In evergreen species, leaf lifespan tends to increase with decreasing temperatures (Wright et al. 2005), probably reflecting the longer payback time for construction costs in such conditions, due to the shorter growing season (Kikuzawa et al 2013). These trends in leaf duration could have contributed to the responses in leaf traits observed in the present study. However, the increases in leaf lifespan with cold between our sites were undetectable for the two oak species and only marginally significant in *P. pinaster*, probably because the temperature gradient here studied was much smaller than those reported in global surveys, such as in Wright et al. (2005). Accordingly, differences in leaf lifespan seem to be too short to be responsible for the clear trends in leaf morphology and chemistry observed in our species.

A greater LMA has traditionally been interpreted as a trait aimed at guaranteeing leaf survival, acting as protection against different environmental factors such as drought or attack by herbivores (Turner 1994, Niinemets 2001). The highest mass per unit leaf area shown by the species studied here in the environments with the coldest winters and subjected to the most intense and continued frosts suggests that the harshest winter conditions also demand greater leaf reinforcement. The increase in LMA with winter harshness seems to be achieved exclusively through an increase in thickness, without changes in density. This increased thickness in colder sites could be a consequence of greater accumulation of photosynthetic biomass per unit leaf area (Niinemets 2015). In contrast, several authors have suggested that the greater thickness of leaves developing in colder climates would be achieved through a thickening of the leaf cell walls, characteristic of leaves growing under these conditions (Griffith and Brown 1982, Kubacka-Zębalska and Kacperska 1999). The amount of cell wall is related to the response capacity of plants to freezing, helping to increase tolerance to cold via a reduction in the freezing rate (Ball et al. 2002). Our results seem to confirm this higher amount of cell wall as being responsible for the greater leaf thickness in environments with harsh winters. All the evergreen species studied here showed a higher concentration per unit leaf mass of structural carbohydrates (cellulose + hemicellulose) in the colder environments. However, we did not note any trend in the lignin concentration associated with the differences in winter temperatures. Despite the traditional belief that lignin contributes to rigidity, and hence to leaf survival (Chabot and Hicks 1982, Cornelissen et al. 1999, Takashima et al. 2004), in recent years, many authors have reported the absence of a relationship between rigidity and leaf hardness and the lignin concentration (Kurokawa and Nakashizuka 2008, Kitajima et al. 2012) and hence between the amount of lignin and leaf duration (Mediavilla et al. 2008). The results of these authors suggest, as is the case here, that it is structural carbohydrates and not lignin that confer the leaves their hardness and thus increase their survival under adverse conditions, such as lower temperatures and greater frost intensity and duration in environments with harsher winters.

Although most leaf N is allocated to the components of the photosynthetic machinery, such as Rubisco and light-collecting complexes (Miyazawa et al. 2004), a significant amount of the N content is invested in other functions such as cell wall construction (Reiter 1998, Hikosaka and Shigeno 2009). If the leaves must reinforce themselves with greater amounts of cell wall material to be able to support lower winter temperatures, it is to be expected that a greater amount of available N must be allocated to the cell walls in environments with cold winters, leading to a reduction in the amount available for photosynthetic compounds. In our oak species, the mass-based concentration of CF, soluble PTs and Rubisco proved to be lower at the sites with the harshest winters. The concomitant variation in LMA tended to reduce to some extent the between-site differences in leaf chemical composition when the concentrations of CF, soluble PTs and Rubisco were expressed per unit area. However, if we assume that a main function of leaves is to deliver a profitable return on the investment that has been made in constructing the leaf (Westoby et al. 2013), mass-based differences may better reflect the costs associated with adaptation to cold. Unlike the increase in the N content of leaves with the decrease in temperatures reported by different authors (Weih and Karlsson 2001, Reich and Oleksyn 2004, Jian et al. 2009), in the present case, we failed to observe any significant trend in the amount of leaf N associated with differences in temperature. At the same sites as those studied in the present work, during the first 2 years of sampling, there was a significant trend towards higher N contents per unit leaf area at the colder sites (Mediavilla et al. 2012), which disappeared in the last 2 years. In fact, although plants grown in laboratory conditions typically contain greater leaf N when grown at low temperatures, field surveys tend to provide much more variable patterns (Reich and Oleksyn 2004, Niinemets 2015).

If the N concentration per unit leaf mass is independent of the differences in winter temperatures between sites, but the concentrations of CF, Rubisco and soluble PTs are lower at the colder sites, this suggests that there is a compromise in the distribution of N between photosynthesis and structural components. Our results, therefore, confirm those of other authors who have reported that the fraction of N allocated to Rubisco declines as LMA increases and the allocation of N to structural functions increases (Ellsworth et al. 2004, Onoda et al. 2004, Takashima et al. 2004). For example, the CF/N ratio in the leaves of the two oak species was significantly higher at the warmer sites (about 0.46 for *Q. suber* and 0.30 for *Q. ilex*) than at the colder sites (about 0.33 for *Q. suber* and 0.21 for *Q. ilex*). Accordingly, for the same amount of N, the amount of CF is higher in environments with milder winters, in parallel with the lower values of LMA, thickness and the concentration of structural carbohydrates. This suggests that the increase in the allocation of N to cell walls in colder environments occurs at the expense of reducing the allocation of N to the photosynthetic apparatus.

Regarding N, therefore, our results conflict with the hypothesis of a compensation of the reduction in the photosynthetic rate and other metabolic processes in colder environments by means of a greater allocation of N to leaves. In the literature on thermal acclimation, this idea is well established (Woods et al. 2003) and has been used to explain global patterns observed in plant leaf N in relation to temperature (Reich and Oleksyn 2004). Higher PT concentrations have been interpreted as an adaptation aimed at compensating the shorter length of the favourable period for growth at sites with lower temperatures. The discrepancy between our own results and this generally accepted idea could be explained in terms of the effect of drought stress during the summer in our Mediterranean environment. If drought stress were more intense at warmer sites, this would reverse the positive effects of higher temperatures and could shorten the growth season at the warmer sites when compared with the colder ones. However, in the present study, differences in summer temperatures were relatively low. At the same time, the warmer sites also received greater rainfall, which could reduce the differences in drought stress. In fact, the Emberger’s pluviothermic index was uncorrelated with temperature (Table 1). Additionally, in a global survey, van Ommen Kloeke et al. (2012) found temperature to be the sole best predictor of the length of the growth season, while water availability (i.e., precipitation and evapotranspiration) had only marginal effects. We believe that the main factor responsible for the reduced allocation of N to the photosynthetic machinery at colder sites is the structural reinforcement detected by us, necessary for the evergreen leaves to cope with the winter conditions at the colder sites. This reinforcement allows leaf longevity to be extended beyond the first growing season and the lower instantaneous productivity to be compensated. As suggested by Kikuzawa et al. (2013), increasingly higher potential photosynthetic rates and nutrient concentrations as the length of the growing season decreases are important adaptations for deciduous leaves, but such an adjustment may not be required for evergreen plants, which can amortize construction costs over multiple seasons.

Contrary to leaf N, the P concentration responded to the changes in temperature in all three evergreen species, increasing among sites with the intensity of their winter harshness. Some authors have suggested changes in the leaf P content to be associated with latitudinal and temperature gradients, with a decrease in the P content with the increase in temperature and nearness to the equator. These authors proposed that this relationship would arise as a result of the differences in the age of the soil substrate, which has been shown to influence soil P availability and leaf P, with lower levels in older soils, closer to the equator, when compared with the younger and less leached soils farther away from the equator (Reich and Oleksyn 2004, Reich 2014). However, in our case, we did not observe any relationship between soil P contents and the P concentration in leaves, and neither did we note any trend in soil P levels associated with latitude or with the temperature at the study sites. In contrast, the highest levels of P found in the leaves of the populations occupying colder sites could respond to the limiting effect that this nutrient seems to exert in the study area. The N/P ratio in plant tissues has been proposed (Koerselman and Meuleman 1996) as a good predictor of limitations of these nutrients in the soil, with a leaf N/P ratio <14 indicating N limitation, a ratio >16 indicating P limitation and a ratio between 14 and 16 indicating that either N or P may limit plant growth or both elements are equally limiting. In our study, the N/P ratio was far above the limit value of 16 in all cases, reflecting P limitation at all our sites. A study conducted on Australian soils, which tend to be particularly P-deficient in comparison with soils from other continents (Atwell et al. 1999), found that leaf P had significance beyond leaf N in predicting the assimilation rate by unit leaf mass (Wright et al. 2001). Since in our case P was the element that supposedly acted as limiting, a higher concentration of this nutrient in the leaves of the trees growing in colder environments would compensate the unfavourable effects of low temperatures on CO_2 assimilation.

In conclusion, our results suggest that an increase in LMA and in the concentration of structural carbohydrates would be an indispensable requirement for the trees to cope with the low winter temperatures and that evergreen species must reinforce their leaves to a greater extent at the coldest sites. This implies that the evergreen habit involves higher costs in these environments, which necessarily presupposes an additional disadvantage for the evergreen habit with respect to the deciduous one. A clear manifestation of these increased costs is that the interspecific differences in LMA and structural carbohydrate concentrations that are usually linked to differences in leaf lifespan are stronger in cold than in warm climates. For example, according to the results of the present article, current-year leaves of the evergreen *Q. ilex* have an average LMA 120% greater than that of the deciduous *Q. pyrenaica* at warm sites, but 135% greater at cold sites. Similarly, the concentration of structural carbohydrates per unit mass is 34% greater in *Q. ilex* with respect to *Q. pyrenaica* at warm sites, but the difference amounts to 45% at cold sites. These increased investments in structural reinforcement in evergreen species involve not only additional construction costs but also a reduced carbon gain (van Ommen Kloeke et al. 2012), and these costs seem to be stronger in colder environments.

CONFLICT OF INTEREST

None declared.

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Capítulo II

EFICIENCIA Y PROFICIENCIA EN LA REABSORCIÓN DE N EN RESPUESTA AL FRÍO INVERNAL EN TRES ESPECIES PERENNIFOLIAS

Este capítulo reproduce íntegramente el siguiente artículo:

González-Zurdo P, Escudero A, Mediavilla S (2015) N resorption efficiency and proficiency in response to winter cold in three evergreen species. Plant and Soil 394: 87-98.

RESUMEN

Antecedentes y objetivos. Los cambios en el estatus nutricio de las hojas a lo largo de gradientes de temperatura han sido abordados por diferentes autores, que sugieren la existencia de diferencias en la disponibilidad y en los patrones del uso de los nutrientes. Sin embargo, rara vez se han estudiado los efectos de la temperatura sobre la reabsorción de los nutrientes a nivel de una especie. Nuestro objetivo es analizar los efectos de las diferencias en las temperaturas invernales sobre la reabsorción de nitrógeno en las hojas de tres especies arbóreas perennifolias.

Métodos. Hojas frescas y senescentes fueron tomadas de ejemplares maduros de las tres especies en 11 sitios con temperaturas invernales contrastadas. Las eficiencias de reabsorción del N se calcularon a partir de las diferencias entre los contenidos de N en hojas frescas y desprendidas, recogidas de los mismos individuos. Las concentraciones mínimas de N en la hojarasca fueron utilizadas como una estimación de la proficiencia de reabsorción. El peso por unidad de superficie foliar (LMA) y las concentraciones de hemicelulosa y celulosa también se determinaron en las hojas frescas.

Resultados. Los contenidos de N en hojas frescas no mostraban ninguna respuesta a los gradientes de temperatura. Por el contrario, los contenidos de N en la hojarasca aumentaban con la disminución de la temperatura. Como consecuencia, la eficiencia y la proficiencia en la reabsorción de N disminuían con el descenso de la temperatura. LMA y las concentraciones de carbohidratos estructurales aumentaban con la disminución de la temperatura.

Conclusiones. Las especies estudiadas tienen un menor potencial de reabsorción de N en ambientes con temperaturas invernales más bajas. La razón principal de esta menor eficiencia parecen ser las mayores cantidades de N inmovilizadas en la mayor cantidad de pared celular necesarias para hacer frente a las temperaturas invernales inferiores. El hábito perennifolio por tanto, estaría asociado con mayores costes en los sitios más fríos, debido a que los rasgos de resistencia al frío implican una eficiencia de reabsorción de N reducida y un incremento de la dependencia del N edáfico.

Palabras clave: reforzamiento estructural, eficiencia de reabsorción del N, *Pinus*, proficiencia, *Quercus*, temperatura invernal.

N RESORPTION EFFICIENCY AND PROFICIENCY IN RESPONSE TO WINTER COLD IN THREE EVERGREEN SPECIES

ABSTRACT

Background and aims Along temperature gradients, changes in leaf nutrient status have been reported by different authors, which suggest the existence of differences in nutrient availability and in the patterns of nutrient use. However, the effects of temperature on nutrient resorption efficiency within a species have rarely been studied. Our aim here is to analyze the effects of the differences in winter temperatures on the nitrogen resorption of the leaves of three evergreen tree species.

Methods Green and senescent leaves were sampled from mature specimens of the three species at 11 sites with contrasting winter temperatures. N resorption efficiencies were calculated from the differences between N contents in green and shed leaves collected from the same tree individuals. Minimum N concentrations in leaf litter were used as an estimation of resorption proficiency. Leaf mass per unit area (LMA) and concentrations of hemicellulose and cellulose were also determined in green leaves.

Results N contents in green leaves did not show any response to temperature gradients. By contrast, N contents in leaf litter increased with decreasing temperature. As a consequence, N resorption efficiency and proficiency declined with decreasing temperature. LMA and the concentrations of structural carbohydrates increased with declining temperature.

Conclusions The species studied have a lower potential for N resorption in environments with lower winter temperatures. The main reason for this lower efficiency seems to be the higher amounts of N immobilized in the greater amount of cell wall needed to cope with lower winter temperatures. The evergreen habit would thus be associated with higher costs at cooler sites, because the cold resistance traits imply reduced N resorption efficiency and

increased dependence on soil N.

Keywords: leaf structural reinforcement, N resorption efficiency, *Pinus*, proficiency, *Quercus*, winter temperature.

INTRODUCTION

Nutrient resorption during leaf senescence is an important mechanism for plant nutrient conservation, and plays a key role in the function and dynamics of ecosystems (Aerts 1996; Aerts et al. 2007; Han et al. 2013). This reutilization of nutrients, which can be expressed either as resorption efficiency (% of the mature leaf nutrient pool that is resorbed) or resorption proficiency (the level to which nutrient concentration is reduced in senesced leaves, Killingbeck 1996), affects key processes such as the carbon cycle and resource use efficiency (Aerts and Chapin 2000; Gleason and Ares 2007) or the decomposition rates (Manzoni et al. 2008, 2010) and may decisively determine competitive relationships and fitness in terms of growth and reproduction (Aerts and Chapin 2000; Berg and McClaugherty 2008; May and Killingbeck 1992).

Owing to the importance of the resorption process, interspecific differences in resorption have been studied exhaustively, although they are still far from being understood. Many factors have been proposed to explain differences in resorption percentages, such as the relative size of sinks (Nambiar and Fife 1991; Silla and Escudero 2003), the phloem transport rate (Chapin and Kedrowski 1983; Chapin and Moilanen 1991), leaf abscission dynamics (Del Arco et al. 1991; Milla et al. 2005) and water availability (Del Arco et al. 1991; Pugnaire and Chapin 1992). Soil nutrient availability and the nutritional status of the leaves are among the determinants of nutrient resorption most studied, although the results are so divergent that they prevent any generalization. Thus, while some evidence suggests that nitrogen resorption can be determined by the availability of this nutrient in the soil, with more resorption in low-fertility soils (Enoki and Kawaguchi 1999; Holub and Tuma 2010; Lovelock et al. 2007; Yuan et al. 2005), in many others cases no relationship has been observed (Aerts 1996; Diehl et al. 2003; Eckstein et al. 1999; Vergutz et al. 2012). Similarly, while some studies failed to find any relationship between resorption efficiency and nutrient concentrations in green leaves (Aerts 1996; Huang et al. 2007; Niinemets and Tamm 2005), other studies have found resorption efficiency to be related to plant nutrient status (Fife et al. 2008; Kobe et al. 2005; Vergutz et al. 2012; Wright and Westoby 2003).

In addition to these controversial findings in the response to the factors most frequently studied, there are still many other factors whose effects have barely been addressed, such as temperature. Numerous studies have demonstrated changes in leaf morphology and chemical composition in response to air temperature, particularly as regards the foliar nutrient concentration (Han et al. 2011; Reich and Oleksyn 2004; Zhang et al. 2012). If it is true that temperature variations determine changes in the composition of the leaves, and if foliar nutrient status indeed finally affects resorption efficiency, one would then also expect to see effects of temperature on resorption percentages. In fact, however, very little is known about this issue, the existing information being limited to a few studies that have either been performed under controlled conditions (and also in this case with often conflicting results), or at a global scale through large environmental gradients. Declines in resorption efficiency with increasing temperature of the environment of origin have been observed, for example, in common-garden experiments conducted by some authors (Oleksyn et al. 2003; Oyarzabal et al. 2008), while others have found only a minor impact on nutrient resorption parameters (Aerts et al. 2007; Welker et al. 2005). Moreover, recent studies that have addressed geographic patterns of nutrient conservation strategies in relation to temperature are consistent in reporting a decrease in the N resorption efficiency in plants occupying warmer environments (Tang et al. 2013; Vergutz et al. 2012; Yuan and Chen 2009). However, these resorption patterns at a global scale include different plant communities and may be influenced by differences in the leaf traits of the different species and in environmental factors, such as the age of the soil substrate and associated soil nutrient availability. Little is known, by contrast, about the effects of temperature on leaf resorption at smaller geographic scales and at the level of a same species. Given the importance of the resorption process for plants and ecosystems, more data are needed to clarify this issue, allowing us to incorporate nutrient resorption into models of plants' responses to climate change (Aerts et al. 2007; Chapin et al. 2011).

In the present work we analyze the effects of the differences in climate harshness during winter on the nitrogen resorption (efficiency and proficiency) of the leaves of three evergreen species widely distributed across the Iberian Peninsula (*Quercus ilex* ssp*. ballota* (Desf.) Samp, *Quercus suber* L. and *Pinus pinaster* Aiton). A previous study with the same species revealed significant differences between sites in several leaf traits related to differences in the intensity of winter harshness, with a similar response in all three species. Thus, the leaves from one species growing in environments with cooler winters had a greater leaf mass per unit area (LMA) than those produced at the sites with milder winters, with no trends in the leaf nitrogen content between environments (Mediavilla et al. 2012). Accordingly, our previous data suggest that winter climate harshness (in particular the intensity and frequency of frosts) contributes to increasing the sclerophyllic character of these evergreen species. Several authors (Chapin and Kedrowski 1983; Pugnaire and Chapin 1993) have proposed that resorption efficiency would depend on the availability of soluble forms of nitrogen, which facilitate remobilization. In particular, a large fraction of N is retained in cell walls (Reiter 1998) and this is only partially retranslocated (Yasumura and Ishida 2011). Thus, according to some authors, the potential for N resorption may vary with the amount of cell wall in the leaves, decreasing with a higher fraction of N associated with structural proteins occluded in the cell wall matrix (Hikosaka 2004; Niinemets and Tamm 2005). Possible differences in the leaf chemical composition associated with differences in the intensity of winter harshness could thus result in differences in resorption efficiencies. Our hypothesis is that if the resorption process varies with the availability of soluble forms of nitrogen for resorption, the amount of N retranslocated during senescence should be lower in colder environments, where evergreen leaves tend to be more sclerophyllic than in warmer environments. Our aim therefore is to check whether in addition to the costs associated with a higher LMA at the coldest sites (Mediavilla et al. 2012), a lower resorption efficiency might also contribute to increasing the costs associated with a higher dependence on soil N in these environments. This information is essential to help understand the possible repercussions of climate change on the distribution of woody species and to improve predictions of vegetation responses to new climatic scenarios.

MATERIALS AND METHODS

Study species and area

Three evergreen tree species were studied: *Pinus pinaster* Aiton, a gymnosperm with an average leaf life span of about 52 months, and *Quercus suber* L. and *Q. ilex* ssp*. ballota* (Desf.) Samp, with 15 and 24 months of mean leaf life span respectively (data taken from Mediavilla and Escudero 2003).

The species were distributed on 11 sites located in the regions of Castilla-León and Extremadura (central-western Spain) between latitudes 41º 45' N and 40º 01' N and between longitudes 6º 22' W and 2º 08' W (Table 1). The sites consisted of flat areas with sparse populations (between 50 and 100 specimens ha^{-1}) of mature (more than 100 years old) individuals. Trunk diameter at 1.3 m height ranged from 20 to 60 cm and mean heights were 4–10 m. Each site was selected so as to include as many study species as possible and to cover a broad gradient of winter temperatures, although taking care that the rest of the climate characteristics would be as homogeneous as possible. Nevertheless, there is a tendency for rainfall levels to be higher at the hottest and southernmost sites, which helps to reduce the differences in the intensity of drought stress between cold and hot sites (Table 1). Owing to its lower tolerance to low temperatures, *Q. suber* was absent at the coldest sites. Annual rainfall data from the station nearest to each stand were provided by the

Table 1. Sites characteristics.

