Universitat de Girona

HOW MEDITERRANEAN PLANT SPECIES ARE ABLE TO COPE WITH INCREASING LEVELS OF UV-B RADIATION AND DROUGHT IN THE CONTEXT OF CLIMATE CHANGE?

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Dipòsit legal: Gi. 1270-2013
http://hdl.handle.net/10803/120560

Doctoral Thesis

How Mediterranean plant species are able to cope with increasing levels of UV-B radiation and drought in the context of climate change?

Memòria presentada per:

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Per optar al grau de doctora per la Universitat de Girona.

La present tesi doctoral ha estat supervisada per:

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I s'inclou dins del programa de doctorat en Ciències Experimentals i de Sostenibilitat.

Universitat de Girona, 2013

Universitat de Girona

Publications derived from the present thesis:

- **Bernal M, Llorens L, Badosa J, Verdaguer D. 2013.** Interactive effects of UV radiation and water availability on seedlings of six woody Mediterranean species. *Physiologia Plantarum* 147: 234-247

- **Bernal M, Verdaguer D, Badosa J, Abadía A, Llusia J, Peñuelas J, Núñez-Olivera E, Llorens L.** Effects of enhanced UV radiation and water availability on performance, biomass production and photoprotective mechanims in *Laurus nobilis* seedlings. In preparation.

- **Bernal M, Llorens L, Julkunen-Tiitto R, Badosa J, Verdaguer D.** Altitudinal and seasonal changes of phenolic compounds in *Buxus sempervirens* leaves and cuticles. Submitted to *Plant physiology and biochemistry*.

Universitat de Girona

La Dra. Laura Llorens Guasch i la Dra. Dolors Verdaguer Murlà del Departament de Ciències Ambientals de la Universitat de Girona,

CERTIFIQUEM:

Que aquest treball titulat "How Mediterranean plant species are able to cope with increasing levels of UV-B radiation and drought in the context of climate change?" que presenta Meritxell Bernal Montolio per a l'obtenció del títol de doctora, ha estat realitzat sota la nostra direcció i que compleix els requeriments per poder optar a Menció Internacional.

I perquè així consti i tingui els efectes oportuns, signem aquest document,

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Girona, març del 2013

Al pare i la mare,

Agraïments

Abans de res, agrair a les meves directores, Laura i Dolors, per donar-me l'oportunitat de fer aquesta tesi així com per totes les ensenyances tant en l'àmbit professional com en el personal. També voldria agrair al Jordi per l'ajut en les mesures de radiació, a la Monona (CSIC Aula dei, Saragossa) per iniciar-me en el món de l'HPLC i dels pigments fotosintètics. A la Encarnación, al Gabi, la Laura i al Javier de la Universitat de la Rioja per tota la ajuda i per fer-me sentir com a casa durant la meva estança a Logronyo, i a la Riitta (Universitat de Joensuu, Finlàndia) per acollir-me durant 6 mesos i per ensenyar-me l'apassionant món dels fenols.

Però sobretot, estic agraïda a tots i totes els que heu estat al meu costat durant aquests anys en els bons i en els mals moments i, el més important, que m'heu ajudat a créixer, perquè, gràcies a vosaltres, el fruit d'aquests anys no queda acotat només en aquesta tesi sinó en molts d'altres àmbits personals.

Per començar, gràcies a l'equip UdG que m'heu acompanyat dia rere dia durant aquests 5 anys. Mercè, Juanita, Laura D, Laura B, Anna, Jordi C, Jordi R, Sergi i Miquel, merci per tots els dinars de taper i els moments cafè, així com per tot el suport incondicional. Gràcies Nuri i Gemma pel suport tècnic i humà.

També us vull agrair als i les "*Biohooligans*" la vitalitat que transmeteu i totes les rialles que desperteu, gràcies per tots els moments de disbauxa, una de les coses que em donen més sentit a la vida.

Gràcies equipo "*muntanyeru*": Laura, Muntsa, Virginia, Carol, Judit i Maira per totes les caminades, pics, estrelles i abraçades que hem compartit, indispensables per carregar pil·les.

Moltes gràcies Carles i Montse per les entrades d'aire fresc dels dimarts, motor per continuar les llargues setmanes d'escriptura, però sobretot, gràcies per mostrar-me que hi ha gironins encantadors.

Gràcies Quim per donar-me l'oportunitat de sentir a través de la guitarra però sobretot, gràcies per ser-hi, per la tranquil·litat que em transmets i perquè de tu, he aprés moltíssim.

Gràcies Enric per escoltar-me, per la paciència que has tingut i per tot el carinyu incondicional que m'has donat.

Gràcies Judit per mostrar-me la importància de buscar dins d'un mateix per tal d'entendre's, valorar-se i estimar-se.