SITES	A	B	C	D	E	F	G	H	I	J	K
Longitude (W)	5º 57´	5º 48´	6º 22'	5º 02´	5º 52´	5º 47´	6º 07´	2º 08´	2º 37´	2º 43´	2º 52´
Latitude (N)	40º 13´	40º 01´	39º 49'	40º11´	41º 14´	41º 08´	40º 55´	40º 59´	41º 45´	41º 32´	41º 43´
Altitude (m.a.s.l.)	619	466	600	449	985	834	832	1246	1189	982	1045
Mean temperature (ºC)											
Annual	16.5	16.6	16.8	16.4	12.3	12.7	13.0	10.1	10.3	11.0	10.6
Spring	18.4	19.3	19.0	19.1	15.0	14.9	15.7	12.0	13.0	14.5	13.2
Summer	25.6	25.8	26.3	26.3	20.8	21.4	22.3	19.4	19.1	19.0	19.1
Autumn	12.6	12.2	13.2	11.5	7.84	9.11	8.32	6.09	6.03	6.51	6.35
Winter	9.55	9.07	9.81	9.2	5.55	5.30	5.86	3.04	3.14	3.99	3.53
Absolute minimum temperature	-2.15	-4.15	-3.55	-5.5	-8.18	-9.82	-7.50	-13.1	-13.7	-15.0	-16.9
Mean daily minimum temperature of the coldest month	4.5	4.06	4.69	3.19	-0.47	-0.90	-1.06	-1.96	-2.93	-3.28	-3.13
Number of days with frost per year	7	15	5	20	57	61	59	98	105	105	112
Mean duration of the period with frosts (days per year)	24	68	55	90	127	154	100	193	222	202	214
Annual precipitation (mm)	729	1055	598	615	385	328	380	383	496	473	506
Emberger's pluviothermic index	72	105	60	60	52	46	48	58	79	69	78
Soil											
Sand content (%)	81.1	77.2	73.9	75.7	84.6	74.3	67.9	57.8	75.1	84.8	84.0
Clay content (%)	8.5	11.7	12.3	10.6	6.4	12.2	16.2	22.8	14.4	8.1	8.3
Silt content (%)	10.4	11.1	13.8	13.7	9.00	13.5	15.9	19.4	10.5	7.10	7.7
Total soil N content (%)	0.082	0.143	0.104	0.126	0.072	0.021	0.078	0.112	0.115	0.040	0.048
P assimilable (ppm)	31	38	5	6	10	4	20	2	7	9	6
M.O. (%)	1.66	4.47	4.10	2.78	2.6	0.37	1.66	1.96	5.12	0.69	1.92
pH	5.7	4.5	4.7	5.1	4.6	4.5	4.4	7.6	4.8	7	6.1
Species	Qi, Qs	Qs	P, Qi, Qs	P, Qi, Qs	P, Qi, Qs	P, Qi, Qs	Qi, Qs	Qi	P, Qi	P, Qi	P, Qi

P *Pinus pinaster*; Qi *Quercus ilex*, Qs *Quercus suber*

Spanish National Meteorological Institute. Air temperature data were obtained for each site by means of data loggers (Hobo Pendant temperature/light datalogger, Part UA-002-08, Onset Computer Corporation, Pocasset, MA, USA). The data loggers were programmed to obtain temperature data every 10 min and they were kept at each site for four years (October 2008 – October 2012). In spring, soil samples were taken down to a depth of 20 cm (excluding the forest floor) from each stand. Determinations of soil granulometry, pH and N and P concentrations were carried out at the Soils Laboratory of the Institute of Natural Resources and Agrobiology in Salamanca according to the methods described by Chapmann and Pratt (1973) and Walkley and Black (1934).

Collection and processing of samples

For each species, 5 individual trees were selected at random at each site. To obtain freshly fallen leaf litter, 10 leaves of each of the two oldest leaf cohorts present in the crown of each individual were held in place via a clip attached by a thread to the stem that allowed them to be retained on the plant after their abscission. Only one flush of leaf growth was observed in all species. Accordingly, all the leaves born in one particular year were considered to belong to the same age class. The tagged leaves were always situated at a mid-height in the sun-lit portion of the crown. The reduced size of the clips allowed the surface of the leaf covered by them to be sufficiently small for the damage to be almost negligible, as checked in previous assays. In any case, to minimize damage the clips were always emplaced in situ slightly before the start of the typical abscission period of each species. Although this method is obviously more time consuming than the “classic” litter-trap method, it does allow the errors associated with intra-canopy variability in leaf traits to be reduced, because the estimation of resorption is based on the comparison between green and senesced leaves collected from the same branches and with a similar size and mass per unit area. Once the clips had been set up, monthly samplings of the leaves shed were performed until all leaves selected had been collected. The sampling frequency was increased to once a week between late spring and late summer to suitably record the periods of abscission typical of these evergreen species (Escudero and Del Arco 1987). Depending on the individual tree, most of the leaves were shed at ages between 1 and 2 years for *Q. suber*, 2-3 years for *Q. ilex* and between 3 and 6 years in *P. pinaster*. In spring of each year, before the abscission period, 25 green leaves of each of the two oldest leaf cohorts were collected from the same individuals and the same branches in which leaves were marked to collect leaf litter. The N content of these green leaves was then used to estimate N resorption efficiency. This sampling procedure was repeated along 3 years (2011-2014).

At the laboratory, leaf thickness was measured with a digital micrometer (Digimatic micrometer, Mitutoyo, Japan) as a mean of three measurements taken at random positions on each leaf or needle, avoiding the main ribs on flat leaves. The total projected leaf and needle areas were determined by an image analysis system (Delta-T Devices LTD, Cambridge, UK). The samples were then oven-dried at 70 ºC to constant weight and the total dry mass was determined. From the data thus obtained, we calculated the leaf dry mass per area (LMA). Once dried, for each species on each sampling date all senesced and green leaves of the same age class collected from each individual were ground together to obtain a sufficient amount of sample for chemical analyses. Leaf N concentrations were determined with a CE-Instruments NA-2100 autoanalyzer (ThermoQuest, Milan, Italy). After the N analyses, the remaining material of the green leaves was used to analyze the fibre content (hemicellulose and cellulose) with an Ankom Analyzer (A220, New York, USA), following the method of Goering and Van Soest (1970). The nitrogen and fiber contents of leaves were expressed per unit dry mass (as milligrams of N or fiber per gram of leaf dry mass), and per unit of leaf area (g m^{-2}), obtained as the concentration per unit dry mass multiplied by LMA.

Data analysis

For each individual tree we obtained a value of the resorption efficiency for each date on which the leaf litter was collected as (100* (N in green leaves – N in shed leaves)/N in green leaves). For the N content in living leaves, we used that of the green leaves collected from the same tree, which was compared with that of the shed leaves of the same age class. Leaf mass loss during senescence was calculated according to a similar equation. Resorption efficiency (RE) was calculated from the nitrogen concentrations expressed both per unit leaf mass (RE_{mass}) and per unit leaf area (RE_{area}). A single resorption efficiency value was estimated for each individual tree as the average of the values obtained on all the dates on which abscission was recorded weighted by the number of leaves shed on each sampling occasion throughout the entire 3-year study period, after checking that there were no significant differences in the average resorption efficiency obtained between the different years of study (data not shown). Finally, a single resorption estimate was calculated for each species and site as the average of the values obtained for each individual tree. To estimate the N resorption proficiency of each species at each site and in each year of study we selected the absolute minimum N content value (expressed per unit mass and per unit leaf area) recorded among all shed leaves for each of the five individuals selected for that species and site. Finally, a single resorption proficiency value was obtained for each species at each site as an average of the fifteen data (five trees and 3 years), after checking that there were no significant differences in the average resorption proficiency obtained for the same species and site between the different years of study.

The relationships between resorption efficiency and proficiency and different temperature measurements were explored by means of linear regression analysis. We assigned a single value to each site for mean monthly temperatures and mean monthly maximum and minimum daily temperatures, obtained as an average of the values measured for each month along the study period. For each site, we also obtained a single value for the absolute maximum and minimum temperatures, the number of days with frost along the study period and the annual rainfall. We used the Emberger's pluviothermic index (Emberger 1930) to analyze the effects of water stress on resorption parameters:

$$Q = \frac{1000 \times P}{(273 + \frac{T_X + T_N}{2}) \times (T_X - T_N)}$$

where P is the annual precipitation (mm), T_X is the average temperature of the hottest month (ºC) and T_N is the average temperature of the coldest month (°C). This index is commonly used in Mediterranean climates (Kunstler et al. 2007). Similarly, to better explore the relationships between N resorption and the different leaf traits analyzed in leaves of the same age class, the data obtained for each trait in each species at each site were pooled over the entire study period, after checking that there were no significant differences in the values obtained for the same species and age class at each site in the different years of study. Linear regression analysis was also used to check the relationships between resorption efficiency and leaf traits. When necessary, data were log-transformed to linearize the relationships. All statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA).

RESULTS

Patterns of leaf nitrogen resorption in relation to winter temperature

Leaf mass loss during senescence ranged between approximately 8 and 20% of the “green” mass and was more marked in the warmer than in the colder environments for all three species (Fig. 1). Due to the decrease in leaf mass accompanying the decrease in N concentrations during senescence, the N resorption percentage was always higher when N contents were expressed on a leaf area basis than on a leaf mass basis. However, for both RE_{mass} and RE_{area} in all species the average resorption efficiency showed a clear negative relationship with the total number of days with frost along the study period (Fig. 1). Within a site, different individual trees tended to have very similar resorption efficiencies, as demonstrated by the low standard errors calculated (Fig. 1). Resorption efficiency per unit area was also related to other indicators of the intensity of winter harshness (mean monthly

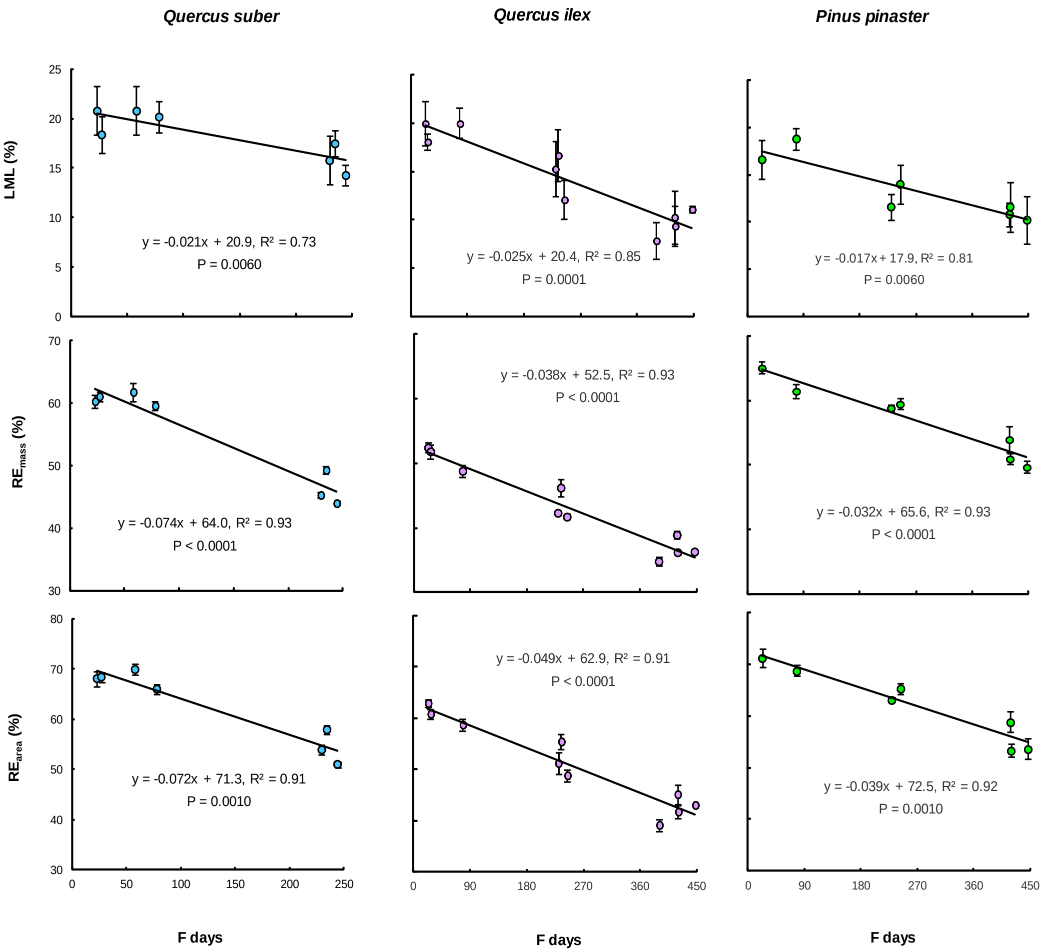

Fig. 1 Leaf mass loss during senescence (LML) and nitrogen resorption efficiency on a per unit leaf mass (RE$_{mass}$) and per unit leaf area (RE$_{area}$) basis as a function of the total number of days with frost along the study period (F days) at each site. Each data point is the mean and standard error of the 5 individual trees sampled at each site.

minimum daily temperatures or absolute minimum winter temperature) (Table 2). Maximum temperatures, by contrast, either had no influence or the effect was less significant than that of winter harshness (Table 2). In most cases, the rainfall levels and the Emberger's pluviothermic index did not reveal significant effects on resorption efficiencies. Total soil nitrogen contents showed no trend along the temperature gradient (Table 1). Accordingly, there was no relationship between the N resorption values at each site and the levels of N recorded in the soils of the same sites for any of the species (Table 2).

These differences in resorption efficiency in response to winter harshness were due mainly to differences in the amount of N in leaf litter, since no trend was observed for the nitrogen

Table 2. Summary of linear regressions relating N resorption efficiency per unit area (RE_{area}, %) and per unit mass (RE_{mass}, %) to different environmental characteristics and to leaf N content.

Dependent variable	Independent variables	*Q. suber* (*n* = 7)				*Q. ilex* (*n* = 10)				*P. pinaster* (*n* = 7)			
		Intercept	Slope	R^2	P	Intercept	Slope	R^2	P	Intercept	Slope	R^2	P
RE_{area}	Mean m min T (ºC)	36.68	2.79	0.889	0.0015	33.94	2.42	0.880	0.0001	48.53	2.07	0.868	0.0023
RE_{area}	Min T (ºC)	77.66	2.65	0.899	0.0012	65.67	1.57	0.867	0.0001	75.67	1.32	0.876	0.0019
RE_{area}	Mean m max T (ºC)	-32.03	4.23	0.596	0.0420	-21.45	3.45	0.743	0.0013	11.17	2.47	0.680	0.0224
RE_{area}	max T (ºC)	-27.07	2.13	0.371	0.1465	-59.61	2.69	0.387	0.0549	-9.94	1.76	0.234	0.2716
RE_{area}	Annual rainfall (mm)	38.81	0.043	0.918	0.0007	31.78	0.039	0.336	0.0791	54.16	0.0161	0.057	0.6070
RE_{area}	Emberger's index	46.54	0.241	0.416	0.1179	62.81	-0.195	0.076	0.4394	88.55	-0.42	0.574	0.0484
RE_{area}	Total soil N content (%)	48.21	172.9	0.519	0.0677	46.62	50.72	0.044	0.5599	58.84	42.11	0.059	0.5996
RE_{mass}	N_{mass} (mg g^{-1})	-88.36	8.99	0.493	0.0785	-38.84	6.38	0.340	0.0767	40.67	2.03	0.138	0.3644
RE_{area}	N_{area} (g m^{-2})	135.6	-26.31	0.561	0.0527	108.3	-18.19	0.095	0.3873	85.72	-5.83	0.101	0.4423

mean m min T Mean monthly minimum daily temperature, *min T* Minimum winter temperature, *mean m max T* Mean monthly maximum daily temperature, *max T* Maximum summer temperature, N_{mass} N concentration per unit mass in green leaves, N_{area} N content per unit area in green leaves content in green leaves in response to the number of frosts, with the exception of N content per unit area in *Q. suber* (Fig. 2). By contrast, N concentrations in leaf litter showed a clear positive relationship with the number of frosts at each site (Fig. 2).

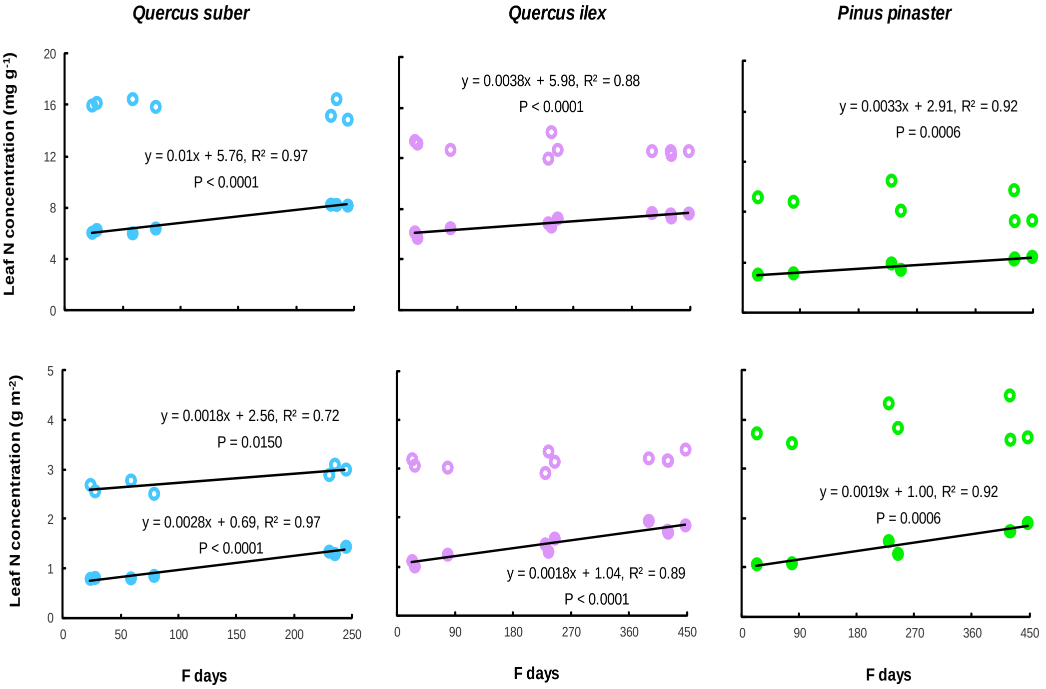

Fig. 2 Nitrogen contents in green (*open symbols*) and senesced (*closed symbols*) leaves as a function of the total number of days with frost along the study period (F days) at each site. Error bars are omitted for clarity

Relationships between nitrogen resorption and different leaf traits

In the three species, LMA, leaf thickness and the structural carbohydrate content (cellulose + hemicellulose) showed a pronounced response to changes in temperature, increasing between the sites with the intensity of their winter harshness (higher total number of days with frost and lower minimum temperatures) (data not shown). Accordingly, the reinforcement of the leaves with a greater LMA as the harshening of winter conditions progresses seems to occur through leaf thickening as a result of a greater amount of structural carbohydrates. Indeed, there was a strong positive relationship between the structural carbohydrate content and the leaf thickness for the three species (Fig. 3). This

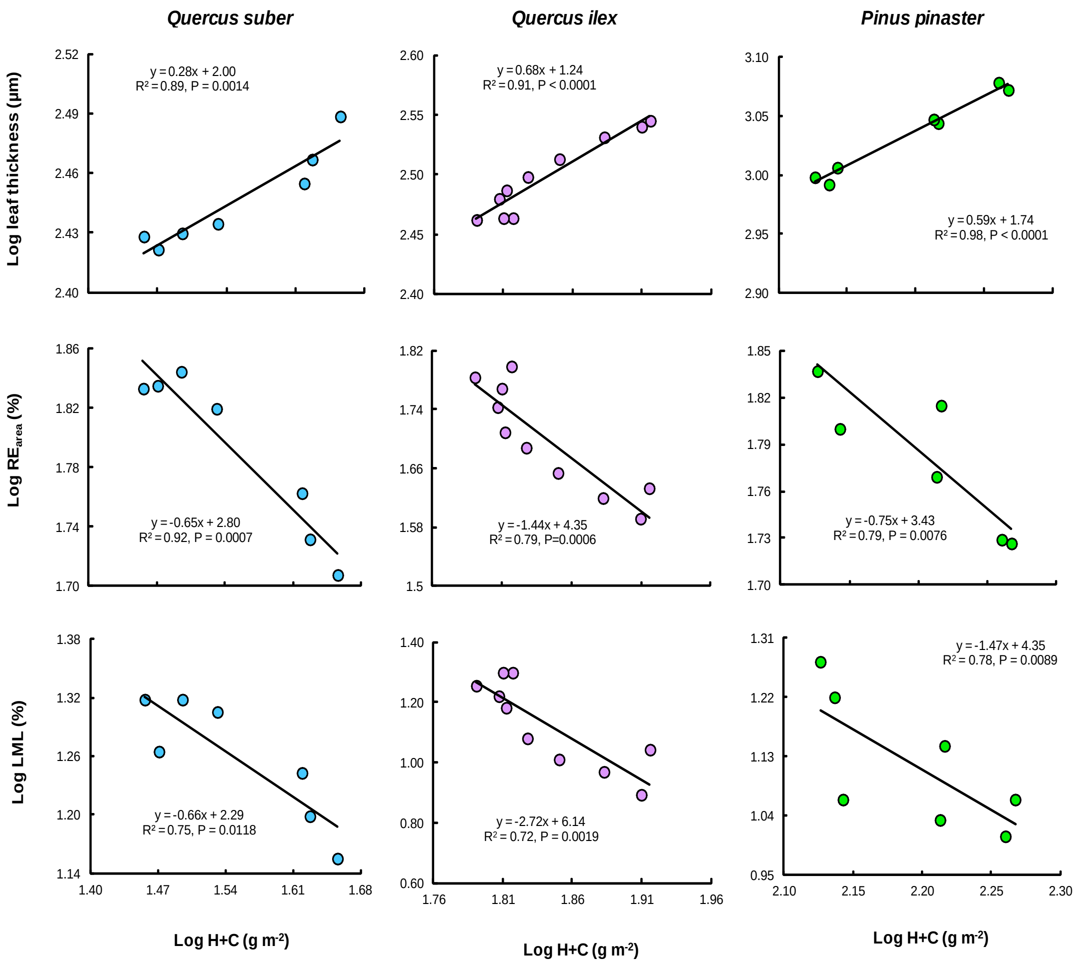

Fig. 3 Green leaf thickness, nitrogen resorption efficiency on a per unit leaf area (RE_{area}) basis and leaf mass loss during senescence (LML) and as a function of hemicellulose and cellulose (H + C) contents in green leaves at each site. Error bars are omitted for clarity.

leaf reinforcement at the coldest sites resulted in significant negative effects of the structural carbohydrate content on N resorption efficiency and leaf mass loss during senescence (Fig. 3). Since no trend was observed for the nitrogen content in green leaves in response to temperature, no significant relationships between leaf N content and resorption efficiency were observed (Table 2). Minimum N concentrations in leaf litter increased (proficiency decreased) with the increase in LMA and the structural carbohydrate content in all three species (Table 3). No significant relationships were observed between the N concentration in green leaves and the resorption proficiency for any of the species (Table 3).

Table 3. Linear regression parameters for N content (mg g^{-1}) in leaf litter depending on leaf mass per unit area (LMA, g m^{-2} hemicellulose + cellulose content (H + C, mg g^{-1}) and N content (mg g^{-1}) in green leaves.

Dependent variable	Independent variables	*Q. suber* (*n* = 7)			*Q. ilex* (n = 10)			*P. pinaster* (n = 7)		
		y	R^2	P	y	R^2	P	y	R^2	P
N (leaf litter)	LMA	0.06x - 2.94	0.82	0.0050	0.05x - 4.70	0.82	0.0003	0.01x - 1.13	0.59	0.0449
	H + C	0.04x - 1.48	0.72	0.0162	0.03x - 1.81	0.51	0.0197	0.03x - 5.91	0.60	0.0424
	N (green leaves)		NS			NS			NS	

DISCUSSION

All the leaves studied underwent a decrease in mass per unit area during senescence. Mass loss can be due to several processes, including resorption, leaching and in situ decomposition between the time of leaf abscission and the collection of the fallen leaves (Vergutz et al. 2012). In the present case, losses due to decomposition and leaching were unlikely, because most leaves were shed during the dry season and remained suspended in the air until they were sampled thanks to the retention clips used. Accordingly, the losses of biomass during senescence must essentially be the consequence of the remobilization of carbon, which -as demonstrated by several authors- represents an important source for growth and reproduction (Eckstein et al. 1998; Karlsson 1994; Vergutz et al. 2012). Evidently, these leaf mass losses affect the total content of N of the senescent leaves and, accordingly, also the estimation and interpretation of the resorption data (Fife et al. 2008; Franklin and Ågren 2002; van Heerwaarden et al. 2003). This is because if leaf mass declines during senescence, nutrient resorption, expressed as a percentage of mass, is underestimated (Chapin and Kedrowski 1983; van Heerwaarden et al. 2003). Indeed, in our study in all cases the N resorption percentages proved to be higher when expressed on a leaf area basis. However, regardless of the mode of expression in all cases a decrease was observed in the N resorption efficiency in the colder environments. In addition, leaf mass losses during senescence were also more marked in warmer environments, which exacerbated the between-site differences in N resorption when expressed per unit leaf area. Killingbeck (1996) proposed that resorption is highly proficient in plants that have reduced their leaf N during senescence to concentrations lower than 7 mg g^{-1}. Thus, according to this proposed resorption threshold, only *P. pinaster* would reach complete N resorption along the whole temperature gradient, with minimum N values in senesced leaves significantly lower than those of the other species. In both *Quercus* species, however, minimum N concentrations in senesced leaves were only below this threshold in the warmer environments, incomplete resorption efficiency being observed at the cooler sites. The lower resorption efficiency at colder sites was mainly due to the effect of minimum winter temperatures. Between-site differences in winter temperatures and in the frequency and intensity of frosts were much more marked than the differences in summer maximum temperatures. As a consequence summer temperatures showed much weaker effects on resorption efficiencies. Differences in the intensity of drought stress is one of the factors previously reported as determinants of the differences in resorption efficiency (Del Arco et al. 1991; Pugnaire and Chapin 1992). Higher summer temperatures could thus have an effect on N resorption if they determine more severe drought at the warmer sites. However, in the present study the warmer sites also receive greater rainfall, which could reduce the differences in drought stress. In fact, the Emberger’s pluviothermic index was not related to summer temperatures (Table 1). Accordingly, rainfall levels and the pluviothermic index tended to have weak and inconsistent effects on resorption, which suggests that the between-site differences in N withdrawal were a direct effect of minimum winter temperatures and not of differences in the levels of drought stress during summer.

According to our results, in environments subjected to a higher intensity of winter harshness the leaves of the three species were reinforced with a greater LMA due to the increased thickness achieved with greater amounts of structural carbohydrates. The increased leaf thickness and cell wall content appear to be necessary to reduce the risk of freezing in leaves in cold environments (Ball et al. 2002; Rajashekar and Lafta 1996). Unlike other studies, which have reported increases in foliar N contents with decreases in temperature (Jian et al. 2009; Reich and Oleksyn 2004; Weih and Karlsson 2001), we did not find any trend in the amounts of this nutrient in green leaves associated with changes in temperature, as in fact has also been observed in other cases (Chen et al. 2013; Hultine and Marshall 2000; Premoli and Brewer 2007). The changes in leaf morphology and chemical composition were also accompanied by a significant decrease in N resorption efficiency and proficiency with lower winter temperatures.

Recent studies have reported lower resorption efficiencies in plant communities occupying warmer environments (Tang et al 2013; Vergutz et al. 2012; Yuan and Chen 2009). Yuan and Chen (2009) suggested that this response of resorption efficiency to temperature can be explained mainly in terms of changes with temperature in the N concentrations in green leaves, which decrease in warmer environments, with relatively smaller changes in the N concentrations in senesced leaves. Their results are consistent with those reported in the literature suggesting that resorption efficiency is modulated by the N status of plants (Chapin and Kredowski 1983; Escudero et al. 1992; Fife et al. 2008), with more resorption in plants with more nitrogen in green leaves, probably because the amount of highly retranslocatable N is higher than that found in N-poor leaves (Chapin and Kredowski 1983; Milla et al. 2005). In the present study, however, the differences in resorption efficiency between environments were related to changes in the N content in senesced leaves, with no associated trends in the N content in green leaves. Thus, the lower efficiency within a same species found in colder environments may instead be explained by the higher amounts of N immobilized in the greater amount of cell wall needed to cope with lower winter temperatures in these environments. Our results therefore support the hypothesis that the potential for N resorption varies with the amount of cell wall in the leaves, decreasing with a higher fraction of N associated with structural proteins occluded in the cell wall matrix (Hikosaka 2004; Niinemets and Tamm 2005). The need for stronger leaf reinforcement in colder areas inevitably leads to lower N resorption efficiency. Changes in leaf chemical composition in response to temperature must therefore be incorporated into the range of factors that can be invoked to explain the intraspecific variations in N resorption efficiency.

The differences in structural carbohydrate contents along the temperature gradient also explain the differences in leaf mass loss during senescence, since C resorption is determined by the availability of retranslocatable nonstructural C fractions (Vergutz et al. 2012).

Our results are therefore not in agreement with the conclusions of previous analyses of geographic patterns of nutrient resorption. These discrepancies might be explained by the fact that most previous studies describe resorption differences among different plant communities, which may be affected by differences in the leaf traits of the different species, such as differences in leaf life span (Vergutz et al. 2012), whereas in the present study temperature effects were measured within a same species. However, Oleksyn et al. (2003) also found a trend towards higher resorption efficiencies in northern latitudes in Scots pine populations, i.e., a result opposite to that found in the present study. In principle, we do not have any explanation for this discrepancy. The resorption efficiencies in Oleksyn et al. (2003) were expressed on a concentration per unit mass basis, which may lead to biased results if, as observed here, leaf mass loss during senescence varies with temperature. In addition, the survey of those authors was performed in colder areas than those addressed in the present study and along broader geographical gradients. These differences might involve additional uncontrolled factors that could explain the mentioned discrepancy.

Although it is difficult to extrapolate the responses observed here to a new scenario of climate change, it is evident that all these temperature-related changes must have profound ecological implications. The evergreen habit demands that leaves must be present throughout the year, overcoming unfavorable seasons, among them winter. As suggested by our observations, evergreen species must reinforce their leaves to a greater extent at the coldest sites in order to maintain their leaf longevity. This necessary greater leaf reinforcement at the coldest sites results in lower N resorption efficiency, thereby increasing the dependence on soil N. This must contribute to increasing the costs of the evergreen habit at the coldest sites. If climate change eventually leads to an increase in temperatures and a decrease in winter harshness (IPCC 2007), this could reduce the unfavorable effects associated with the leaf traits shown by evergreen species under conditions of low temperatures. It is clear in any case that the effect of temperature on the resorption process must also be incorporated into predictive models of the implications of climate change on vegetation.

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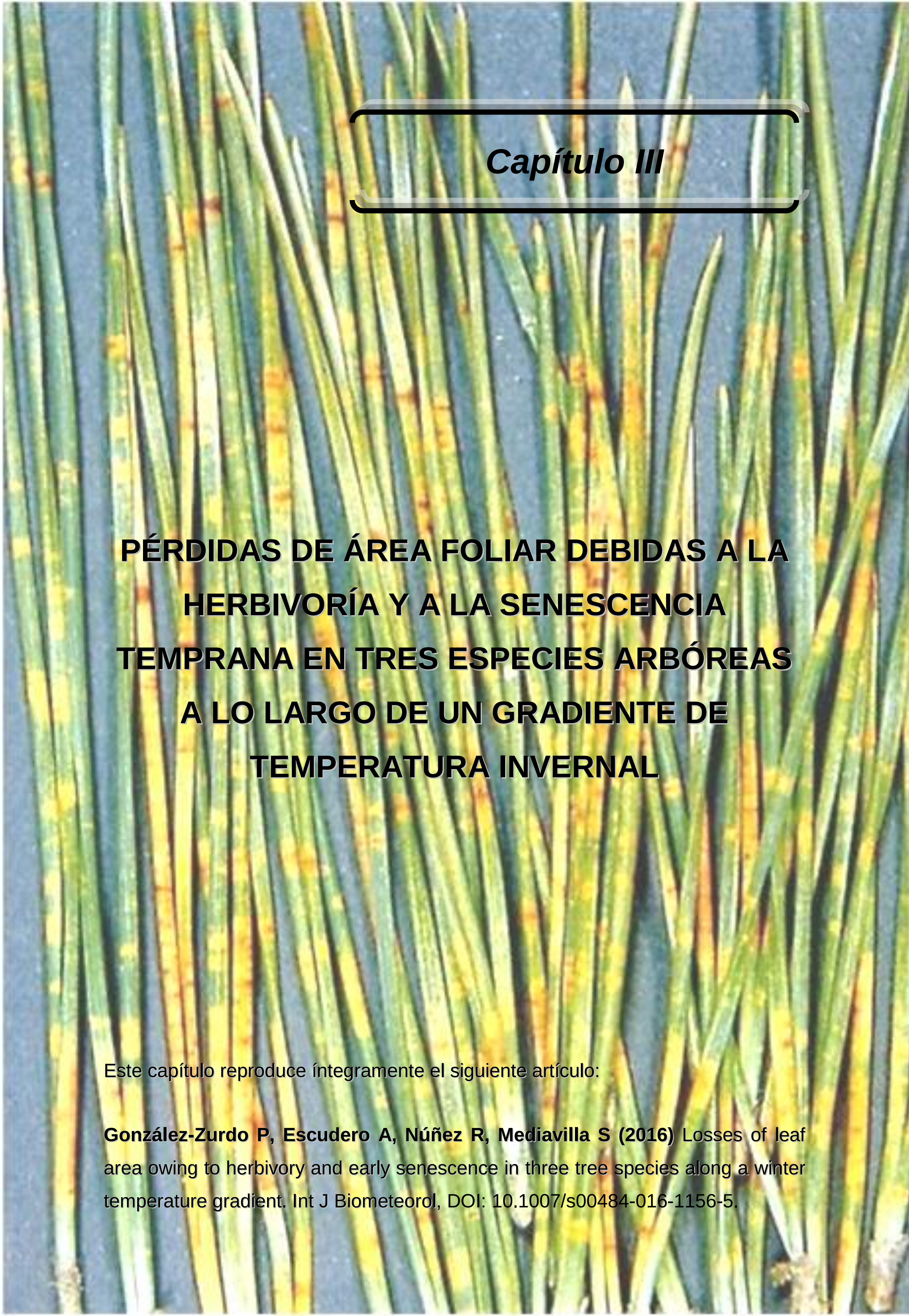

Capítulo III

PÉRDIDAS DE ÁREA FOLIAR DEBIDAS A LA HERBIVORÍA Y A LA SENESCENCIA TEMPRANA EN TRES ESPECIES ARBÓREAS A LO LARGO DE UN GRADIENTE DE TEMPERATURA INVERNAL

Este capítulo reproduce íntegramente el siguiente artículo:

González-Zurdo P, Escudero A, Núñez R, Mediavilla S (2016) Losses of leaf area owing to herbivory and early senescence in three tree species along a winter temperature gradient. Int J Biometeorol, DOI: 10.1007/s00484-016-1156-5.

RESUMEN

En los climas templados, las hojas perennes tienen que sobrevivir a lo largo de periodos invernales con bajas temperaturas. Los daños por congelación y frío pueden provocar la senescencia acelerada de una parte de la superficie de la hoja, lo que contribuye a la reducción de la vida útil de la maquinaria fotosintética y del tiempo disponible para la asimilación de carbono. Las bajas temperaturas también están asociadas a cambios en la morfología y composición química foliar que afectan al consumo por los herbívoros. Por lo tanto, la intensidad de las pérdidas de área foliar debidas a la senescencia anticipada y la herbivoría puede cambiar a lo largo de gradientes de temperatura invernal. El objetivo de este estudio es analizar dichas respuestas en las hojas de tres especies perennifolias (*Quercus ilex*, *Q. suber* y *Pinus pinaster*) a lo largo de un gradiente climático. Las hojas de las tres especies presentaban un mayor peso por unidad de área foliar (LMA) y mayores concentraciones de carbohidratos estructurales en las zonas más frías. Sólo las dos especies de *Quercus* mostraban síntomas visibles de daño causado por herbivoría, siendo menos intenso en los lugares más fríos. Las hojas de las tres especies mostraban manchas por clorosis y necrosis que se incrementaban con la edad de las hojas. La superficie foliar afectada por clorosis y necrosis era mayor en los sitios con los inviernos más fríos. Por lo tanto, los efectos del frío invernal sobre la vida útil de la maquinaria fotosintética eran contradictorios: las pérdidas de área foliar debidas a la senescencia anticipada se incrementaban, pero descendían las pérdidas causadas por herbivoría. Las consecuencias finales para la asimilación de carbono dependen en gran medida del momento exacto de la aparición del daño resultante de las bajas temperaturas y del consumo por los herbívoros.

Palabras clave: estrés abiótico, herbivoría, nitrógeno foliar, reforzamiento estructural, temperatura invernal.

LOSSES OF LEAF AREA OWING TO HERBIVORY AND EARLY SENESCENCE IN THREE TREE SPECIES ALONG A WINTER TEMPERATURE GRADIENT

ABSTRACT

In temperate climates, evergreen leaves have to survive throughout low temperature winter periods. Freezing and chilling injuries can lead to accelerated senescence of part of the leaf surface, which contributes to a reduction of the lifespan of the photosynthetic machinery and of leaf lifetime carbon gain. Low temperatures are also associated with changes in foliar chemistry and morphology that affect consumption by herbivores. Therefore, the severity of foliar area losses caused by accelerated senescence and herbivory can change along winter temperature gradients. The aim of this study is to analyse such responses in the leaves of three evergreen species (*Quercus ilex*, *Q. suber* and *Pinus pinaster*) along a climatic gradient. The leaves of all three species presented increased leaf mass per area (LMA) and higher concentrations of structural carbohydrates in cooler areas. Only the two oak species showed visible symptoms of damage caused by herbivory, this being less intense at the coldest sites. The leaves of all three species presented chlorotic and necrotic spots that increased in size with leaf age. The foliar surface affected by chlorosis and necrosis was larger at the sites with the coldest winters. Therefore, the effects of the winter cold on the lifespan of the photosynthetic machinery were contradictory: losses of leaf area due to accelerated senescence increased, but there was a decrease in losses caused by herbivory. The final consequences for carbon assimilation strongly depend on the exact timing of the appearance of the damage resulting from low temperature and grazing by herbivores.

Key words: abiotic stress, herbivory, leaf nitrogen, evergreen species, structural reinforcement, winter temperature.

INTRODUCTION

The study of the changes in foliar traits along environmental gradients has become a useful tool to assess the effects of climate change on the productivity and distribution patterns of the different tree species (Mediavilla et al. 2012; Klein et al. 2013). The analysis of changes in leaf traits across different environments shows that there is a trend towards an increase in leaf mass per unit leaf area (LMA) in colder environments (Ogaya and Peñuelas 2007; Atkin et al. 2008). Similarly, several authors reported increases in N contents in leaves produced in colder sites (Weih and Karlsson 2001; Jian et al. 2009), although in other studies the patterns in N content proved inconsistent (Hultine and Marshall 2000; Chen et al. 2013). These changes in LMA and, possibly, in nitrogen content along temperature gradients should affect productivity in different environments, given the effects of both traits on instantaneous photosynthesis per unit leaf mass (Reich et al. 1997; Mediavilla et al. 2001; Niinemets and Sack 2006).

Temperature gradients also affect other productivity-determining factors, for example, the effects of temperature on plant-herbivore interactions have been frequently reported (Veteli et al. 2002; Jamieson et al. 2012). Temperature effects on the growth of herbivore populations are often direct. Higher temperatures promote higher metabolic rates that lead to higher consumption, growth and development rates (Jamieson et al. 2012). Moreover, warmer late winter and early spring temperatures tend to enhance insect survival (Bale et al. 2002). Furthermore, differences in foliar nitrogen content affect herbivores' preferences and, therefore, higher leaf N concentrations lead to stronger attack rates (White 1984; Agrawal 2004). On the other hand, leaf mass per unit area is known to correlate positively with leaf rigidity and hardness (Kudo 1996), often being considered an adaptation aimed at reducing vulnerability to herbivore attacks and other stress factors (Wright and Cannon 2001; Shipley et al. 2006). Thus, LMA and nitrogen changes along climatic gradients might interact with the direct effects of temperature on herbivore activity, leading to differences in the levels of herbivory. Herbivory is a major determinant of leaf mortality rates, and has been shown to affect plant fitness (Moles and Westoby 2000; Mothershead and Marquis 2000). Leaf area losses resulting from herbivory usually take place at the early stages of leaf development and can significantly reduce the photosynthetic surface for a large part of the leaf's lifespan (Coley 1983; Kudo 1996; Moles and Westoby 2000).