Gràcies Mariona per compartir amb mi aquest camí de primera mà i per entendre'm, animar-me i recolzar-me en tot moment.

Gràcies Laurins per entendre'm tan bé en els moments d'estrès i per donar-me la tranquil·litat de saber que tinc algú amb qui comptar passi el que passi.

Moltes gràcies Míriam per ensenyar-me a convertir els mals moments en un pretext de noves il·lusions.

I gràcies Marta per donar-me una mica de calor en la freda Finlàndia, però també, per ser al meu costat en el moment necessari recordant-me qui sóc i què vull i donant-me l'empenta que em calia per continuar endavant.

Per últim, vull agrair a qui més complicat és acotar en unes línies tot el que sento, a vosaltres pares, gràcies per tot el suport, amor i valoració que sempre m'heu donat però sobretot, per fer-me sentir increïblement afortunada de poder comptar amb uns pares que sempre m'estimaran i estaran al meu costat.

Content

Resum

Durant les properes dècades, a la Regió Mediterrània es preveu un augment de la radiació ultraviolada (UV) que arriba als ecosistemes bàsicament com a conseqüència de la disminució de la nuvolositat. A més a més, la migració altitudinal de les espècies deguda a l'escalfament global també podria incrementar els nivells d'UV als quals es veuen exposades les espècies mediterrànies. La radiació UV-B pot ser un factor oxidatiu per les plantes affectant la seva activitat fisiològica i la seva morfologia. Tot i així, la majoria dels estudis sobre els efectes de la radiació UV realitzats fins ara s'han dut a terme en espècies de latituds altes, essent pocs els basats en especies mediterrànies. Els pocs estudis realitzats en espècies mediterrànies suggereixen que la radiació UV podria incrementar la resistència de les plantes a la sequera. Per tant, tenint en compte que a la Regió Mediterrània es preveu una intensificació de la sequera estival per a les properes dècades, l'augment d'UV previst per a aquesta regió podria beneficiar de manera indirecta les espècies mediterrànies. En aquest context pretenem investigar els efectes de la radiació UV-B sobre les espècies llenyoses mediterrànies així com la seva interacció amb una baixa disponibilitat hídrica. Per tal d'assolir aquest objectiu hem desenvolupat tres experiments. En el primer experiment hem fet créixer sis espècies autòctones mediterrànies en un hivernacle sota tres condicions diferents de llum ultraviolada (sense UV, amb UV-A o amb UV-A+UV-B). A més a més, dins de cada condició de llum ultraviolada una part de les plantes rebien l'aigua necessària per a mantenir el sòl en capacitat de camp mentre que l'altra part rebien la meitat de l'aigua aplicada a les primeres. Al segon experiment hem fet créixer plàntules de llorer (*Laurus nobilis*) a l'exterior també sota tres condicions de llum ultraviolada (UV ambiental, augment d'UV-A i augment d'UV-A+UV-B respecte dels nivells ambientals). Igual que en el primer experiment, les plàntules de cada condició d'UV estaven exposades a dos règims hídrics. En aquests dos primers experiments hem analitzat els efectes dels tractaments a diversos nivells: morfològic (biomassa i morfologia foliar), fisiològic (intercanvi de gasos i pigments fotosintètics) i bioquímic (compostos que absorbeixen la llum ultraviolada). Per últim, en el tercer experiment, hem analitzat per a cada estació de l'any el contingut de fenols, compostos produïts per les plantes

per fer front a la radiació UV, en fulles i cutícules foliars de *Buxus sempervirens* crescudes de manera natural al llarg d'un gradient altitudinal per tal de determinar si els canvis en aquests compostos poden ser produïts per les variacions naturals en la radiació UV-B i alhora poder trobar un bioindicador dels nivells ambientals d'UV-B.

Els nostres resultats mostren que l'augment dels nivells d'UV-A i d'UV-A+UV-B incrementa, en algunes especies, la producció de biomassa quan les plantes creixen sota condicions de baixa disponibilitat hídrica. Aquest efecte beneficiós podria ser degut a una millora de les relacions hídriques de la planta produïda principalment per la radiació UV-A. Els canvis en la morfologia foliar observats en resposta a l'augment en la radiació UV (fulles més gruixudes o amb un major índex de massa per àrea depenent de l'experiment) poden haver contribuït a aquesta millora de les relacions hídriques. Per altra banda però, hem observat en el llorer que l'augment en la radiació UV-A sembla comportar un excés d'energia lumínica dins la fulla, ja que les plantes suplementades amb UV-A presenten una reducció en el contingut foliar dels pigments captadors de llum, un augment de l'índex de desepoxidació del cicle de les xantofil·les i un augment de l'energia dissipada en forma de calor (mesurada com la extinció no fotoquímica, NPQ).