Apart from the damage caused by herbivory, abiotic stress produces different physiological reactions and structural changes resembling natural senescence. These processes occur before the age of programmed senescence and may thus be interpreted as accelerated cell senescence (Günthardt-Goerg and Vollenweider 2007). Among other abiotic stress factors, low temperatures are known to produce irreversible damage that accumulates as the leaf ages (Günthardt-Goerg and Vollenweider 2007). Accordingly, the intensity of leaf area losses should also vary along temperature gradients, provided that other stress factors remain similar. Frequently, abiotic stress, such as drought, frost or chilling, results in necrotic areas bordered by green tissue (Vollenweider and Günthardt-Goerg 2005), which implies that only part of the leaf area is affected and that the individual leaf may remain alive until it reaches its age of programmed senescence. Similarly, for species with a long leaf lifespan, a large amount of herbivory damage may occur before the leaves are shed (Poorter et al. 2004). Therefore, leaf area losses resulting from herbivory and anticipated senescence may lead to a significant reduction in the amount of photosynthetic machinery before the end of the leaf's lifespan. As a result, individual leaves may have a longer life span than any unit of leaf mass or nutrients (Mediavilla and Escudero 2003a). Obviously, the shorter duration of the photosynthetic machinery renders the estimates of leaf-level carbon balance inaccurate, when they are based on the lifespan of individual leaves (Mediavilla and Escudero 2003b; Kikuzawa and Lechowicz 2006). In evergreen species, leaf lifespan tends to increase with decreasing temperatures (Wright et al. 2005), probably reflecting the longer payback time for construction costs in such conditions, due to the shorter growing season. However, this trend could be compensated by anticipated losses of leaf area under conditions of cold stress.

We are not aware of any studies devoted to the analysis of changes in the intensity of foliar damage due to abiotic factors along climatic gradients. Conversely, there are numerous studies on the damage caused by herbivory along different environmental gradients, albeit with contradictory results. Thus, for example, while there are cases where higher levels of herbivory were detected in warmer environments (Bale et al. 2002; Bjorkman et al. 2011), other authors' results show higher levels of consumption in colder environments (Coley and Aide 1991; Aizen and Williams 1995) or no trends along temperature gradients (Andrew and Hughes 2007; Adams et al. 2009). In most studies, the sampled species changed along the climatic gradients, which makes it difficult to identify the factors responsible for the trends in levels of herbivory. The task of analysing leaf damage caused by herbivory and other factors is very time-consuming, which could be one of the reasons why this aspect has not been integrated into previous production models. There is a clear need for studies that analyse differences in leaf area losses comparing different environments, so that we might understand the impacts of climate change on the potential distribution of tree species.

The purpose of this study is to analyse damage to the leaf area caused by different stress factors in three evergreen tree species differing in leaf life span (*Quercus ilex* ssp*. ballota* (Desf.) Samp, *Quercus suber* L. and *Pinus pinaster* Aiton) located at different sites along a temperature gradient and to determine the effects of differences in foliar traits on possible differences in damage intensity. An earlier study using the same species and locations showed that there were significant site-related differences in several leaf traits. Thus, the leaves of the analysed species exhibited higher LMA, thickness and structural carbohydrate content in colder areas, with no trends in nitrogen content (Mediavilla et al. 2012; González-Zurdo et al. 2015). These differences among sites suggest the need for greater structural reinforcement under harsher winter conditions, which would involve higher costs in the coldest areas, mainly due to the negative relationship between LMA and instantaneous photosynthesis (Reich et al. 1997; Niinemets and Sack 2006). These costs could be compensated, however, if the greater reinforcement were to involve a decrease in herbivory-related leaf area losses in colder environments. Therefore, our aim is to test whether the greater leaf structural reinforcement in cold environments involves lower costs in terms of losses of photosynthetic area associated with herbivory. By contrast, we anticipate that losses of photosynthetic area caused by accelerated senescence would be higher in colder environments.

MATERIALS AND METHODS

Species and study areas

Three evergreen species were selected for the study: *Pinus pinaster* Aiton, *Quercus suber* L. and *Q. ilex* ssp. *ballota* (Desf.) Samp. The species were distributed over six sites located in the regions of Castilla-Leon and Extremadura (central-western Spain) between latitudes 41º 45' N and 39º 49' N and longitudes 6º 22' W and 2º 37' W (Table 1). Altitudes range between 466 and 1189 m a.s.l. Because of the differences in geographical location and altitude, two of the selected sites may be categorized as cold, with the lowest minimum temperatures and the highest number of frost days; two sites could be identified as warm and the remaining two as having intermediate winter conditions. The sites consisted of flat areas with sparse populations (between 50 and 100 specimens ha^{-1}) of mature (over 100 years old) individuals. Each site was selected so as to include as many study species as possible and to cover a wide winter temperature gradient, at the same time making sure that the rest of climatic characteristics were as homogeneous as possible. Nevertheless, there is a tendency for rainfall levels to be higher in the hottest and southernmost sites, which helps to reduce the differences in the intensity of drought stress between cold and hot sites (Table 1). Owing to its lower tolerance to low temperatures, *Q. suber* was absent at the coldest sites. Rainfall data were obtained from the digital climatic atlas of the Iberian Peninsula (Ninyerola et al. 2005): a set of digital climatic maps of mean air temperature, precipitation

Table 1. Sites characteristics.

STANDS	A	B	C	D	E	F
Longitude (W)	5º 48´	6º 22'	5º 52´	5º 47´	2º 37´	2º 52´
Latitude (N)	40º 01´	39º 49'	41º 14´	41º 08´	41º 45´	41º 43´
Altitude (m.a.s.l.)	466	600	985	834	1189	1045
Climate characteristics						
Mean annual temperature (ºC)	16.6	16.8	12.3	12.7	10.3	10.6
Mean spring temperature (ºC)	19.3	19.0	15.0	14.9	13.0	13.2
Mean summer temperature (ºC)	25.8	26.3	20.8	21.4	19.1	19.1
Mean autumn temperature (ºC)	12.2	13.2	7.84	9.11	6.03	6.35
Mean winter temperature (ºC)	9.07	9.81	5.55	5.30	3.14	3.53
Absolute minimum temperature (ºC)	-4.15	-3.55	-8.18	-9.82	-13.7	-16.9
Mean daily minimum temperature of the coldest month (ºC)	4.06	4.69	-0.47	-0.90	-2.93	-3.13
Number of days with frost per year	15	5	57	61	105	112
Mean duration of the period with frosts (days per year)	68	55	127	154	222	214
Precipitation (mm)						
Annual	986	672	495	460	567	646
Summer	54	51	60	54	107	111
Emberger's index	105	60	52	46	79	78
Ozone indicator AOT40 (µg m^{-3} h)	23576	22192	21676	23318	20895	22928
Soil						
Sand content (%)	77.2	73.9	84.6	74.3	75.1	84.0
Clay content (%)	11.7	12.3	6.40	12.2	14.4	8.30
Silt content (%)	11.1	13.8	9.00	13.5	10.5	7.70
Total soil N content (%)	0.143	0.104	0.072	0.021	0.115	0.048
P assimilable (ppm)	38	5	10	4	7	6
Organic matter (%)	4.47	4.10	2.60	0.37	5.12	1.92
pH	4.5	4.7	4.6	4.5	4.8	6.1
Species	Pp, Qi, Qs	Pp, Qi, Qs	Pp, Qi, Qs	Pp, Qi, Qs	Pp, Qi	Pp, Qi

Pp *Pinus pinaster* ; Qi *Quercus ilex*, Qs *Quercus suber* and solar radiation elaborated with 200-m resolution by using data from climate stations and a combination of geographical variables (altitude, latitude, continentality, solar radiation and terrain curvature). Temperature data were obtained for each site by means of data loggers (Hobo Pendant temperature/light datalogger, Part UA-002-08, Onset Computer Corporation, Pocasset, MA, USA) programmed to obtain temperature data every 10 min and kept at each site for 6 years (October 2008 – October 2014). Data on ozone concentrations were obtained from The European Environment Agency (EEA) (The European air quality database and http://www.eea.europa.eu/data-and-maps/data/airbase-the-european-air-qualitydatabase-8), using the information from the nearest control stations. In all cases the stations were situated at an altitude similar to the study sites. Ozone levels were expressed according to the index AOT40 (accumulated ozone over threshold 80 µg m^{-3}) along years 2012-2014 (Table 1). Soil samples were taken up to a depth of 20 cm (excluding the forest floor) from each stand. Determinations of soil granulometry, pH and N and P concentrations were carried out at the Soils Laboratory of the Institute of Natural Resources and Agrobiology of Salamanca, according to the methods described by Chapmann and Pratt (1973) and Walkley and Black (1934).

Sample collection and processing

At each site, a branch from each of five mature specimens of each species selected randomly was taken at the top of the crown. The trees chosen were sun-exposed mature individuals whose trunk diameter at a height of 1.3 m ranged from 40 to 60 cm, mean heights being 8-10 m. All the samples were gathered in mid-October 2014.

The samples were taken immediately to the laboratory and the branches were separated into annual segments (shoots) of different age classes. Only one flush of leaf growth was observed in all species. Accordingly, all the leaves sprouting in a given year were considered to belong to the same age class. All the leaves from each species and age were visually analyzed and classified into different categories depending on whether they presented visible symptoms of damage or not. The damaged leaves were further classified according to damage type: leaves attacked by herbivores, leaves with symptoms of abiotic stress and leaves with both types of damage (herbivory and abiotic factors). There are many studies that offer plentiful graphic information that allows for identifying both biotic and abiotic stress symptoms in leaves (Vollenweider and Günthardt-Goerg 2005; Günthardt-Goerg and Vollenweider 2007). Samples from *Pinus pinaster* were only separated into two categories (healthy leaves and leaves presenting abiotic damage) because the herbivory levels detected for this species were practically negligible. After sorting, a count of the number of leaves in each category was made for each of the selected tree individuals of each species. A total of around 54,000 leaves were examined to detect damage and its type.

To check possible differences in the traits of the leaves of a same species according to the different environments and damage categories, 50 leaves of each species, leaf age class and category (healthy, with damage by herbivores and with abiotic damage) from each site (10 leaves of each category from each of the 5 selected specimens) were randomly selected. Leaf thickness was measured with a digital micrometer (Digimatic micrometer, Mitutoyo, Japan) as a mean of three measurements taken at random positions on each leaf or needle, avoiding the main ribs on flat leaves. The total projected leaf and needle areas were determined by an image analysis system (Delta-T Devices LTD, Cambridge, UK). In the case of *P. pinaster*, we also measured needle length with the digital micrometer. The samples were then oven-dried at 70 ºC to constant weight and the total dry mass was determined. From the data thus obtained, we calculated the leaf dry mass per area (LMA). Once all the data had been collected, a value for each specimen and leaf age class was calculated as the average of 10 leaves of each damage category. The 10 individual leaves taken from each specimen and leaf age class were ground together to obtain a sufficient amount of sample for the chemical analyses. Leaf N concentrations were determined with a

CE-Instruments NA-2100 auto analyser (ThermoQuest, Milan, Italy). After the N analyses, the remaining material was used to analyze the fibre content (hemicellulose, cellulose and lignin) with an Ankom Analyzer (A220, New York, USA), following the method of Goering and Van Soest (1970). The nitrogen and fibre contents of leaves were expressed per unit dry mass (as milligrams of nutrient or fibre per gram of leaf dry mass), and N also as per unit of leaf area, obtained as the N content per unit dry mass multiplied by LMA.

The foliar surface lost to herbivores or damaged by abiotic stress was also determined based on 10 leaves per tree individual up to a total of 50 from each species and age class, from each site, using the ImageJ programme (http://rsb.info.nih.gov/ij/; Abràmoff et al. 2004). The leaves were first subjected to high-resolution scanning (2300 x 3300 pixels). The ImageJ programme allowed for the analysis of the images obtained, measuring both the whole area of each leaf and those areas with a different colouration due to necrosis and chlorosis caused by abiotic stress damage. The area lost to herbivores was estimated by reconstructing leaf outlines and calculating the difference between the reconstructed leaf and the actual area. Thus, the percentage of surface loss caused by herbivory or other types of damage was estimated for each of the 50 leaves analysed in each case.

Additional branches were sampled on a 2-monthly basis to estimate leaf lifespan. Five mature specimens of each species were selected randomly at each site during each sampling session. A composite sampling of sun-exposed branches with leaves from different crown positions in each canopy was undertaken for each individual selected. The number of leaves or needles per shoot was counted for each age class and the data were used to construct static life tables, which made it possible to estimate the mean leaf life span for each species according to standard methods.

Data analysis

Once the leaves of each type had been counted up, we calculated the percentage of damaged leaf area for each tree. A final estimate of damage was obtained for each species and leaf age class from each site as a mean of the five trees selected in each case. One-way analysis of variance (ANOVA) was used to examine the differences among sites within each species. Linear regression analysis was used to explore the relationships within each species between levels of herbivory and leaf traits at the different sites. Regression analysis was also used to test the relationship between climate and level of damage at each site. Each site was given a single value for the average temperature and mean daily maximum and minimum temperatures, obtained as the average of the mean values measured each month over 4 years. A value for the maximum and minimum temperatures recorded was also obtained. In addition to temperature, the total annual and summer precipitation of each site and the Emberger's pluviothermic index (Emberger 1930) were used to analyse the effect of possible differences in water availability on the level of foliar damage recorded at each site. Emberger's index, commonly used in Mediterranean climates (Kunstler et al. 2007), was obtained through the following equation:

$$Q = \frac{1000 \times P}{(273 + \frac{T_X + T_N}{2}) \times (T_X - T_N)}$$

where P is the annual precipitation (mm), T_X is the average temperature of the hottest month (ºC) and T_N is the average temperature of the coldest month (°C). All statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA).

RESULTS

Intraspecific variation in the traits of healthy leaves along the climatic gradient

No significant differences were found between the two sites selected in each of the three climatic environments for any of the leaf traits analysed. Conversely, ANOVA results revealed differences among the three climatic environments for most of the leaf traits (Table

2). There was a tendency for leaves of a same species and age class produced at the warmest sites to be larger (larger area in the two *Quercus* and longer needles in *P. pinaster*), but thinner, with lower LMA and fibre content than in the coldest sites, with significant differences among sites in practically all of the cases (Table 2). The leaves from sites with intermediate climates also yielded intermediate values for thickness, LMA and fibre contents (Table 2). By contrast, between-sites differences in lignin concentrations were non-significant (not shown). Similarly, differences in nitrogen concentration per unit mass (N_{mass}) were small and non-significant in most of the cases. The differences in N content per unit area (N_{area}) tended to be more pronounced because of the increase in LMA at the coldest sites, although, in most cases, these differences did not reach significance either (Table 2). Leaf life span showed a non-significant trend to decrease with the increase in temperature in *P. pinaster*, but there were no trends for the other two species. The LMA, thickness and fibre content increased with the leaf longevity of the different species, whereas the opposite happened with nitrogen content per unit mass, which tended to be lower in leaves with longer life spans (Table 2).

Intraspecific differences in the levels of foliar damage along the climatic gradient

Most herbivorous damage was due to leaf area loss by leaf chewers and scars on leaf blades mainly by skeletonizers. The two species that presented significant damage caused by herbivory showed significant differences in level of damage in the different areas of the climatic gradient, the percentage of herbivore-attacked leaves and average area consumed per leaf always being significantly higher in the warmest sites (Table 3). In the case of *Q. ilex*, leaves also showed intermediate levels of consumption at the sites with intermediate climate characteristics (Table 3). The differences among environments could be observed in all leaf age classes. There were no differences in the intensity of herbivore damage among different age classes, the leaves of the different cohorts showing similar proportions of attacked leaves, as well as of consumed areas in both species (Table 3). Between the two

Table 2. Traits of the healthy leaves (SE in parentheses, *n* = 10 trees per species) and mean leaf life span of the three species under different climatic conditions.

Species	Leaf age class	Sites temperatures	Leaf size	Leaf thickness (µm)	LMA (gm^{-2})	H+C ($mg\ g^{-1}$)	Total fibre ($mg\ g^{-1}$)	Nmass ($mg\ g^{-1}$)	Narea (gm^{-2})	LL (days)
Q. suber	1	Warm	5.23 (0.33) a	252 (7.29) b	166 (6.21) b	165 (2.48) b	315 (5.40) b	16.6 (0.33) a	2.74 (0.09) a	365 (0.5) a
		Intermediate	4.21 (0.24) b	274 (8.14) a	190 (5.96) a	233 (2.84) a	387 (4.54) a	15.8 (0.47) a	3.02 (0.15) a	382 (4.0) a
Q. ilex	1	Warm	3.02 (0.10) a	269 (3.92) c	202 (6.83) c	214 (3.73) b	350 (6.72) c	14.3 (0.42) a	2.91 (0.16) b	817 (62) a
		Intermediate	2.64 (0.10) ab	304 (7.05) b	236 (8.62) b	260 (2.54) a	403 (296) b	12.7 (0.60) b	2.99 (0.16) b	814 (41) a
		Cold	2.10 (0.09) b	353 (4.90) a	267 (12.9) a	284 (3.56) a	433 (3.83) a	14.2 (0.21) a	3.80 (0.16) a	820 (26) a
	2	Warm	2.96 (0.11) a	289 (4.18) c	215 (5.92) c	226 (3.38) c	371 (3.99) c	11.8 (0.47) b	2.54 (0.13) b	
		Intermediate	2.67 (0.12) ab	323 (16.4) b	247 (5.30) b	269 (3.91) b	421 (5.97) b	10.8 (0.25) c	2.66 (0.04) b	
		Cold	1.92 (0.14) b	362 (6.01) a	278 (8.97) a	308 (5.01) a	456 (7.53) a	12.8 (0.39) a	3.58 (0.20) a	
P. pinaster	1	Warm	133 (4.89) a	919 (20.0) c	355 (14.6) b	303 (5.36) c	456 (4.95) b	9.26 (0.42) a	3.29 (0.24) a	1099 (50) a
		Intermediate	117 (6.77) a	984 (35.2) b	369 (3.00) ab	336 (6.69) b	503 (8.20) a	10.1 (0.92) a	3.77 (0.42) a	1169 (70) a
		Cold	95 (2.56) b	1065 (16.0) a	393 (13.0) a	365 (3.04) a	514 (3.86) a	9.39 (0.70) a	3.68 (0.28) a	1290 (101) a
	2	Warm	139 (2.72) a	951 (12.7) b	367 (23.3) b	321 (5.33) b	474 (4.75) b	8.92 (0.46) a	3.26 (0.18) b	
		Intermediate	124 (6.21) ab	1076 (32.7) ab	391 (6.89) ab	351 (5.80) a	510 (10.2) a	9.58 (0.52) a	3.78 (0.66) ab	
		Cold	106 (4.46) b	1117 (38.3) a	414 (13.3) a	364 (8.60) a	520 (9.71) a	9.80 (0.43) a	4.05 (0.12) a	
	3	Warm	122 (7.67) a	978 (5.56) b	379 (3.89) b	312 (3.31) c	473 (3.75) c	7.60 (0.84) b	2.89 (0.34) b	
		Intermediate	106 (10.5) ab	1015 (31.9) b	410 (4.05) a	338 (2.92) b	506 (3.72) b	8.70 (0.69) a	3.57 (0.30) a	
		Cold	89 (6.20) b	1068 (8.19) a	423 (16.0) a	368 (2.12) a	538 (3.36) a	8.09 (0.60) ab	3.43 (0.32) ab	

For each leaf type, means with different letters indicate significant differences (*P* = 0.05) between climatic categories.

Leaf size area (cm^2) in oaks and needle lenght (mm) in pine, *LMA* leaf mass per unit area, *H+C* cellulose + hemicellulose concentration, *Nmass* N concentration per unit leaf mass, *Narea* N content per unit leaf area, *LL* mean leaf life span.

oaks, the species with the shortest-lived leaves, *Q. suber*, showed the highest percentage of damaged leaves and consumed area in each leaf (Table 3).

Table 3. Percentage of leaves attacked by herbivores and damaged by abiotic factors (SE in parentheses, *n* = 10 trees per species).

Species	Leaf age class	Sites temperatures	No. of leaves examined	Leaves attacked by herbivores (%)	Area lost per leaf (%)	Leaves damaged by abiotic stress (%)	Area damaged per leaf (%)
Q. suber	1	Warm	3200	55.2 (0.99) a	15.69 (1.38) a	33.6 (0.84) b	9.64 (0.75) b
	1	Intermediate	4487	49.5 (1.59) b	10.80 (0.57) b	45.1 (2.13) a	17.3 (0.81) a
	2	Warm	100	56.5 (6.50) a	14.75 (1.49) a	44.0 (6.00) b	18.3 (1.61) b
	2	Intermediate	865	48.7 (3.09) b	11.39 (1.34) b	51.2 (3.09) a	25.0 (0.66) a
Q. ilex	1	Warm	6492	50.9 (0.80) a	7.48 (0.81) a	8.11 (0.67) a	6.17 (0.53) c
	1	Intermediate	4056	44.1 (0.63) ab	6.25 (0.64) ab	9.74 (0.29) a	11.0 (0.67) b
	1	Cold	5893	41.0 (4.10) b	4.87 (0.49) b	11.9 (2.27) a	18.9 (1.78) a
	2	Warm	2110	49.0 (0.41) a	7.34 (0.58) a	17.7 (0.79) b	9.35 (1.07) c
	2	Intermediate	2216	45.8 (0.82) b	6.34 (0.45) ab	20.2 (0.54) ab	16.4 (1.45) b
	2	Cold	1989	42.2 (1.13) c	5.06 (0.46) b	23.5 (3.13) a	22.5 (0.98) a
	3	Warm	210	48.2 (0.63) a	7.61 (0.65) a	23.5 (0.53) c	13.5 (3.33) c
	3	Intermediate	828	44.8 (0.84) b	6.61 (0.66) a	25.3 (0.58) b	22.0 (2.05) b
	3	Cold	214	42.4 (1.34) b	4.92 (0.32) b	30.5 (0.43) a	28.6 (1.97) a
P. pinaster	1	Warm	3079	-	-	18.84 (0.49) c	1.03 (0.12) b
	1	Intermediate	2849	-	-	21.97 (0.31) b	1.99 (0.06) a
	1	Cold	2552	-	-	24.92 (0.88) a	2.38 (0.18) a
	2	Warm	2513	-	-	25.39 (1.17) c	2.23 (0.21) b
	2	Intermediate	2532	-	-	31.99 (0.93) b	2.91 (0.27) ab
	2	Cold	2335	-	-	37.10 (0.79) a	3.63 (0.21) a
	3	Warm	1420	-	-	44.66 (1.26) c	3.11 (0.35) b
	3	Intermediate	1257	-	-	52.21 (1.36) b	3.92 (0.33) b
	3	Cold	1309	-	-	62.97 (2.67) a	4.91 (0.32) a
	4	Warm	212	-	-	78.28 (1.67) c	3.84 (0.42) b
	4	Intermediate	461	-	-	85.35 (1.43) b	4.48 (0.15) b
	4	Cold	968	-	-	91.03 (1.31) a	5.50 (0.33) a

Average loss of area (%) per individual leaf (*n* = 10). Means with different letters indicate significant differences (*P* = 0.05) between climatic categories.