En relació als compostos que absorbeixen la radiació UV, encara que no hem trobat un efecte de la radiació UV sobre el contingut total d'aquests a les fulles, sí que hem observat canvis en el contingut foliar de compostos específics a *Laurus nobilis*. Per exemple, hem vist que l'augment d'UV-A disminueix el contingut foliar d'alguns kaempferols i quercetines. Dels nostres resultats també deduïm que les variacions estacionals o altitudinals en el contingut de compostos fenòlics foliars de *Buxus sempervirens* estan més subjectes a l'ontogènia foliar o a altres factors ambientals que no pas a les variacions naturals en la radiació UV-B.

En conclusió, els nostres resultats suggereixen que l'augment en la radiació UV que s'espera per a les properes dècades no tindrà un efecte negatiu sobre les espècies mediterrànies estudiades. De fet, fins i tot podria tenir un efecte positiu (sobretot com a conseqüència de l'augment en la radiació UV-A) si, tal i com es preveu, va acompanyat d'una reducció en la precipitació.

Resumen

En la Región Mediterránea se prevé un aumento del flujo de la radiación ultravioleta (UV) durante las próximas décadas como consecuencia de la disminución de la nubosidad. Además, la migración altitudinal de las especies debida al calentamiento global también podría incrementar los niveles de radiación UV a los cuales están expuestas las especies mediterráneas. La radiación UV-B puede ser un factor oxidativo para las plantas afectando su actividad fisiológica y su morfología. Sin embargo, la mayoría de los estudios sobre los efectos de la radiación UV se han desarrollado en especies de latitudes elevadas mientras que pocos estudios se han basado en especies mediterráneas. La escasa literatura en especies mediterráneas sugiere que la radiación UV podría incrementar la resistencia de las plantas a la sequía. Dado que en la Región Mediterránea también se prevé una intensificación del período de sequía estival durante las próximas décadas como consecuencia del cambio climático, el aumento en la radiación UV previsto para esta región podría beneficiar de manera indirecta las especies mediterráneas. En este contexto, nos proponemos investigar los efectos de la radiación UV-B en especies leñosas mediterráneas así como la interacción de estos efectos con una baja disponibilidad hídrica. Para ello, hemos desarrollado tres experimentos. En el primer experimento hemos comparado plántulas de seis especies autóctonas mediterráneas crecidas en un invernadero bajo tres condiciones distintas de radiación ultravioleta (sin UV, con UV-A y con UV-A+UV-B). Además, dentro de cada una de las condiciones de radiación UV, la mitad de las plantas las regamos hasta capacidad de campo mientras que la otra mitad recibían la mitad del agua aplicada a las primeras. En el segundo experimento, plántulas de laurel (*Laurus nobilis*) crecieron en el exterior bajo tres condiciones distintas de radiación UV (UV ambiental, aumento de UV-A y aumento de UV-A+UV-B respecto de los niveles ambientales). Igual que en el primer experimento, las plántulas de cada condición de radiación UV estaban expuestas a dos regímenes hídricos. En estos dos experimentos hemos analizado los efectos de los tratamientos aplicados a distintos niveles: morfológico (biomasa y morfología foliar), fisiológico (intercambio de gases y pigmentos fotosintéticos) y bioquímico (compuestos que absorben la radiación UV). Por último, en el tercer experimento,

hemos analizado estacionalmente el contenido en fenoles, compuestos producidos por las plantas para hacer frente a la radiación UV, en las hojas y cutículas foliares de *Buxus sempervirens* crecidas de manera natural a lo largo de un gradiente altitudinal. El objetivo era determinar si los cambios en estos compuestos podrían estar producidos por las variaciones naturales en la radiación UV-B y si alguno de estos compuestos podría usarse como bioindicador de los niveles ambientales de UV-B. Nuestros resultados muestran que un aumento de los niveles de UV-A y de UV-A+UV-B incrementan, en algunas especies, la producción de biomasa cuando las plantas crecen en condiciones de baja disponibilidad hídrica. Este efecto beneficioso podría ser debido a una mejora de las relaciones hídricas de la planta producida principalmente por la radiación UV-A. Los cambios en la morfología foliar observados en respuesta al aumento en la radiación UV (hojas más gruesas o con un mayor índice de masa por área dependiendo del experimento) pueden haber contribuido a dicha mejora de les relaciones hídricas. Por otro lado, sin embargo, hemos observado en el laurel que el aumento en la radiación UV-A podría comportar un exceso de energía lumínica dentro de la hoja ya que las plantas suplementadas con UV-A mostraron una reducción en el contenido foliar de los pigmentos captadores de luz, un aumento del índice de desepoxidación del ciclo de las xantofilas y un aumento de la energía disipada en forma de calor (medida como la extinción no fotoquímica, NPQ). En relación a los compuestos que absorben la radiación UV, a pesar de no haber encontrado un efecto de los tratamientos sobre el contenido total de estos compuestos en hojas, sí que hemos observado cambios en el contenido foliar en compuestos específicos en *Laurus nobilis*. Por ejemplo, hemos visto que el aumento en la radiación UV-A diminuye el contenido foliar en algunos kaempferoles y quercetinas. De nuestros resultados también deducimos que las variaciones estacionales o altitudinales en el contenido de compuestos fenólicos foliares de *Buxus sempervirens* está más sujeta a la ontogenia foliar o a otros factores ambientales que a las variaciones naturales de radiación UV-B.