Trends of abiotic damage among sites proved opposite to those obtained for herbivory levels. For the two species represented in the three climatic environments (*P. pinaster* and *Q. ilex*), both the percentage of damaged leaves and the percentage of leaf area damaged, tended to be higher at the coldest sites than at the warmest ones, with intermediate values at sites with intermediate temperatures (Table 3). The same results were obtained for *Q. suber*, the intensity of damage caused by abiotic factors being much higher at the sites with lower temperatures (more damaged leaves and larger damaged area per leaf) as compared to the hottest sites. In contrast with herbivory, there were differences in damage caused by abiotic stress among the successive foliar cohorts, the proportion of damaged leaves and of the damaged part increasing with leaf age (Table 3).

Of the three species, the leaves of the one with the lowest leaf lifespan (*Q. suber*) once again showed higher intensities in the damage caused by stress factors when compared to leaves of the same age from the two other species (Table 3). Between *Q. ilex* and *P. pinaster*, *P. pinaster* showed a greater proportion of leaves with visible damage symptoms, although the percentage of damaged area per leaf in each of the environments was much lower than in *Q. ilex* (Table 3).

Leaf traits and environmental factors that determine the differences in levels of foliar damage

Regression analysis including data from the six sites studied was used to detect the foliar traits that determine the differences in the levels of herbivory among sites. Although we had herbivory data for the two oak species, the analysis was restricted to the leaves of *Q. ilex*, since *Q. suber* could only be found at four sites. Since the levels of herbivory were similar for the different leaf cohorts, the analyses were further limited to current-year leaves. There was a close relationship between all the traits related to the reinforcement of leaf structure and the levels of attack, the percentage of leaves consumed (Fig. 1) and the mean leaf area consumed (Fig. 2) becoming lower as LMA, thickness and structural carbohydrates increased along the climatic gradient. However, there was no significant relationship between levels of herbivory and concentrations of lignin and the nitrogen content per unit mass in the leaves from the different sites. There was a trend towards lower levels of herbivory as nitrogen content per unit leaf area increased, although it was only significant for the percentage of leaf area lost (Fig. 2).

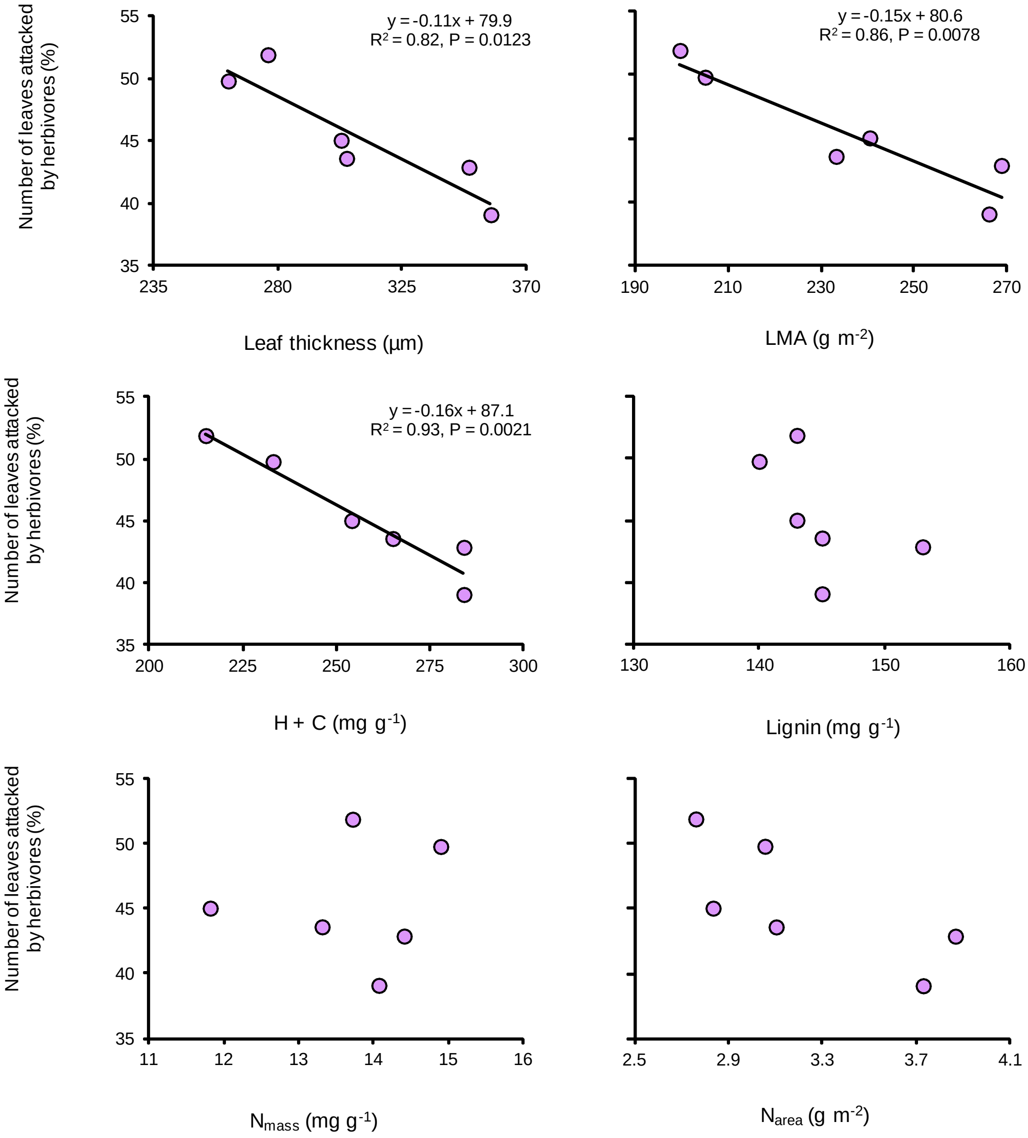

Figure 1. Relationships between the percentage of leaves attacked by herbivores and different leaf traits of current-year leaves of *Quercus ilex* at different sites. *LMA* = leaf mass per unit area, *H+C* = cellulose + hemicellulose concentration, N_{mass} = N concentration per unit leaf mass, N_{area} = N content per unit leaf area.

The comparison between the traits of the healthy and the attacked leaves of each species at each site confirmed the influence of structural strengthening on herbivory levels (Table 4). There were no significant differences in nitrogen content per unit area between leaves with herbivore damage and intact leaves across sites. At the hottest sites, the current *Q. ilex* leaves that had been attacked by herbivores presented lower N_{mass} contents than did the

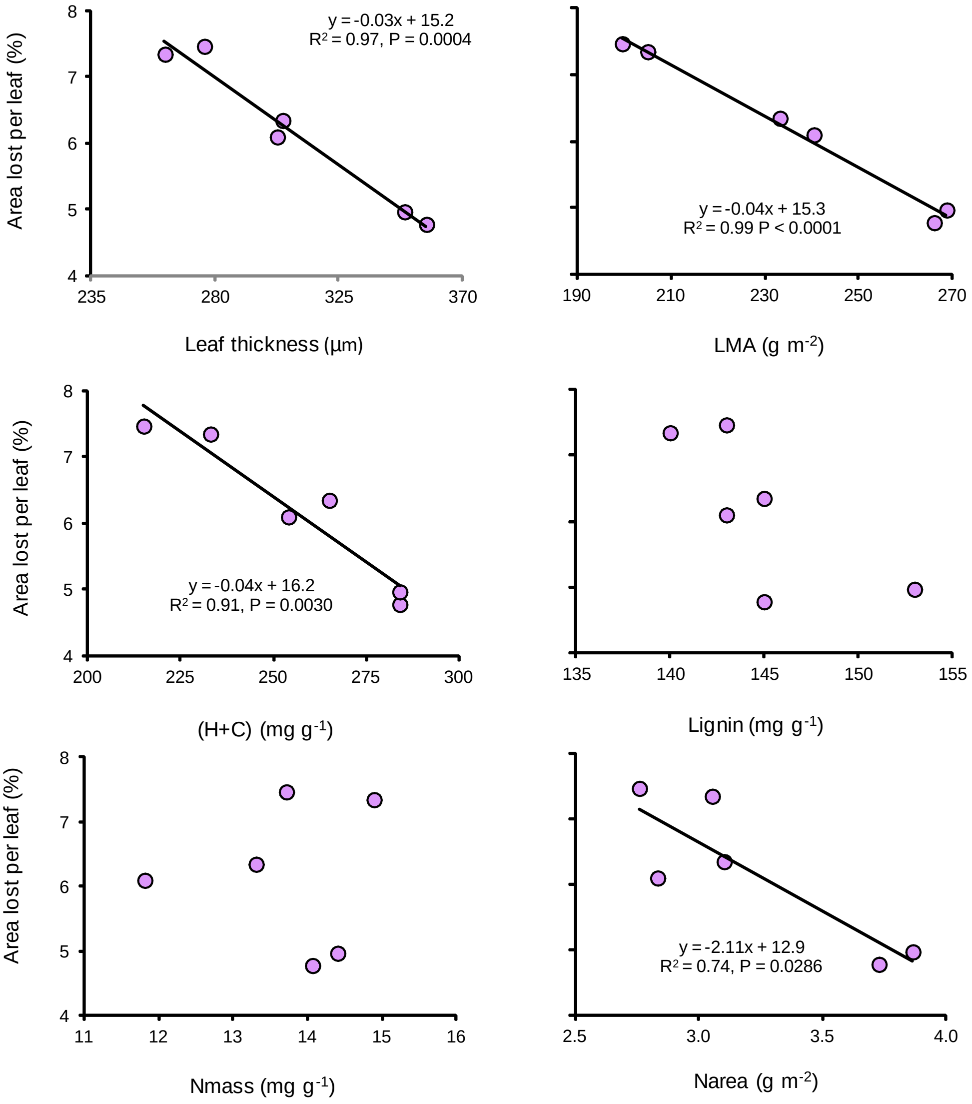

Figure 2. Relationships between the percentage of leaf area lost by herbivory and different leaf traits of current-year leaves of *Quercus ilex* at different sites. Abbreviations as in Fig. 1.

healthy ones. No differences were observed with regard to the individual area of intact and attacked leaves. Conversely, in many cases, there were significant differences in LMA between healthy and damaged leaves, both by herbivory and by abiotic stress factors. The healthy leaves tended to have greater LMA than do the damaged ones, regardless of the type of damage suffered (Table 4).

Table 4. Mean traits of healthy and damaged leaves at the different sites (SE in parentheses, n = 10 trees).

			Warm sites				***Intermediate sites***				***Cold sites***			
	Leaf age class	Category of damage	Leaf size	LMA (gm^{-2})	N ($mg\ g^{-1}$)	N (gm^{-2})	Leaf size	LMA (gm^{-2})	N ($mg\ g^{-1}$)	N (gm^{-2})	Leaf size	LMA (gm^{-2})	N ($mg\ g^{-1}$)	N (gm^{-2})
Q. suber	1	Healthy	5.23 (0.33) a	166 (6.21) a	16.6 (0.33) a	2.74 (0.09) a	4.21 (0.24) a	190 (5.96) a	15.8 (0.47) a	3.02 (0.15) a		-	-	-
		Abiotic stress	5.55 (0.37) a	149 (3.53) ab	16.5 (0.41) a	2.46 (0.07) a	4.77 (0.16) a	168 (4.99) b	15.7 (0.30) a	2.64 (0.10) a	-	-	-	-
		Herbivory	5.49 (0.45) a	146 (4.90) b	16.0 (0.34) a	2.34 (0.09) a	4.65 (0.15) a	164 (3.93) b	15.0 (0.48) a	2.46 (0.11) a	-	-	-	-
Q. ilex	1	Healthy	3.02 (0.10) a	202 (6.83) a	14.3 (0.42) a	2.91 (0.16) a	2.64 (0.10) a	236 (8.62) a	12.7 (0.60) a	2.99 (0.16) a	2.10 (0.09) a	267 (12.9) a	14.2 (0.21) ab	3.80 (0.16) a
		Abiotic stress	2.97 (0.13) a	203 (7.93) a	13.9 (0.49) ab	2.80 (0.12) a	2.69 (0.14) a	213 (4.92) b	12.2 (0.56) a	2.60 (0.15) a	2.09 (0.10) a	241 (7.06) b	13.9 (0.52) b	3.34 (0.18) a
		Herbivory	2.99 (0.12) a	183 (13.7) b	13.3 (0.40) b	2.42 (0.19) a	2.42 (0.14) a	209 (7.60) b	12.4 (0.29) a	2.58 (0.11) a	2.21 (0.13) a	242 (4.97) b	15.0 (0.61) a	3.63 (0.14) a
	2	Healthy	2.96 (0.11) a	215 (5.92) a	11.8 (0.47) a	2.54 (0.13) a	2.67 (0.12) a	247 (5.30) a	10.8 (0.25) a	2.66 (0.04) a	1.92 (0.14) a	278 (8.97) a	12.8 (0.39) a	3.58 (0.20) a
		Abiotic stress	2.84 (0.13) a	205 (7.42) a	10.8 (0.48) b	2.22 (0.14) a	2.41 (0.15) a	237 (7.03) a	10.2 (0.33) a	2.41 (0.06) a	2.10 (0.13) a	256 (7.90) b	13.1 (0.25) a	3.35 (0.13) a
		Herbivory	2.87 (0.14) a	199 (3.35) a	11.6 (0.36) ab	2.31 (0.10) a	2.54 (0.16) a	237 (5.28) a	10.6 (0.29) a	2.52 (0.11) a	2.13 (0.24) a	257 (12.4) b	13.6 (0.39) a	3.51 (0.24) a
P. pinaster	1	Healthy	133 (4.89) a	355 (14.6) a	9.26 (0.42) a	3.29 (0.24) a	117 (6.77) a	369 (3.00) a	10.2 (0.92) a	3.77 (0.42) a	95 (2.56) a	393 (13.0) a	9.39 (0.70) a	3.68 (0.28) a
		Abiotic stress	122 (7.77) a	317 (4.50) b	8.24 (0.52) b	2.61 (0.15) a	124 (6.24) a	339 (12.3) b	9.71 (0.85) a	3.31 (0.36) a	90 (2.04) a	354 (12.8) b	8.78 (0.66) a	3.11 (0.26) a
	2	Healthy	139 (2.72) a	367 (23.3) a	8.92 (0.46) a	3.26 (0.18) a	124 (6.21) a	391 (6.89) a	9.58 (0.52) a	3.78 (0.66) a	106 (4.46) a	414 (13.3) a	9.80 (0.43) a	4.05 (0.12) a
		Abiotic stress	124 (7.93) a	348 (12.0) a	7.65 (0.26) b	2.66 (0.09) a	132 (3.16) a	366 (4.87) b	9.91 (0.72) a	3.63 (0.64) a	104 (3.89) a	383 (11.0) b	9.38 (0.96) a	3.56 (0.26) a
	3	Healthy	122 (7.67) a	379 (3.89) a	7.60 (0.84) a	2.89 (0.34) a	124 (6.21) a	410 (4.05) a	8.70 (0.69) a	3.57 (0.30) a	89 (6.20) a	423 (16.0) a	8.09 (0.60) a	3.43 (0.32) a
		Abiotic stress	124 (9.17) a	359 (4.46) a	6.55 (0.47) b	2.36 (0.18) a	102 (13.4) b	389 (6.24) a	8.39 (0.96) a	3.25 (0.38) a	74 (3.19) a	396 (10.6) b	8.18 (0.77) a	3.21 (0.22) a

Means with different letters indicate significant differences (P = 0.05) between categories of damage. Leaf size: area (cm2) in oaks and needle lenght (mm) in pine. *LMA* leaf mass per unit area.

The climatic factors responsible for the differences among sites in foliar damage caused by abiotic stress were determined by means of linear regression analysis between the percentage of leaves with damage symptoms and the percentage of damaged area per leaf against the different climatic variables recorded at each site (Table 5). Once again, *Q. suber* was excluded from the analysis because this species was only found in four plots. The

Table 5. Linear regression parameters for mean levels of damage by abiotic stress depending on different climatic variables at each site (C = current-year leaves).

	Number of leaves damaged (%)			Area damaged per leaf (%)		
Q.ilex (C) (*n* = 6)	*y*	R^2	*P* value	*y*	R^2	*P* value
Mean monthly temperature (ºC)	y = -0.58x + 17.6	0.81	0.0148	y = -1.89x + 37.1	0.88	0.0059
Maximum summer temperature (ºC)	y = -0.47x + 29.3	0.23	NS	y = -1.56x + 76.3	0.25	NS
Minimum winter temperature (ºC)	y = -0.28x + 7.30	0.74	0.0257	y = -1.08x + 1.91	0.96	< 0.0001
Number of days with frost per year	y = 0.04x + 7.78	0.77	0.0219	y = 0.13x + 4.41	0.97	< 0.0001
Total annual rainfall (mm)	y = -0.01x + 12.1	0.31	NS	y = -0.01x + 18.0	0.23	NS
Summer precipitation (mm)	y = 0.07x + 7.11	0.58	NS	y = 0.22x + 2.29	0.68	NS
Emberger's index	y = -0.01x + 10.6	0.01	NS	y = 0.01x + 11.8	0.01	NS
Q.ilex (C+1) (*n* = 6)						
Mean monthly temperature (ºC)	y = -0.93x + 32.9	0.8	0.0157	y = -2.03x + 42.9	0.96	< 0.0001
Maximum summer temperature (ºC)	y = -0.71x + 49.9	0.2	NS	y = -2.03x + 99.5	0.41	NS
Minimum winter temperature (ºC)	y = -0.48x + 16.1	0.71	0.0342	y = -1.09x + 5.88	0.93	0.0018
Number of days with frost per year	y = 0.06x + 17.0	0.82	0.0136	y = 0.13x + 8.25	0.98	< 0.0001
Total annual rainfall (mm)	y = -0.01x + 23.8	0.25	NS	y = -0.01x + 23.8	0.37	NS
Summer precipitation (mm)	y = 0.11x + 15.8	0.64	NS	y = 0.20x + 7.17	0.55	NS
Emberger's index	y = -0.01x + 20.9	0.01	NS	y = -0.04x + 18.6	0.02	NS
P. pinaster (C) (*n* = 6)						
Mean monthly temperature (ºC)	y = -0.95x + 34.4	0.94	0.0012	y = -0.22x + 4.67	0.99	< 0.0001
Maximum summer temperature (ºC)	y = -0.85x + 56.7	0.32	NS	y = -0.26x + 12.4	0.59	NS
Minimum winter temperature (ºC)	y = -0.48x + 17.4	0.82	0.0123	y = -0.11x + 0.77	0.87	0.007
Number of days with frost per year	y = 0.06x + 18.3	0.93	0.0019	y = 0.03x + 0.99	0.93	0.0017
Total annual rainfall (mm)	y = -0.01x + 25.0	0.26	NS	y = -0.01x + 2.70	0.44	NS
Summer precipitation (mm)	y = 0.10x + 17.4	0.64	NS	y = 0.02x + 1.02	0.39	NS
Emberger's index	y = -0.01x + 22.2	0.01	NS	y = -0.01x + 2.25	0.05	NS
P. pinaster (*C+1*) (*n* = 6)						
Mean monthly temperature (ºC)	y = -1.83x + 55.6	0.98	0.0002	y = -0.21x + 5.77	0.87	0.0061
Maximum summer temperature (ºC)	y = -1.84x + 107	0.42	NS	y = -0.19x + 10.7	0.29	NS
Minimum winter temperature (ºC)	y = -0.96x + 22.5	0.91	0.0032	y = -0.11x + 1.90	0.76	0.0234
Number of days with frost per year	y = 0.12x + 24.5	0.98	0.0002	y = 0.01x + 2.10	0.86	0.0072
Total annual rainfall (mm)	y = -0.01x + 37.9	0.32	NS	y = -0.01x + 3.75	0.31	NS
Summer precipitation (mm)	y = 0.18x + 23.5	0.56	NS	y = 0.02x + 1.90	0.59	NS
Emberger's index	y = -0.02x + 32.9	0.01	NS	y = -0.01x + 3.12	0.01	NS

results for the two younger leaf age classes of each species were included in this case because of the fact that foliar damage caused by abiotic stress increased with leaf age. Only differences in temperatures showed significant effects on damage levels. From the different temperature estimates, in most cases, the lowest temperatures and the number of frost days per year showed the highest percentages of explained variance (Table 5). Average temperatures also showed highly significant effects, mainly because of the strong effect of the lowest temperatures on the means. On the other hand, absolute maximum temperatures showed no significant effects on abiotic damage symptoms. No significant effects of the estimates of water availability in the different sites were observed in practically any of the cases (Table 5).