En conclusión, los resultados sugieren que el aumento en la radiación UV que se espera para las próximas décadas no afectará negativamente las especies mediterráneas estudiadas. De hecho, incluso podría beneficiarlas (como consecuencia, sobretodo, del aumento en la radiación UV-A) si, tal y como se prevé, se produce un reducción de la precipitación.

Abstract

Climatic models predict higher fluxes of UV radiation in the near future for the Mediterranean region mainly due to a decrease in the mean cloudiness. Furthermore, the altitudinal migration of species in response to global warming will probably also lead to Mediterranean species being exposed to higher UV radiation. The UV-B radiation can be an oxidative stress factor for plants affecting their physiological activity and morphology. However, most of the studies about UV radiation effects have been performed in species from high latitudes being the studies on Mediterranean woody species scarce. Some of these previous studies on Mediterranean species have suggested that UV-B radiation may increase plant resistance to drought stress. Taking into account that an intensification of drought conditions during summer is also expected in the Mediterranean region in the coming years, the predicted increase in UV-B radiation in this region could indirectly benefit Mediterranean species. In this context, we aim to investigate the effects of UV-B radiation on Mediterranean woody species and its interaction with low water availability. Accordingly, three experiments were designed and carried out as follows. In the first one, seedlings of six Mediterranean species were grown in a greenhouse under three different UV radiation conditions (without UV, with UV-A and with UV-A+UV-B). Within each UV condition, half of the plants were watered to saturation while the other half received half of the water applied to the first ones. In the second experiment, seedlings of *Laurus nobilis* were grown outdoors under three different UV radiation conditions (ambient UV, enhanced UV-A and enhanced UV-A+UV-B). As in the first experiment, seedlings from each UV condition were exposed to two watering regimes. In both of these experiments the effects of the treatments applied were examined at three different levels: morphological (biomass production and leaf morphology), physiological (gas exchange parameters and photosynthetic pigments) and biochemical (UV-absorbing compounds). In the third experiment seasonal changes in the content of phenols, compounds synthesized by plants to cope with UV-B radiation, in leaves and foliar cuticles of *Buxus sempervirens* growing along an altitudinal gradient in the field were measured during one year. The aim was to investigate whether the changes in the levels of phenolic compounds of this species

followed the natural variation in the levels of UV-B radiation and whether any of these compounds could be used as a biomarker for ambient levels of UV-B radiation.

The results showed that UV-A and UV-A+UV-B supplementations caused an increase in the plant biomass of some species when plants were grown under a low water supply. This beneficial effect on plant biomass seemed to be mediated by a UV-A-induced improvement of plant water relations. Increases in leaf sclerophylly or leaf thickness, depending on the experiment, in response to enhanced UV radiation might have contributed to the amelioration of plant water deficit. Nevertheless, for *Laurus nobilis* an excess of light energy under UV-A radiation supplementation promoted a higher de-epoxidation state of the violaxanthin cycle and a greater energy dissipation as heat (measured as non-photochemical quenching, NPQ) compared to plants grown under ambient levels of UV radiation.

Although a general effect of UV radiation on total leaf content of phenols or UV-B-absorbing compounds was not observed, some compound-specific responses were found, at least in *Laurus nobilis*. More specifically, enhanced UV-A radiation decreased the foliar content of specific kaempferol and quercetin derivatives. In the case of *Buxus sempervirens*, results pointed out that UV-B radiation was not the main factor modulating the observed seasonal and altitudinal changes in leaf and cuticle content of phenolic compounds.

In conclusion, the results of the present thesis suggest that the increase in UV radiation expected in the near future for the Mediterranean region will not have a damaging effect on the studied woody Mediterranean species. In fact, under low water availability, it could even have a positive effect on these species mainly as a consequence of UV-A radiation exposure.