DISCUSSION

The results obtained confirm those of previous studies that conclude that LMA is not exclusively a specific protection against drought and attack by herbivores (Niinemets 2001; Wright et al. 2002), but rather a response to a wide range of environmental stress factors, including low temperatures (Ogaya and Peñuelas 2007; Mediavilla et al. 2012; González-Zurdo et al. 2015). The tendency of the leaves of all three species was for LMA, contents of structural carbohydrates and thickness to be higher in colder than in warmer environments. In the two oak species, these differences in foliar traits among environments were accompanied by differences in levels of consumption by herbivores, with a lower percentage of attacked leaves and a smaller part of consumed area per leaf at the colder sites.

Differences in levels of herbivory at the different sites could be due to a direct effect of temperatures on the growth of herbivore populations (Bale et al. 2002; Jamieson et al. 2012). However, within a same site, the herbivore-attacked leaves presented lower LMA and thickness than the healthy ones did, which suggests a direct effect of these leaf traits on herbivores’ preferences. At each site, herbivores seemed to first choose the species with lower leaf thickness and LMA and, within each species, the leaves with these same characteristics. On the other hand, and again within each species, herbivores seem not to discriminate because of N concentrations.

Many leaf traits have been related to defence against herbivory. Foliar nitrogen content has been suggested as the main determinant for interspecific and intraspecific differences in levels of attack by several authors (White 1984; Agrawal 2004). Unlike the increase in the N content of leaves with the decrease in temperatures reported in some studies (Weih and Karlsson 2001; Jian et al. 2009), in the present study no trends in leaf N contents related to temperature changes were observed, which is, on the other hand, consistent with other studies (Hultine and Marshall 2000; Chen et al. 2013). Therefore, the between-sites differences in levels of herbivory observed in the species analysed showed no relationship to differences in foliar nitrogen contents.

Conversely, the level of herbivory significantly decreased with the increase in LMA, probably because of the positive effect of this trait on leaf rigidity (Kitajima and Poorter 2010; Onoda et al. 2011). These results suggest that, as defended by other authors (Poorter et al. 2004; Carmona et al. 2011; Cárdenas et al. 2014), leaf rigidity would be the best predictor of differences in the rate of herbivory. The higher LMA at the coldest sites was a result of a thickening caused by higher amounts of structural carbohydrates in the cell wall, with no changes in density (Mediavilla et al. 2012; González-Zurdo et al. 2015). Both thickness and cellulose and hemicellulose contents had a negative effect on the levels of herbivory at the different sites, while lignin content had no significant effects. Different authors (Coley 1983; Kitajima et al. 2012; Cárdenas et al. 2014) have proved the relevance of cellulose content as a predictor of herbivory-related damage. The lack of effects of lignin contents on levels of herbivory has also been observed in diverse studies (Kurokawa and Nakashizuka 2008; Kitajima et al. 2012). Conversely, our results are in contrast with those of other authors who state that the levels of herbivory depend on leaf density (Kitajima et al. 2012), while leaf thickness (Cárdenas et al. 2014) or hemicellulose content (Kitajima et al. 2012) show no significant effects. The reason for these discrepancies could be the fact that the conclusions of other authors are based on interspecific comparisons, while in this study responses are analysed at the intraspecific level.

Harsher climatic conditions during winter resulted in greater foliar damage by abiotic factors, with more leaves damaged by the effects of low temperatures and larger fractions of damaged area per leaf at the coldest sites for the three studied species. Accordingly, in contrast with its role in the protection against herbivores, the structural reinforcement in cold sites does not seem to contribute to protection against cold stress. Differences in rainfall and in Emberger's pluviothermic index had no effect on the level of damage of the leaves from the different sites. Other authors (Grossoni et al. 1998; Munné-Bosch and Peñuelas 2004) have found that drought stress also produces visible leaf damage symptoms. Mediterranean environments almost always suffer drought stress during the summer (Lionello et al. 2006). Therefore, the fact that rainfall levels have no effect on the intensity of abiotic damage suggests that, even though the rainfall levels recorded at each site were different, there were no differences in the intensity of drought stress during summer. In fact, Emberger's index varied little from site to site and showed no relation to the temperatures at each of them. Among the different environmental variables studied, minimum winter temperatures and the number of frost days per year had the strongest effects on the intensity of abiotic damage. On the other hand, the effects of maximum temperatures were non-significant.

Accelerated foliar senescence is usually related to oxidative stress (Günthardt-Goerg and Vollenweider 2007), so the most likely explanation for the higher levels of damage in cold climates is oxidative stress caused by excess light absorption in periods when temperatures represent a constraint for photosynthesis. In cold Mediterranean climates, during winter there are frequently periods of relatively high atmospheric pressure and clear skies when air temperature remains low during the whole day while leaves receive high levels of solar radiation. It is well-known that, if the energy absorbed from light that reaches the reaction centres exceeds the amount of energy that can be used, as is the case with low temperatures, photoinhibition may cause damage to the photosynthetic apparatus (Demming-Adams and Adams 1992; Lucinski and Jackowski 2006). This damage could be the main cause for the appearance of necrotic and chlorotic spotting in the leaves of the studied species. Depending on atmospheric conditions, UV irradiance may be expected to increase with altitude at a rate of between 3 and 7% km^{-1} (Pfeifer et al. 2006). Accordingly, the differences in altitude between our sites could contribute to the responses observed in our species. In fact, increases in leaf thickness in response to UV-B radiation have been found in different experimental studies (Filella and Peñuelas 1999). The combination of low temperatures and high UV-B irradiances could exacerbate the appearance of visible symptoms of injury at higher altitudes. Damages by ozone have been reported at the Iberian Peninsula particularly in Mediterranean and other coastal areas (Saavedra et al 2012). However, to our knowledge, there are no reports of ozone damages in rural areas at the interior of the Iberian Peninsula for the three species included in the present study. Sclerophylly is considered as an adaptation that protects leaves against the effects of ozone, UV-B radiation and other pollutants (Bussotti and Ferretti 1998). Given the sclerophyllic character of the species studied, current ambient ozone levels in southern Europe are probably not high enough to induce visible injury in this species (Sanz and Calatayud 2016, http://www.ozoneinjury.org/). In any case, levels of ozone were very similar during the study years for the different sites (Table 1). Accordingly, differences in ozone effects cannot be responsible for the strong differences in visible symptoms observed between the different sites.

According to the results of the present study, in the environments with lower temperatures, evergreen tree species produced leaves with higher LMA and structural carbohydrates concentration. This structural reinforcement would have a negative impact on instantaneous C assimilation (Reich et al. 1997; Mediavilla et al. 2001; Niinemets and Sack 2006), which should be interpreted as a cost for the evergreen leaves produced at cold sites. This study shows that, in addition to this unfavourable effect on photosynthesis, damage caused by abiotic factors was also higher at the sites with lower winter temperatures, which should contribute to a decrease in total CO_2 fixation across the entire lifespan of a leaf. On the contrary, at cold sites, losses resulting from herbivory were lower in both oak species. Moreover, lifespan of *Pinus pinaster* needles was slightly longer in colder than in warmer areas, although the trend was not significant. Therefore, the negative effects on leaf area persistence of the adaptation to cold might be partially counteracted by the positive ones. If these two types of effect are compared, in *Q. suber* the percentage of leaves attacked by herbivores was approximately 10% lower at the colder sites, which adds to 31% lower area consumed per leaf. Nevertheless, levels of damage by abiotic factors inversely increased in a proportion of 34% more damaged leaves and 79% more damaged area per leaf at the coldest sites. For current-year *Q. ilex* leaves, the decrease in levels of herbivory in the coldest plots in comparison with the warmest ones was of 19% less leaves attacked and 35% less average leaf area consumed. On the other hand, the intensity of damage caused by abiotic factors increased in around 47% more damaged leaves and 26% more average damaged area per leaf in the coldest environments compared to the warmest. Therefore, the reduction of anticipated losses of photosynthetic area caused by herbivory in cold environments would not, in principle, be enough to compensate the higher losses in photosynthetic area undergone in the coldest environments with respect to the warmest ones. Losses caused by herbivorous insects occur mainly during leaf formation, since herbivores show a preference for young leaves as opposed to old ones, which have lower concentrations of nutrients and higher rigidity (Coley and Aide 1991; Coley and Barone 1996). This means that the negative effects of area losses caused by herbivores take place over the leaf’s whole lifespan. As opposed to this, damage caused by abiotic factors increases with leaf age, which allows the young leaves to maintain their photosynthetic area in healthy conditions during the most productive phases of their life cycles, because photosynthetic rates tend to decline with leaf age (Mediavilla and Escudero 2003b; Niinemets et al. 2005). Therefore, the timing of the appearance of both types of damage would mean that in equal amounts of damaged leaf area, the effect of herbivory on productivity would be more negative than that of abiotic damage. However, losses caused by herbivory occur at very early phases of the leaf expansion period. If leaves are still small at the time of the attack, the volume of resource lost might be also relatively small. In fact, there were no significant differences in individual area between healthy leaves and those attacked by herbivores within a same species and site, which suggests that losses of the photosynthetic area due to this kind of attack are compensated during growth, so that the final leaf size is independent of herbivore attack. The differences between both types of leaf damage thus make it difficult to assess their final effects on leaf-level carbon balance. In any case, for both oak species, the increase in the intensity of abiotic damage at the coldest sites in relation to the warmest was stronger than the decrease in herbivory, which suggests that the negative impact of cold temperatures on the duration of the photosynthetic surface would be stronger than the positive effects. As far as *P. pinaster* is concerned, the percentage of leaves damaged by abiotic stress was very high, especially after their second year of life, although the percentage of damaged leaf area was small (around 4-5%). Therefore, the reduction in the photosynthetic area at cold sites was relatively small and lower than the lengthening of leaf lifespan in the coldest locations.

To our knowledge, this is the first study to provide data on the differences in anticipated losses of leaf area in populations of a same species located in different environments. Further study of other species is undoubtedly required to confirm our results. In any case, the anticipated loss of leaf area owing to herbivory and abiotic stress is a key factor that should be incorporated into models for estimating carbon sequestration capacity of the different tree species with the purpose of predicting future changes in ecosystem carbon balance.

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Capítulo IV

RESPUESTAS DE LOS PARÁMETROS FOTOSINTÉTICOS A LAS DIFERENCIAS EN LAS TEMPERATURAS INVERNALES A LO LARGO DE UN GRADIENTE DE TEMPERATURA EN DOS ESPECIES ARBÓREAS PERENNIFOLIAS

Este capítulo reproduce íntegramente el siguiente artículo:

Mediavilla S, González-Zurdo P, Babiano J, Escudero A (2016) Responses of photosynthetic parameters to differences in winter temperatures throughout a temperature gradient in two evergreen tree species. Remitido a European Journal of Forest Research.

RESUMEN

La dependencia de los parámetros fotosintéticos respecto a la temperatura ha sido objeto de interés durante los últimos años, debido a sus profundas implicaciones en el nuevo escenario climático. Muchos son los estudios que se han ocupado de las respuestas a corto plazo de los parámetros fotosintéticos a los cambios en temperatura. Menos atención se ha prestado a la variabilidad intraespecífica en los parámetros bioquímicos de la fotosíntesis en respuesta a las diferencias en temperatura durante el crecimiento. Este estudio explora los efectos del rigor invernal sobre los rasgos foliares de dos especies arbóreas perennifolias (*Quercus ilex* y *Q. suber*). El peso por unidad de superficie foliar (LMA) y las concentraciones de fibra, nitrógeno (N), proteínas solubles, clorofila y ribulosa 1,5-bisfosfato carboxilasa/oxigenasa (Rubisco) fueron determinados en ambas especies a lo largo de un gradiente de temperatura. Varios parámetros fotosintéticos [la máxima tasa de carboxilación (V_{cmax}), la máxima tasa de transporte de electrones (J_{max}), la tasa de respiración en oscuridad (R_d), la limitación estomática de la fotosíntesis (RSL), y la fracción aparente de nitrógeno invertida en Rubisco (P_R) y en las proteínas limitantes de la velocidad del transporte fotosintético de electrones (P_B)] fueron evaluados midiendo las curvas de respuesta de la asimilación neta de carbono con respecto a la presión parcial de CO_2 en los espacios intercelulares. LMA y las concentraciones de carbohidratos estructurales aumentaban con el descenso de las temperaturas invernales, mientras que las concentraciones de N no mostraban patrones definidos. La clorofila, las proteínas solubles, la Rubisco, V_{cmax}, J_{max}, P_R y P_B disminuían con el descenso en las temperaturas invernales, mientras que R_d a una temperatura común determinada (25ºC) era mayor en los sitios más fríos. Nuestros resultados sugieren que un incremento en LMA y en la concentración de carbohidratos estructurales en ambientes fríos está asociado con una menor asignación de N a la maquinaria fotosintética, lo que conduce a una menor la capacidad fotosintética.

Palabras clave: parámetros fotosintéticos, Rubisco, concentración de fibras, nitrógeno, especies perennifolias, gradiente climático.

RESPONSES OF PHOTOSYNTHETIC PARAMETERS TO DIFFERENCES IN WINTER TEMPERATURES THROUGHOUT A TEMPERATURE GRADIENT IN TWO EVERGREEN TREE SPECIES

ABSTRACT

The temperature dependence of photosynthetic parameters has been a focus of interest during recent years owing to its profound implications in the new climate scenario. Many studies have addressed the short-term responses of photosynthetic parameters to temperature change. Less attention has been given to the intraspecific variability in the biochemical parameters of photosynthesis in response to differences in growth temperature. This study explores the effects of winter harshness on the leaf traits of two evergreen tree species (*Quercus ilex* and *Q. suber*). Leaf mass per unit area (LMA) and the concentrations of fiber, nitrogen (N), soluble protein, chlorophyll and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) were determined in both species throughout a temperature gradient. Several photosynthetic parameters [maximum carboxylation rate (V_{cmax}), maximum light-driven electron flux (J_{max}), dark respiration rate (R_d), relative stomatal limitation to photosynthesis (RSL), and the apparent nitrogen fraction in Rubisco (P_R) and in the rate-limiting proteins of photosynthetic electron transport (P_B)] were assessed by measuring leaf response curves of net CO_2 assimilation versus intercellular CO_2 partial pressure. LMA and structural carbohydrate concentrations increased with the decrease in winter temperatures, whereas N concentrations did not show definite patterns. Chlorophyll, soluble proteins, Rubisco, V_{cmax}, J_{max}, P_R and P_B declined with the decrease in winter temperatures, whereas R_d at a set common temperature (25ºC) was higher at colder sites. Our results suggest that an increase in LMA and in the concentration of structural carbohydrates in cold environments is associated with a reduced N allocation to the photosynthetic machinery, which leads to reduced photosynthetic capacity.

Key words: photosynthetic parameters, Rubisco, fibre concentration, nitrogen, evergreen

species, climatic gradient.

INTRODUCTION

The investigation of the leaf traits that determine the natural variability of photosynthetic capacity is one of the main objectives of the present ecological research, given the large impact of photosynthesis on carbon sequestration and its role in the mitigation of global warming (Friedlingstein et al. 2006, Booth et al. 2012). The biochemical model proposed by Farquhar et al. (1980) and later improved by von Caemmerer and Farquhar (1981), Sharkey (1985) and Harley and Sharkey (1991) has been the most commonly used for characterizing the photosynthetic performance of the different species and for simulating carbon assimilation at different times and spatial scales. Significant variation in the two key parameters of this model (i.e. maximum velocity of carboxylation, V_{cmax}, and maximum rate of electron transport, J_{max}) has been found both across different species (Wohlfahrt et al. 1999, Manter and Kerrigan 2004, Kattge and Knorr 2007, Niinemets et al. 2009a, Lin et al. 2013, Flexas et al. 2014), and within a single species in response to changes in environmental factors such as growth irradiance (Daas-Ghrib et al. 2011, Vaz et al. 2011, Zhang and Yin 2012), nutrient supply (Grassi et al. 2002, Warren et al. 2003, Daas-Ghrib et al. 2011), water availability (Misson et al. 2010, Vaz et al. 2010, Aranda et al. 2012) and canopy position (Montpied et al. 2009, Legner et al. 2014).

Among the different environmental factors, the temperature dependence of photosynthetic parameters has been a focus of interest during recent years because of its profound implications in the new climate scenario. Many studies have addressed the seasonal variability of temperature responses of photosynthetic parameters in different species (Medlyn et al. 2002, Han et al. 2004, Borjigidai et al. 2006, Han et al. 2008, Dillen et al. 2012, Lin et al. 2013). However, less attention has been given to the intraspecific variability in the biochemical parameters of photosynthesis in response to differences in leaf growth temperature. Various authors have analysed the effects of the differences in growth temperature on the photosynthetic capacity of a single species, but most of these studies rely on short-term experiments with seedlings growing in temperature-controlled growth chambers (Harley et al. 1992, Bunce 2000, Ghouil et al. 2003, Yamori et al. 2005, 2006, Warren 2008, Pons 2012). In contrast, there are not many studies addressing the variability in the photosynthetic parameters in adult specimens of a single tree species growing in different temperature environments (Zhang et al 2007, Dusenge et al 2015).

It is known that differences in V_{cmax} and J_{max} are often related to differences in leaf morphology and chemical composition. For instance, strong positive relationships between V_{cmax} and leaf nitrogen content have been observed by many authors (Niinemets and Tenhunen 1997, Wilson et al. 2000, Frank et al. 2001, Yamori et al. 2011). Similarly, decreases in the photosynthetic parameters have been frequently found associated with decreases in the fraction of nitrogen invested in the photosynthetic functions as leaf mass per unit area (LMA) increases (Hikosaka et al. 1998, Poorter and Evans 1998, Onoda et al. 2004, Takashima et al. 2004, Coste et al. 2005). LMA and leaf thickness also affect the internal CO_2 diffusion, with a tendency for leaves with greater LMA and thickness to have lower internal conductance (Syvertsen et al. 1995, Kao and Chang 2001, Niinemets et al. 2005, Zhang et al. 2007, Flexas et al. 2008, Tomas et al. 2013) and a lower photosynthetic capacity. In several studies, changes in the leaf traits that determine the photosynthetic parameters have been reported throughout climatic gradients. Within a single species, LMA tends to be greater in colder environments (Ogaya and Peñuelas 2007, Atkin et al. 2008, Mediavilla et al. 2012). Similarly, several authors have reported increases in N contents in leaves produced in colder sites (Weih and Karlsson 2001, Jian et al. 2009), although in other studies the patterns in N content proved inconsistent (Hultine and Marshall 2000, Chen et al. 2013). Accordingly, changes in the photosynthetic capacity of a species could be expected in response to changes in leaf growth temperature.

In the present paper we analyse the variations in the photosynthetic capacity of mature specimens of two evergreen tree species (*Quercus ilex* ssp*. ballota* (Desf.) Samp and

Quercus suber L.) throughout a winter temperature gradient. Our objective was to assess the extent of the differences in V_{cmax} and J_{max} in specimens of the same species growing in the field under contrasting winter conditions, and to determine the factors controlling these possible differences. Previous studies with the same species revealed significant differences between sites in several leaf traits related to differences in the intensity of winter harshness. Thus, the leaves from one species growing in environments with cooler winters showed a greater leaf mass per unit area (LMA) and structural carbohydrates concentrations, but lower chlorophyll, soluble proteins and Rubisco, with no trends in the leaf nitrogen content between environments (Mediavilla et al. 2012, González-Zurdo et al. 2016). By contrast, these trends were not observed in deciduous species (González-Zurdo et al. 2016), which suggests that the differences between sites in winter conditions were the cause of the changes observed in leaf traits for the evergreen species. Most authors addressing the effects of temperature on photosynthetic capacity have reported significantly higher capacities in the cooler temperatures than in warmer temperatures (Medlyn et al. 2002, Yamori et al. 2005, Dusenge et al. 2015, Kaluthota et al. 2015). These observations are in line with the hypothesis that plants growing in cool environments make relatively larger N investment in photosynthetic enzymes, compared to plants growing in warmer environments, in order to compensate for the low enzyme activity and slower membrane processes at low temperatures (Berry and Björkman 1980, Hikosaka 1997, von Caemmerer 2000). However, our previous data suggest that in evergreen species the cold resistance traits imply reduced N allocation to the photosynthetic machinery, associated with a stronger structural reinforcement at colder sites (Mediavilla et al. 2012, González-Zurdo et al. 2016). These large differences in leaf structure and leaf N allocation between sites could be associated with significant differences in photosynthetic capacity, with lower V_{cmax} and J_{max} values at the colder sites. In addition, lower mesophyll conductance to CO_2 might be expected in the colder environments as a consequence of the higher LMA and thickness of the leaves produced under these conditions. Our hypothesis, then, is that evergreen trees should exhibit lower photosynthetic capacity at colder sites than at warmer sites, as a consequence of the structural reinforcement of the leaves produced in colder environments.