Chapter I. General introduction

1.1) The ultraviolet-B radiation

Ultraviolet (UV) radiation covers the range of wavelengths of the solar spectrum from 100 to 400 nm and is divided into three bands: UV-C (100-280 nm), UV-B (280-315 nm) and UV-A (315-400 nm) (International Commission on Non-Ionizing Radiation Protection, ICNIRP 2004). Since UV-C is blocked by the terrestrial atmosphere, UV-B is the UV band with the shortest wavelength that reaches the Earth's surface. Hence, it is the most energetic component of the sunlight reaching the biosphere and can be a photo-oxidative stress factor for plants affecting their physiological activity and morphology (Stratmann 2003, Caldwell *et al.* 2007).

Levels of UV-B radiation reaching the Earth's surface are primarily determined by the sun emittance and the orbital position of the Earth which determines the path through the atmosphere that UV-B crosses (Bais *et al.* 2007). Therefore, UV-B fluxes vary among latitudes, with the highest levels being at the lowest latitudes (Seckmeyer *et al.* 2008). UV-B radiation also increases with altitude because of the shorter optical path that radiation has to cross to reach the surface and a lower degree of scattering and absorption. In addition, UV-B radiation is positively related to surface albedo[1] (Calbó *et al.* 2005), which is particularly high over strongly reflecting surfaces, such as ice, snow or sand (Bais *et al.* 2007).

In the atmosphere, many compounds such as ozone, aerosols and other gaseous air pollutants (e.g. O_3, S_2, NO_2, N_2O_5, NOx, aldehydes, acetone, nitrated aromatics and certain organic acids) absorb UV-B radiation attenuating its incidence on the Earth's surface (Bais *et al.* 2007). Among them, stratospheric ozone is of great importance, since a decrease in the amount of ozone at the upper atmosphere usually results in higher levels of UV-B radiation reaching the Earth's surface. For this reason, the depletion of the stratospheric ozone registered during approximately the last three decades, mainly due to the emissions of chlorofluorocarbons (CFCs), has led to a considerable concern about the effects of enhanced UV-B radiation on organisms. Mainly due to the success of the Montreal protocol (1987) in reducing the

[1] The surface albedo is the ratio between the shortwave irradiance reflected from a system and the incident irradiance.

use of ozone-depleting substances, the ozone layer is recovering, with the amount of ozone in most regions being expected to be greater than 1980 levels by the end of this century (McKenzie *et al.* 2011).

However, in spite of the recovering of the ozone layer, models predict an increase in the surface UV-A and UV-B levels by the end of this century in some regions mainly driven by changes in other climatic factors rather than stratospheric ozone (Bais *et al*. 2007). For example cloudiness is an important factor attenuating UV-B incidence on the earth surface because UV-B radiation is scattered when passing through the water droplets or ice crystals that form clouds. Therefore, changes in cloud cover can be translated in changes in surface UV-B radiation levels (Calbó *et al.* 2005, Mateos *et al.* 2011). On the other hand, the altitudinal migration of species in response to global warming (Peñuelas *et al.* 2007) will lead some species, such as Mediterranean ones, to be exposed to higher UV radiation since UV radiation increases with altitude (McKenzie *et al.* 2001).

Since climate change effects differ at regional scales, future changes in the surface UV-B radiation are expected to differ between regions. Indeed, at high latitudes is predicted a decrease in erythemal[2] UV radiation (UV_E) and hence, in UV-B, due to reduced area of snow which reduces surface albedo and an increase in cloud cover, while at low- and mid-latitudes is expected an increase in UV-B radiation mainly due to reduced cloudiness (Fig. 1) (Bais *et al.* 2007, McKenzie *et al.* 2011). Therefore, the study of the effects that UV-B changes in the coming decades can have on the biosphere should be regionally focused.

[2] UV_E is an index of the UV irradiance weighted by the erythema action spectrum that is a measure of the "sunburnig" effects on human skin (McKinlay and Diffey 1987). Since UV-B present more "sunburning" effects on human skin than UV-A, in the UV_E index the contribution of the UV-B range is 87% being the contribution of UV-A range only 13%. Therefore, UV_E could be used as an indirect measure of UV-B.