MATERIALS AND METHODS

Study species and sites

Two evergreen species were selected for the study: *Quercus suber* L. and *Q. ilex* ssp. *ballota* (Desf.) Samp. The two species were distributed on six sites located in the regions of Castilla & Leon and Extremadura (central-western Spain) between latitudes 41º 45' N and 39º 49' N and between longitudes 6º 22' W and 2º 37' W (Table 1). Owing to the differences in altitude and to the effects of continentality, there were great between-site differences in temperature, which were especially pronounced for the minimum winter temperatures and the number of frosts per year (Table 1). In contrast, the differences in summer temperatures were less intense. Accordingly, the annual temperature range was higher in colder sites mainly because of the effects of continentality (Ninyerola et al. 2000). According to differences in winter temperatures, two sites (A, B) may be classified as warm and two (E, F) as cold. Sites C and D had intermediate temperatures. The sites consisted of flat areas with sparse populations (between 50 and 100 specimens ha^{-1}) of mature (more than 100 years old) tree individuals. Each site was selected to cover a wide gradient in winter temperatures, although it was also ensured that the rest of climate characteristics were as homogeneous as possible. Nevertheless there was a tendency for rainfall levels to be higher in the hottest and southernmost sites, which helped to reduce the differences in the intensity of drought stress between cold and hot sites (Table 1). Owing to its lower tolerance to low temperatures, *Q. suber* was absent at the coldest sites. Rainfall data were obtained from the digital climatic atlas of the Iberian Peninsula (Ninyerola et al. 2005): a set of digital climatic maps of mean air temperature, precipitation and solar radiation elaborated with 200 m resolution by using data from climate stations and a combination of geographical variables (altitude, latitude, continentality, solar radiation and terrain curvature).

Table 1. Sites characteristics.

STANDS	**A**	**B**	**C**	**D**	**E**	**F**
Longitude (W)	5º 48´	6º 22'	5º 52´	5º 47´	2º 37´	2º 52´
Latitude (N)	40º 01´	39º 49'	41º 14´	41º 08´	41º 45´	41º 43´
Altitude (m.a.s.l.)	466	600	985	834	1189	1045
Climate characteristics						
Mean annual temperature (ºC)	16.6	16.8	12.3	12.7	10.3	10.6
Mean spring temperature (ºC)	19.3	19.0	15.0	14.9	13.0	13.2
Mean summer temperature (ºC)	25.8	26.3	20.8	21.4	19.1	19.1
Mean autumn temperature (ºC)	12.2	13.2	7.84	9.11	6.03	6.35
Mean winter temperature (ºC)	9.07	9.81	5.55	5.30	3.14	3.53
Absolute minimum temperature (ºC)	-4.15	-3.55	-8.18	-9.82	-13.7	-16.9
Mean daily minimum temperature of the coldest month (ºC)	4.06	4.69	-0.47	-0.9	-2.93	-3.13
Number of days with frost per year	15	5	57	61	105	112
Mean duration of the period with frosts (days per year)	68	55	127	154	222	214
Annual rainfall (mm)	986	672	495	460	567	646
Daily mean radiation (W m^{-2})	185	184	186	183	177	179
Emberger's index	105	60	52	46	79	78
Soil						
Sand content (%)	77.2	73.9	84.6	74.3	75.1	84.0
Clay content (%)	11.7	12.3	6.40	12.2	14.4	8.30
Silt content (%)	11.1	13.8	9.00	13.5	10.5	7.70
Total soil N content (%)	0.143	0.104	0.072	0.021	0.115	0.048
P assimilable (ppm)	38	5	10	4	7	6
Organic matter (%)	4.47	4.10	2.60	0.37	5.12	1.92
pH	4.5	4.7	4.6	4.5	4.8	6.1
Species	Qi, Qs	Qi, Qs	Qi, Qs	Qi, Qs	Qi	Qi

Qi *Quercus ilex,* Qs *Quercus suber*

Temperature data were obtained for each site by means of data loggers (Hobo Pendant temperature/light datalogger, Part UA-002-08, Onset Computer Corporation, Pocasset, MA, USA). The data loggers were programmed to obtain temperature data every 10 minutes and were kept at each site for six years (2008–2014).

We used the Emberger's pluviothermic index (Emberger 1930) to analyse possible differences in water stress:

$$Q = \frac{1000 \times P}{(273 + \frac{T_X + T_N}{2}) \times (T_X - T_N)}$$

where P is the annual precipitation (mm), T_X is the average temperature of the hottest month (ºC) and T_N is the average temperature of the coldest month (°C). This index is commonly used in Mediterranean climates (Kunstler et al. 2007).

Soil samples were taken up to a depth of 20 cm (excluding the forest floor) from each stand. The determination of soil granulometry, pH and N and P concentrations was carried out at the Soils Laboratory of the Institute of Natural Resources and Agrobiology in Salamanca according to the methods described by Chapmann and Pratt (1973) and Walkley and Black (1934).

Gas exchange measurements and biochemical modeling

Leaf gas exchange was measured with a portable steady-state photosynthetic system (CIRAS, PP-system, Hitchin, UK) equipped with a Parkinson leaf chamber (2.5 cm^2). Measurements were conducted between 7:00 and 13:00 h local solar time during late spring and early summer, before the onset of summer drought, over a period of two years (2012-2013), on fully expanded sunlit leaves from the current year spring flush of both species. The A/C_i curves were produced under saturating light intensity (1800 µmol photons m^{-2} s^{-1}), a temperature of 25ºC and a relative humidity of around 60%. After allowing the gas exchange rates to stabilize for 15 minutes at 400 ppm CO_2 concentration (C_a), gas exchange rates were recorded over a range of intercellular CO_2 concentrations (C_i) resulting from changing the CO_2 supply in ten steps from 50 to 1800 ppm. The supply of CO_2 was reduced step-wise to the minimum value, then returned to 400 ppm again, and increased step-wise from that concentration to complete the A/C_i curve at the high C_a end. At each C_a, photosynthesis was allowed to stabilize for a minimum of 4 minutes and three successive measurements were made at 30-s intervals, to ensure stability. A full A/C_i response curve usually took about 1.5 h to complete. In total, around 80 A/C_i curves were performed over the two growing seasons in all sites where *Q. ilex* was present and 60 curves were performed for *Q. suber*.

Photosynthetic parameters were estimated by fitting the model equations of Farquhar et al. (1980) and later modified by Sharkey (1985) and Harley and Sharkey (1991) to the measurements of leaf gas exchange by nonlinear least squares regression. For characterizing the photosynthetic capacity of the leaves, we chose light-saturated net photosynthesis rate (A_{max}, measured at the CO_2 concentration close to 400 µmol mol^{-1} and 1800 µmol m^{-2} s^{-1} PPFD), maximum carboxylation rate (V_{cmax}) and maximum electron transport rate (J_{max}) as parameters. In addition, day respiration rate (R_d) was calculated from the A/C_i curves. The Michaelis-Menten constants for CO_2 and $O_{2,}$ and the CO_2 compensation point in the absence of mitochondrial respiration were taken from Kosugi et al. (2003). We assumed an infinite CO_2 transfer conductance from the intercellular air spaces to the chloroplasts, i.e. the CO_2 partial pressure in the chloroplasts was assumed to be equal to C_i, as have numerous other researchers (Daas-Ghrib et al. 2011, van de Weg et al. 2012, Dillen et al. 2012, Lin et al. 2013, Dusenge et al. 2015). Estimation of internal conductance requires a considerable experimental effort using a combination of gas exchange measurements together with either carbon isotope fractionation or fluorescence techniques, which were not available in the present study. This assumption could induce underestimation of true V_{cmax} values (Ethier and Livingston 2004), and therefore only apparent values of maximum carboxylation rates (V_{cmax}, C_i) were displayed. Limitations of RuBP regeneration arising from the availability of inorganic phosphate for photophosphorylation (Sharkey 1985) were not considered in the present study.

The relative stomatal limitation to photosynthesis (RSL), an estimate of the proportion of the reduction in photosynthesis attributable to CO_2 diffusion between the atmosphere and the site of carboxylation, was calculated from A/C_i curves according to the method established by Farquhar and Sharkey (1982), where R = $(1-A/A_o)100$ and A is the net photosynthetic rate when C_i equals ambient C_a. Under these conditions, A_o is the rate of photosynthesis that would occur if there were no diffusive limitation to CO_2 transfer from the bulk atmosphere to the site of carboxylation. For this calculation, mesophyll conductance was also considered to be infinitely large. The intrinsic water use efficiency was calculated as the ratio of net CO_2 assimilation and stomatal conductance.

Leaf chemical and morphological analyses

Leaf morphology and nitrogen concentration were measured in the same leaves used for measuring photosynthesis. At the laboratory, leaf thickness was measured with a digital micrometer (Digimatic micrometer, Mitutoyo, Japan) and calculated as a mean of three measurements, taken at random positions on each leaf, but avoiding the main ribs on flat leaves. The total projected leaf areas were determined by an image analysis system (Delta-T Devices LTD, Cambridge, UK). The samples were then oven-dried at 70ºC to constant weight and the total dry mass was determined. From the data obtained we calculated the leaf dry mass per area (LMA). Leaf N concentrations were determined with a CE-Instruments NA-2100 autoanalyser (ThermoQuest, Milan, Italy) and later expressed as per unit dry mass (N_{mass}, mg g^{-1}), and per unit of leaf area (N_{area}, g m^{-2}), calculated as the concentration per unit dry mass multiplied by LMA.

LMA and leaf nitrogen content per dry mass (N_{mass}) values were used together with V_{cmax} and J_{max} estimates to calculate the apparent nitrogen fraction in Rubisco (P_R), and in the rate-limiting proteins of photosynthetic electron transport (P_B), according to Niinemets and Tenhunen (1997):

$$P_R = V_{cmax} / (6.25 \times V_{cr} \times N_{mass} \times LMA)$$

$$P_B = J_{max} / (8.06 \times J_{mc} \times N_{mass} \times LMA)$$

where V_{cr} is the maximum rate of ribulose-1,5-bisphosphate carboxylation per unit Rubisco protein (V_{cr} = 20.5 µmol CO_2 (g Rubisco)$^{-1}$ s^{-1} at 25ºC), J_{mc} is the capacity for photosynthetic electron transport per unit cytochrome f (J_{mc} = 156 mol e^{-} (mol cyt f)$^{-1}$ s^{-1} at 25ºC), and scaling coefficients of 6.25 and 8.06 are based on the stoichiometry of nitrogen content of proteins and rate-limiting proteins (Niinemets and Tenhunen 1997).

The concentrations of chlorophyll, Rubisco, soluble protein and fibre (hemicellulose, cellulose and lignin) were determined in leaf samples collected in close proximity to the leaves used in the gas-exchange measurements. The samples used to determine the fibre concentration were harvested on the same dates as the gas-exchange measurements. Fibre concentrations were determined with an Ankom Analyzer (A220, New York, USA), following the method of Goering and Van Soest (1970). Since determining the concentration of Rubisco, chlorophyll and soluble protein is costly in terms of both time and money, we limited our analyses to samples taken on one single occasion (end of spring - beginning of the summer of 2013) from the same specimens sampled for the photosynthesis measurements. The samples were transported to the laboratory in a fridge. At the laboratory, the plant material was weighed and immediately plunged into liquid nitrogen and kept at -80ºC until further analysis. Protein extraction and the determination of protein and chlorophyll concentrations were conducted using the Agrisera method (Sweden). Chlorophyll was measured according to Whatley and Arnon (1963) and total soluble protein was measured according to Bradford (1976). The dry mass of the leaves used for the analyses were also determined, and the chlorophyll (CF) and protein (PT) contents of leaves were expressed per unit dry mass. For Western blotting and Rubisco analysis we used the Agrisera method, with minor modifications (see Vicente et al. 2011). The relative amount of the Rubisco large subunit was calculated by densitometric scanning of PVDF membranes by image analysis using the Scion ImagePC software (Scion, MD, USA) and expressed in arbitrary units (AU).

Statistical analysis

The data concerning the two plots selected for each climatic category as a function of the intensity of their winter harshness (two warmer plots, two intermediate plots and two plots with the coldest winters) were pooled after checking that there were no significant differences in the mean values obtained for each of them in any of the variables (data not shown). Between-site differences in the parameters analysed were explored using one-way analysis of variance. All statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). The Levenberg-Marquardt estimation technique of SPSS was used for all non-linear regression analyses of A/Ci curves.

RESULTS

Leaf morphology and chemistry: intraspecific variability related to a winter temperature gradient

In response to the temperature gradient, the two species studied showed significant between-site differences in several leaf traits. LMA, leaf thickness and the structural carbohydrates concentration (cellulose + hemicellulose) tended to be greater at the coldest sites, while the lignin concentration did not show differences between sites (Fig. 1). Also N_{area} tended to increase across the sites in correlation with the intensity of their winter harshness. However, the differences in N_{area} were merely the result of the increase in LMA associated with the decrease in temperatures, since N_{mass} did not vary significantly among sites in both species (Fig.1). The concentrations of chlorophyll and soluble proteins fell with the reduction in winter temperatures when they were expressed per unit of leaf mass, with significantly higher values in the leaves produced in warmer environments with respect to the colder sites (Fig. 2). The differences, however, tended to disappear when both constituents were expressed per unit leaf area, due to increased LMA in the coldest places.

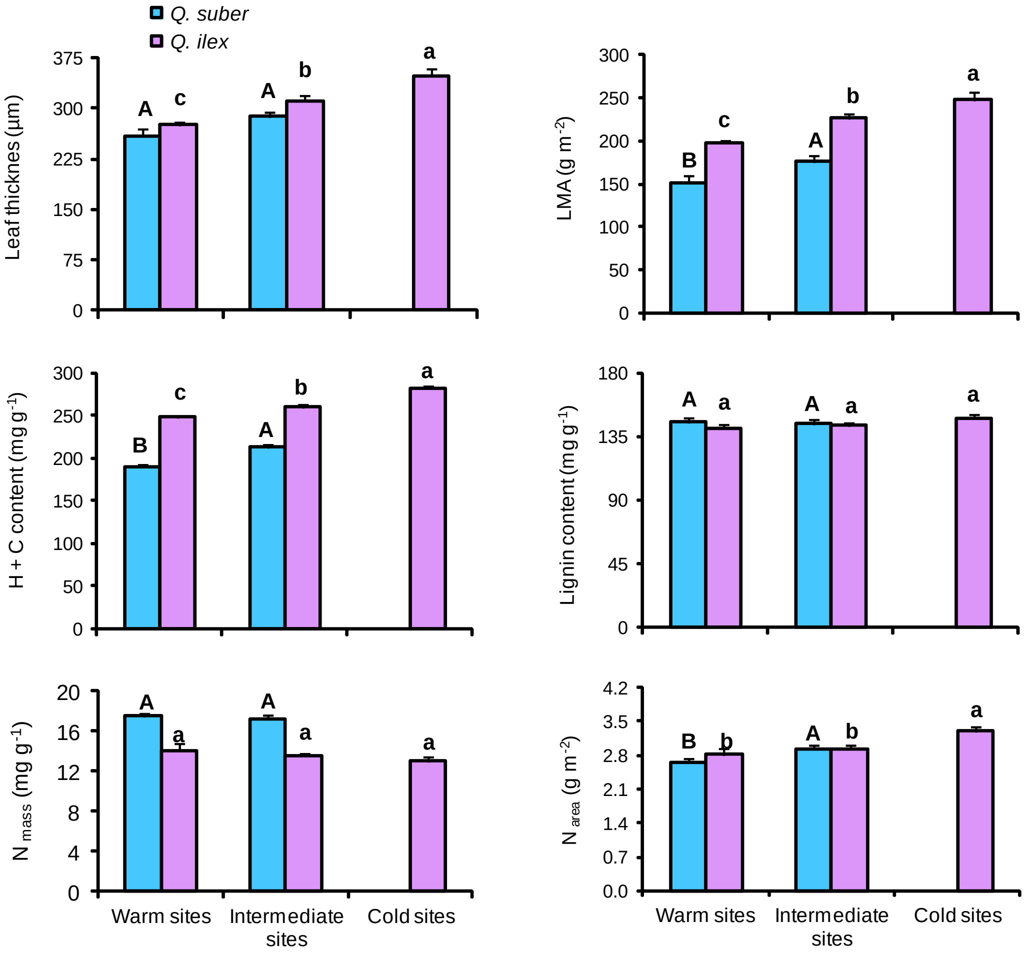

Figure 1. Mean (±SE, n = 20-30) leaf traits at the different sites. The significant differences among sites are marked with different letters (Fisher LSD test, $P < 0.05$). LMA, leaf mass per unit area; H+C, hemicellulose + cellulose.

For *Q. ilex*, the sites with intermediate conditions were not always significantly differentiated from the warmer or colder sites, although they consistently showed intermediate values between both. In both species the relative amounts of Rubisco decreased with the increase in the intensity of winter harshness (Fig. 2).

Intraspecific variability in photosynthetic parameters

The large between-environment differences in leaf structure and chemical composition were paralleled by significant variability in photosynthetic capacity as measured by apparent V_{cmax}

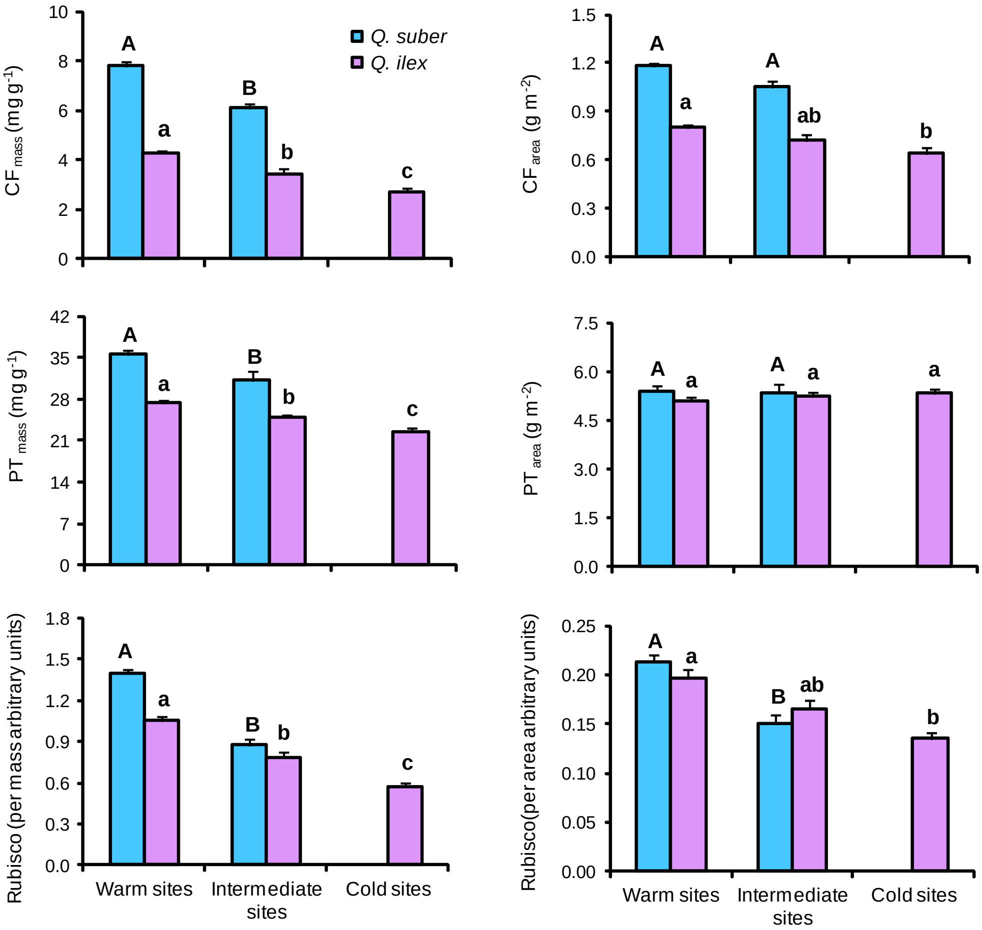

Figure 2. Mean (±SE, n = 10) concentration of chorophyll (CF), soluble protein (PT) and Rubisco at the different sites. The significant differences among sites are marked with different letters (Fisher LSD test, $P < 0.05$).

and J_{max}. The two parameters reached higher values at the warmer sites both when expressed per unit leaf area and per unit leaf mass (Fig. 3). By contrast, the day respiration rates (R_d) measured at 25ºC were higher at the colder sites for both species (Fig. 3). No significant differences were observed for J_{max}/V_{cmax} between the different sites (Fig. 3).

Due to the differences in V_{cmax} and J_{max}, the light-saturated photosynthetic rates at an ambient CO_2 concentration around 400 µmol mol^{-1} were always higher at the warmer sites (Table 2). The between-site differences were especially marked when photosynthetic rates were expressed on a per leaf mass basis. The relative stomatal limitation to photosynthesis

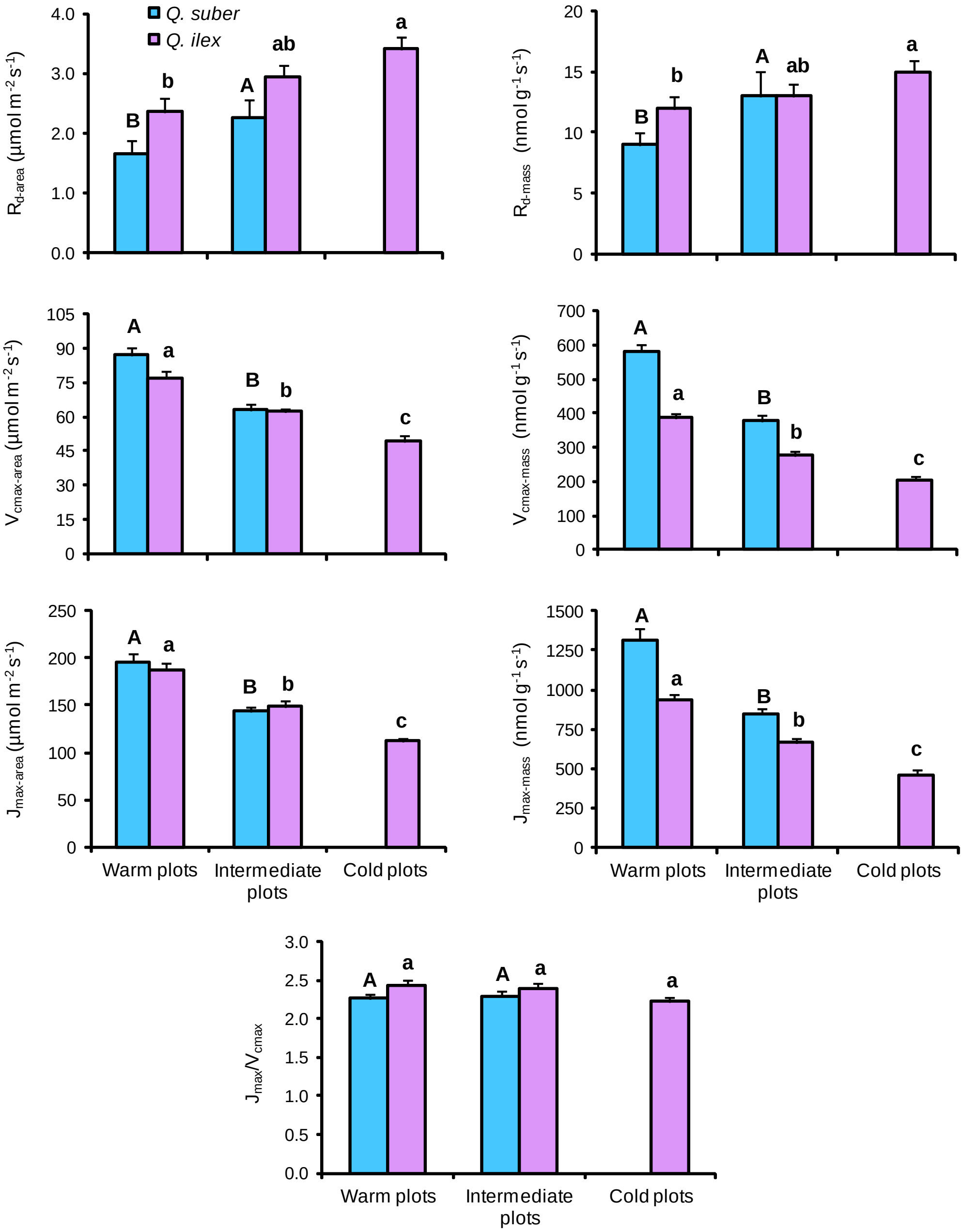

Figure 3. Mean (±SE, n = 20-30) day respiration rates and photosynthetic parameters at the different sites. The significant differences among sites are marked with different letters (Fisher LSD test, $P < 0.05$).

(RSL) was stronger in the warmer environments. Finally, the intrinsic water-use efficiency also reached significantly higher values at the warmer sites (Table 2). Under comparable temperature conditions, *Q. suber* exhibited higher values per unit leaf mass of V_{cmax}, J_{max} and light-saturated photosynthetic rate (Table 2 and Fig. 3).

Table 2. Light-saturated photosynthetic rates per unit leaf area (Aarea) and per unit leaf mass (Amass), intrinsic water use efficiency (A/g) and relative stomatal limitation (RSL) at the different sites. Standard errors in parentheses (n=20-30). The significant differences among sites are marked with different letters (Fisher LSD test, $P < 0.05$)

Species	Sites	A area (μmol m^{-2} s^{-1})	Amass (nmol g^{-1} s^{-1})	A/g (μmol C mol^{-1} H_2O)	RSL (%)
Q. suber	Warm sites	11.22 (0.55) a	75 (4.05) a	96 (3.80) a	37 (2.72) a
	Intermediate sites	9.30 (0.31) b	54 (0.95) b	71 (4.04) b	30 (1.77) b
Q. ilex	Warm sites	9. 00 (0.37) a	46.0 (1.81) a	109 (9.14) a	43 (1.80) a
	Intermediate sites	8.14 (0.31) ab	36.4 (1.42) b	90 (4.77) b	38 (2.23) a
	Cold sites	7.05 (0.56) b	29.2 (2.27) c	63 (4.53) c	31 (1.96) b

Relative investment of leaf nitrogen in photosynthesis

The estimated apparent nitrogen fraction in Rubisco (P_R) and in the rate-limiting proteins of photosynthetic electron transport (P_B) varied significantly among the different sites occupied by each species. Apparent leaf nitrogen investment in Rubisco (P_R) amounted to approximately 26% (*Q. suber*) and 21% (*Q. ilex*) at the sites with higher winter temperatures, but decreased to 17 and 12%, respectively, in the coldest locations for each species (Table 3). The higher fractional investment of foliar N in Rubisco in the warmer environments was also accompanied by a stronger investment in bioenergetics (P_B). Again, sites with intermediate temperatures exhibited intermediate values of P_B and P_R in *Q. ilex*

Table 3. Apparent nitrogen fraction in Rubisco (P_R) and in the rate-limiting proteins of photosynthetic electron transport (P_B) and photosynthetic nitrogen use efficiency (PNUE) at the different sites. Standard errors in parentheses (n=20-30). The significant differences among sites are marked with different letters (Fisher LSD test, $P < 0.05$)

Species	Sites	P_R (mg g^{-1})	P_B (mg g^{-1})	PNUE (μmol g^{-1} N s^{-1})
Q. suber	Warm sites	257 (10.2) a	59.2 (2.70) a	4.36 (0.23) a
	Intermediate sites	173 (8.55) b	40.2 (1.51) b	3.21 (0.14) b
Q. ilex	Warm sites	208 (5.89) a	52.9 (1.74) a	3.40 (0.12) a
	Intermediate sites	160 (1.73) b	39.1 (1.27) b	2.78 (0.09) b
	Cold sites	122 (4.46) c	29.4 (1.43) c	2.12 (0.15) c

(Table 3). As a consequence, both species achieved significantly higher photosynthetic nitrogen-use efficiencies at the warmer sites (Table 3). Within each species, the decrease in P_R in response to the decrease in winter temperatures was of a similar order of magnitude to the decrease in P_B, and in both species P_B and P_R exhibited percentage reductions similar to those observed for V_{cmax} and J_{max} respectively. Under the same temperature conditions, *Q. ilex* maintained lower fractional investments of its leaf N in Rubisco and in the rate-limiting proteins of photosynthetic electron transport, as well as lower PNUE than *Q. suber* (Table 3).

DISCUSSION

Confirming previous reports, in both species the leaves that had developed in cooler winter environments had a greater thickness, LMA and a higher concentration of structural carbohydrates than those that had developed at sites with milder winters. These results suggest that a greater leaf structural reinforcement is required to cope with more severe winter conditions. The between-site differences in leaf traits have important repercussions on photosynthesis, with large differences in the parameters of photosynthesis across the temperature gradient in both species. Many authors have reported greater V_{cmax} and J_{max} values in the leaves of a species grown at cooler temperatures than for those grown at warmer temperatures (Bunce 2000, Fan et al 2011, Dusenge et al 2015). These responses to temperature would support the hypothesis that plants growing in cool environments make relatively larger N investments into photosynthetic enzymes compared to plants growing in warmer environments in order to compensate for the low enzyme activity at low temperatures (Dusenge et al. 2015).

In contrast, in the present study, we have shown that the two species had lower values of V_{cmax} and J_{max} and light-saturated photosynthetic rates at the sites with colder winter temperatures. Given the strong correlations often observed between N_{area}, V_{cmax} and J_{max} across different species, the differences in leaf N content are usually considered as the main determinant of the variations in the photosynthetic parameters within a single species (Grassi et al. 2002, Medlyn et al. 2002, Warren et al. 2003, Whitehead et al. 2004, Misson et al. 2006, Zhang et al. 2007, Vaz et al. 2011, Zhang and Yin 2012). Several authors have reported higher N concentrations in the leaves produced under low temperatures (Reich and Oleksyn 2004), which also supports the hypothesis that higher investments in enzymes constitute an adaptation aimed at compensating for the shorter length of the favourable growth period at sites with lower temperatures. However, in the present study, the between-site differences in N_{area} were small. Accordingly, we did not observe any trend in increased N investments in colder sites.

Leaf structure, especially leaf mass per area (LMA), has been proposed by different authors as one of the determinants of photosynthetic rates, owing to the effects of LMA on the patterns of nitrogen allocation (Field and Mooney 1986, Evans 1989, Poorter et al. 2009). Greater allocation of leaf nitrogen to cell walls and reduced investment in Rubisco as LMA increased has been observed in different studies (Onoda et al. 2004, Takashima et al. 2004). In this study, the larger LMA at the colder sites was accompanied by lower concentrations of chlorophyll, soluble protein and Rubisco than in warmer environments. Despite the similar leaf N concentrations at the different sites, the fractional investments of foliar N in Rubisco (P_R) and in the rate-limiting proteins of photosynthetic electron transport (P_B) always reached significantly lower values at the colder sites. The greater thickness and LMA at colder sites is probably a consequence of the thickening of the cell walls, which explains the higher concentrations of structural carbohydrates, a characteristic of leaves growing in cold climates (Kubacka-Zebalska and Kacperska 1999, Stefanowska et al. 1999). It is known that cell walls accumulate a significant amount of nitrogen compounds: up to 10% of the cell content (Reiter 1998, Hikosaka and Shigeno 2009). Accordingly, it can be expected that a greater amount of available nitrogen would be allocated to cell walls in environments with harsher winters, leading to a reduction in the amount available for allocation to chlorophyll or photosynthetic proteins. This lower allocation is the most plausible explanation for the lower values of photosynthetic capacity, V_{cmax} and J_{max} in the colder environments. In addition to the lower values of the photosynthetic parameters, leaf dark respiration rates at 25ºC tended to be higher in the colder than in the warmer habitats, a trend previously reported by several authors (Mooney 1963, Oleksyn et al. 1998). These higher R_d values may reflect an inevitable increase in maximum respiratory capacity associated with repair functions under cold, bright conditions that may induce photoinhibition (Atkin et al. 2000). Our results have suggested that the within-leaf N allocation is a stronger determinant of the intraspecific variation in photosynthetic capacity than absolute leaf N content. Owing to the differences in the allocation of N to photosynthetic enzymes, leaf structural components and repair functions, the two species achieved higher N-use efficiency at the warmer sites.

The between-site differences in leaf structure should determine important differences in mesophyll conductance (g_m). Decreases in the internal diffusion conductance have been associated with increases in LMA and in the cell wall fraction in several species (Syvertsen et al. 1995, Kao and Chang 2001, Niinemets et al. 2005, 2006, Flexas et al. 2008, Tomas et al. 2013), which should contribute to limiting the supply of CO_2 to the chloroplasts, and to reducing the photosynthetic capacity. We made our calculations assuming infinite conductance of CO_2 transfer between the intercellular spaces and sites of carboxylation. Rates of photosynthesis are related to the concentration of CO_2 at the sites of carboxylation (Cc), not the more easily measured Ci, and it is possible that the draw-down from Ci to Cc varies among sites and this affects the results. Different authors have demonstrated that assuming an infinite mesophyll conductance leads to a large underestimation of V_{cmax} and to a lesser extent of J_{max} (Ethier and Livingston 2004, Manter and Kerrigan 2004, Flexas et al. 2008, Niinemets et al. 2009b). If leaf mesophyll conductance is lower in leaves with the high LMA and cell wall content typical of the colder environments, Cc will be much lower than Ci and will result in a more significant underestimation of V_{cmax} than that obtained in the warmer environments. The changes in mesophyll conductance could thus attenuate the differences in the actual values of the photosynthetic parameters. However, the decrease in V_{cmax} and J_{max} with the decrease in winter temperatures observed in the present study was very strong (more than 30% in *Q. suber* and 35% in *Q. ilex*). It is hard to believe that this strong reduction in the photosynthetic parameters would be explained solely by calculation errors associated with the differences in g_m. Furthermore, the percent reduction in J_{max} was of similar magnitude to that of V_{cmax}, despite the fact that J_{max} is much less affected by g_m than V_{cmax} (Ethier and Livinston 2004, Flexas et al. 2008, Niinemets et al. 2009b).

Owing to the parallel variation in both photosynthetic parameters, we did not find significant between-site differences in the J_{max}/V_{cmax} ratio. Other authors that have analyzed between-site differences in the photosynthetic parameters have reported decreases in the J_{max}/V_{cmax} ratio at the warmer sites in several controlled experiments (Bernacchi et al. 2003, Yamori et al. 2005), as well as in other field studies with different species (Onoda et al. 2005, Kattge and Knorr 2007, Leuning 1997, Hikosaka et al. 1999, Bernacchi et al. 2001, Leuning 2002, Yamori et al. 2010). This would suggest that these species invest more nitrogen in RuBP regeneration processes (electron transport, ATP synthase, and Calvin cycle except for Rubisco) than in Rubisco as growth temperatures decrease. The higher apparent investment in J_{max} compared to V_{cmax} at the colder sites has been interpreted as an adaptation to the lower PAR levels at higher latitudes (Yamori et al. 2010, van de Weg et al. 2012). Obviously, this explanation is only valid when the temperature gradients are associated with large latitude gradients that imply differences in the available radiation levels. In our case, however, the differences in latitude of our sites are small, and, accordingly, the solar radiation levels were similar (Table 1). The constancy in the values of the J_{max}/V_{cmax} ratio suggests that this ratio is insensitive to changes in temperature, which is consistent with the results obtained by other authors (Ferrar et al. 1989, Bunce 2000).

In conclusion, our results reveal that, owing to the leaf structural reinforcement typical of colder climates, the proportion of N allocated to the photosynthetic machinery in these environments is lower than in warmer locations. This lower allocation contributes to decreasing the photosynthetic N-use efficiency at the colder sites with respect to the populations of the same species located in warmer areas. Although the relative stomatal limitation to photosynthesis (RSL) was lower in the colder environments, this lower limitation was not sufficient enough to compensate the apparent lower activity of the photosynthetic machinery, which results in lower rates of light-saturated photosynthesis at ambient CO_2 concentrations. It has been suggested that low photosynthetic rates of evergreen species in colder climates may be compensated by a longer leaf lifespan (Wright et al. 2005). However, for the two species included in the present study, differences in leaf lifespan were not significant across the temperature gradient (González-Zurdo et al. 2016). It could be argued that under water-limited conditions, as in Mediterranean environments, the lower photosynthetic capacity typical of colder environments is compensated by lower stomatal limitations during the drought-stressed part of the growth season. If drought stress was more intense at warmer sites, this would reverse the positive effects of higher temperatures and could shorten the growth season at the warmer sites when compared with the colder ones. However, in the present study, differences in summer temperatures were relatively low. At the same time, the warmer sites also received greater rainfall, which could reduce the differences in drought stress. In fact, the Emberger's pluviothermic index was uncorrelated with temperature (Table 1). Accordingly, under the conditions of the present study, the photosynthetic performance of evergreen leaves is significantly lower at colder sites and this disadvantage is not compensated for by other leaf traits.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Conclusiones

Ocupar ambientes con inviernos más fríos exige a las especies reforzar sus hojas con un mayor peso por unidad de superficie (LMA), el cual se consigue a través de un mayor espesor, sin cambios asociados en densidad. Nuestros resultados revelan que es el contenido de carbohidratos estructurales (celulosa y hemicelulosa) y no la lignina quien confiere a las hojas ese mayor refuerzo, incrementando su supervivencia bajo condiciones adversas, como son las menores temperaturas y mayor intensidad y duración de las heladas en los ambientes con inviernos más duros.

Ninguna tendencia era observada en el contenido de nitrógeno foliar asociada a las diferencias en temperatura a lo largo del gradiente de rigor invernal. Sin embargo la concentración de clorofila, Rubisco y proteínas solubles es siempre más baja en los sitios más fríos. Este resultado sugiere que el incremento en el reparto de nitrógeno a las paredes celulares en los ambientes más fríos ocurre a expensas de reducir la asignación al aparato fotosintético, poniendo de manifiesto una vez más la existencia de un compromiso en la distribución del nitrógeno entre fotosíntesis y persistencia.

Contrariamente al nitrógeno, la concentración foliar de fósforo responde a los cambios en temperatura, incrementándose entre sitios con la intensidad del rigor invernal. Puesto que en nuestro caso el fósforo es el elemento que supuestamente actúa como limitante, una mayor concentración de este nutriente en las hojas de los árboles creciendo en ambientes más fríos podría ayudar a compensar los efectos desfavorables de las bajas temperaturas sobre la asimilación de carbono.

Todas las especies de estudio muestran menor potencial para la reabsorción de nitrógeno en ambientes con temperaturas invernales más bajas, como consecuencia de las mayores cantidades de nutriente inmovilizadas en el gran volumen de pared celular necesaria para hacer frente a estas condiciones más extremas. Los cambios en composición química foliar en respuesta a la temperatura deben, por tanto, ser incorporados en la serie de factores que permiten explicar las diferencias intraespecíficas en la eficiencia de reabsorción de nitrógeno.

El mayor reforzamiento foliar en los ambientes más fríos contribuye a reducir la intensidad de los daños por herbivoría, pero no resulta suficiente como para reducir las pérdidas de área por efecto de las bajas temperaturas, siempre mayores en los ejemplares sometidos a condiciones invernales más adversas. El incremento en la intensidad de los daños abióticos en los sitios más fríos respecto a los más cálidos es mayor que el descenso en herbivoría, lo que sugiere un impacto final negativo de las bajas temperaturas sobre la duración de la superficie fotosintética, que, en principio, supondría una reducción de la fijación total de C respecto a la obtenida por las mismas hojas en ambientes más cálidos.

Debido a la mayor inversión de N en paredes celulares, y consecuente descenso en la inversión en Rubisco y proteínas implicadas en la cadena de transporte de electrones, las hojas de una misma especie muestran siempre menor V_{cmax} y J_{max} y por tanto capacidad fotosintética y eficiencia en el uso del N en los ambientes más fríos. La menor limitación estomática que también muestran nuestras especies en estos ambientes no es suficiente para compensar la menor actividad de la maquinaria fotosintética, lo que resulta finalmente en menores tasas de fotosíntesis a concentración ambiente de CO_2.

Si, como sugiere nuestro estudio, ocupar ambientes con inviernos más fríos conlleva una menor eficiencia de retranslocación de N, menor capacidad fotosintética y mayores pérdidas prematuras de área foliar, esto supondría que el hábito perennifolio implica mayores costes en estos ambientes, lo cual necesariamente supone una desventaja adicional con respecto a las caducifolias, que no es compensada por otros rasgos como pudiera ser un incremento en la duración de sus hojas.

Si el cambio climático finalmente conlleva un aumento de las temperaturas y una disminución del rigor invernal, esto podría reducir los efectos desfavorables asociados al mayor espesor y LMA que hemos visto parecen tener que presentar las hojas de las perennifolias para hacer frente a las bajas temperaturas. En vista de nuestros resultados está claro entonces que el efecto de los cambios en temperatura durante el invierno deben ser tenidos muy en cuenta en los modelos predictivos de la posible composición de especies de nuestros bosques en el nuevo escenario climático previsto.