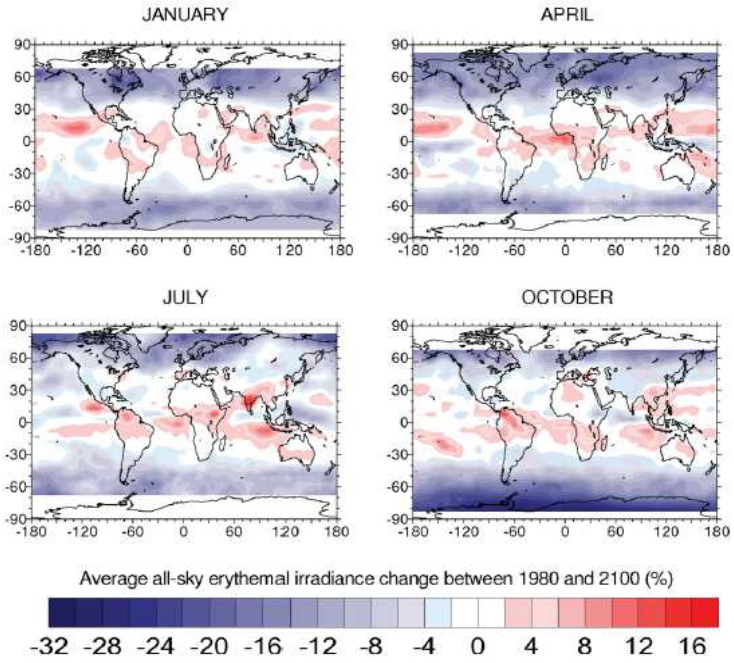


Fig. 1 Multi-model average changes in surface erythemal UV irradiance (UV_E) from 1980 (1975-1985) to 2100 (2089-2099) for four months (from McKenzie et al. 2011).

1.2) The effects of UV-B on plants

UV-B radiation has historically been considered to be a photo-oxidative stress factor (Stratmann 2003, Caldwell *et al.* 2007). However, it has recently been suggested that UV-B could also be an environmental regulator controlling gene expression, cellular and metabolic activities as well as growth and development, mediated, at least partially, by the UV-B-specific photoreceptor UV RESISTANCE LOCUS 8 (UVR8) (Hideg *et al.* 2013). Furthermore, the effects of UV-B on plants depend on many factors such as doses of UV-B, background intensity of UV-A and/or photosynthetically active radiation (PAR), water and nutrient availability and plant genotype and acclimation state (Hideg *et al.* 2013). For example, in plants acclimated to high light conditions, like those in the Mediterranean Basin, natural UV-B radiation could be considered, rather than as a stress factor, an element that drives morphogenetic processes on plants to adapt better to the oxidative stresses typical of this environment (Pollastrini *et al.* 2011).

The damaging effects of high UV-B radiation doses on plants have been studied widely during the last decades, but relevant studies have been mainly focused on species typical of high latitudes or of agricultural interest (Jansen *et al.* 1998, Barnes *et al.* 2000, Caldwell *et al.* 2003 and 2007, Kakani *et al.* 2003, Chartzoulakis and Psarras 2005, Reboredo and Lidon 2012). Previous studies have shown that UV-B effects differ greatly between plant life forms (Li *et al.* 2010a) and species (Kakani *et al*. 2003, Yao *et al.* 2006). Furthermore, UV-B radiation can affect plants at multiple levels since it is absorbed by many biological molecules (Paul and Gwynn-Jones 2003). For instance, UV-B exposure can damage DNA generating pyrimidine dimers which may cause mutations during replication (Britt 1999). UV-B can also react with lipids and proteins in the presence of oxygen producing lipid peroxy radicals and hydroperoxides that can damage plant structures and metabolites (Foyer *et al.* 1994, Yao *et al.* 2006). UV-B radiation can also negatively affect some hormones related to growth, as well as the photosynthetic apparatus (for a review see Hollósy 2002 and Lidon *et al.* 2012, Shine and Guruprasad 2012). Photosynthetic rates might decrease under UV-B radiation due to photo-oxidation of the photosynthetic pigments, a decrease in Rubisco activity or photodamage in the D1 protein of the PSII (Friso *et al*. 1995, Andersson and Aro 2001). A reduction in photosynthesis has also been related to UV-B-induced stomatal closure (Nogués *et al.* 1999). Therefore, due to these effects on photosynthesis and/or on the amount of hormones, changes in UV-B levels can alter plant growth, although these effects would strongly depend on the UV-B doses applied (see Searles *et al.* 2001 and Li *et al.* 2010a for reviews).

1.3) Plant response mechanisms to UV-B radiation

Plants have been exposed to UV-B radiation over millions of years and, thus, they have developed mechanisms to cope with this type of radiation, although these mechanisms vary among species and life forms. Mechanisms displayed by plants to cope with higher doses of UV-B radiation are mainly directed to a) prevent or minimize the penetration of UV-B radiation through plant tissues, b) prevent oxidative

stress, and c) repair the damage caused by UV-B to plant molecules and metabolites.

Plants are able to reduce UV-B penetration into photosynthetic tissues mainly by changing morphology, and/or by accumulating UV-B radiation absorbing compounds (UACs). In order to decrease UV-B-exposed surfaces, plants reduce their leaf area and inhibit stem elongation or axillary branching. In addition, some plants increase their leaf thickness to lengthen the UV-B path inside the leaf and diminish the probability that UV-B would be absorbed by most internally located molecules (Jansen 2002, Fagerberg and Bornman 2005). An increase in the production of leaf waxes and hairs have also been observed in some species to promote UV-B reflection (Skalsta *et al.* 1994). However, the accumulation of UACs, mainly in the leaf epidermal cells, to avoid or attenuate UV-B penetration into the internal leaf tissues, has been described as the main response to UV-B increases in indoor experiments (Reboredo and Lidon 2012). Among UACs, phenols are the most important compounds (Dixon and Paiva 1995) and, because of their importance in plant tolerance to UV-B, not only for their role in UV-B-screening but also for their antioxidant function (Edreva 2005), they will be explained in an independent section (see below).

Plants have evolved other mechanisms to decrease light absorption or, once absorbed, to dissipate excess energy as heat in order to avoid reactive oxygen species (ROS) production and photo-oxidative stress. Previous studies have reported degradation of chlorophylls under enhanced UV-B radiation (Núñez-Olivera *et al.* 2006, Doupis *et al.* 2012), which would decrease the amount of photons absorbed by leaves, as well as an increase in the carotenoid content of leaves, which can help plants to dissipate excess energy as heat and can also act as antioxidants (Munné-Bosch and Alegre 2000).

In higher plants, thermal dissipation of excess light energy can be mediated by two xanthophyll cycles, the violaxanthin cycle (V-cycle) and the lutein epoxide cycle (Lx-cycle). However, the role of UV-B in the stimulation of V-cycle is still unclear (Bischof *et al.* 2002, Šprtová *et al.* 2003, Sobrino *et al.* 2005, Nuñez-Olivera *et al.* 2006, Martz *et al.* 2007, Láposi *et al.* 2009), and is unknown for Lx-cycle. The V-cycle consists on the formation of zeaxanthin by the de-epoxidation of violaxanthin via the

intermediate antheraxanthin (Fig. 2). The leaf content in zeaxanthin, and to a lesser extent in antheraxanthin, has been related to the thermal dissipation of energy as heat, which can be measured as non-photochemical quenching (NPQ) of chlorophyll fluorescence (Demmig-Adams 2003, Müller *et al.* 2001). The Lx-cycle has been described in some species (Bungard *et al.* 1999, Llorens *et al.* 2002, García-Plazaola 2002), but it seems to be taxonomically restricted (García-Plazaola *et al.* 2007). As for the V-cycle, a correlation between the energy dissipated as heat measured as NPQ and the level of lutein, the de-epoxidated product of lutein-epoxide, has been found (Llorens *et al.* 2002, García-Plazaola *et al.* 2003). Nevertheless, little is known about the regulation of this cycle (García-Plazaola *et al.* 2007).

Furthermore, plants have different enzymatic (e.g. photolyases) and non-enzymatic systems (such as ascorbate, glutathione, α-tocopherol and phenolic compounds) to scavenge ROS and to repair UV-B-damaged structures and metabolites (Hollósy 2002). Several studies have suggested that UV-A radiation can activate specific photorepair mechanisms that can avoid UV-B damage on plants (Wilson *et al.* 2001, Jayakumar *et al.* 2004). Photosynthetically active radiation (PAR) can also have a protective effect against UV-B, but in this case by increasing leaf thickness and the concentration of phenolic compounds that act as ultraviolet screens (Krizek 2004).

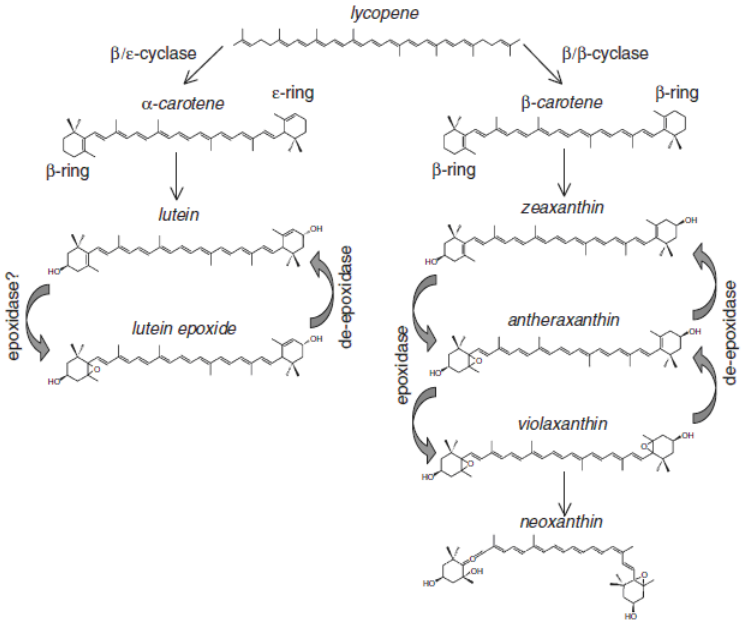


Fig. 2 Pathways of V-cycle and Lx-cycle pigments biosynthesis (from García-Plazaola et al. 2007)

1.4) Phenolic compounds

Phenolic compounds are the most abundant secondary metabolites in plants and include a large and diverse group of molecules that bear at least one functional hydroxyl group attached to an aromatic ring (C_6). These compounds are chemically heterogeneous and can be broadly divided in two groups: a) simple phenols, such as phenolic acids, and b) polyphenols, such as flavonoids (Fig. 3). While phenolic acids and flavonoids are soluble in water or organic solvents, other phenols, such as condensed tannins, lignins or cell-wall bound hydroxycinammic acids, are non-soluble (Treutter 2010).

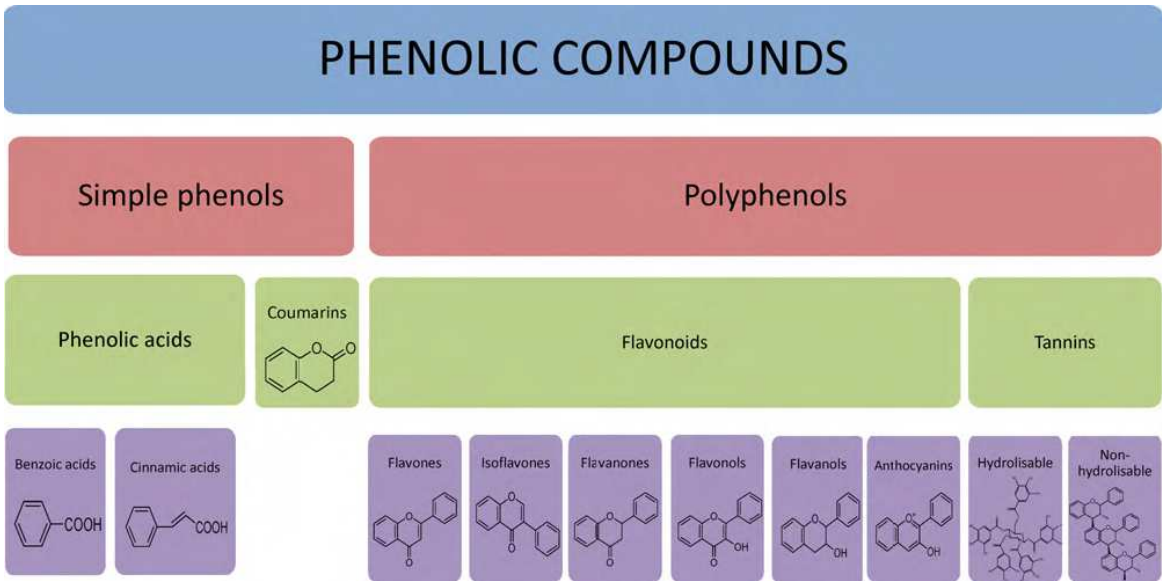


Fig. 3 A simplified classification of phenolic compounds and representative structures (adapted from Hurtado-Fernández et al. 2010).

1.4.1) The biosynthesis of phenolic compounds

Two basic pathways are involved in the biosynthesis of phenolic compounds: a) the shikimic acid pathway, which is the pathway used for the biosynthesis of most plant phenolics, and b) the malonic acid pathway, with less significance in higher plants. In the shikimic acid pathway simple carbohydrate precursors are converted into the

three aromatic aminoacids: phenylalanine, tyrosine and tryptophan. Most phenolic compounds in plants are derived from phenylalanine. The reaction is catalyzed by phenylalanine ammonia lyase (PAL) which eliminates an ammonia molecule of phenylalanine to form cinnamic acid. From cinnamic acid and via a series of hydroxylation, methylation and dehydration reactions, simple phenolic compounds, such as phenolic acids (e.g. ferulic and caffeic acids) are produced. From the basic carbon skeletons of these phenolic compounds, plants can synthesize more complex products such as flavonoids. The malonic acid pathway is known to be involved in the synthesis of flavonoids, being acetyl-CoA the precursor of this pathway (Fig. 4) (Taiz and Zeiger 2008).

Recent evidences suggest that the biosynthesis of flavonoids is up-regulated by a wide range of biotic and abiotic stresses, which have in common the generation of ROS. In particular, it has been shown that enhanced UV-B radiation can increase the activity of the enzymes involved in the shikimic and malonic pathways and, thus, the synthesis of phenolic compounds (Treutter, 2005).

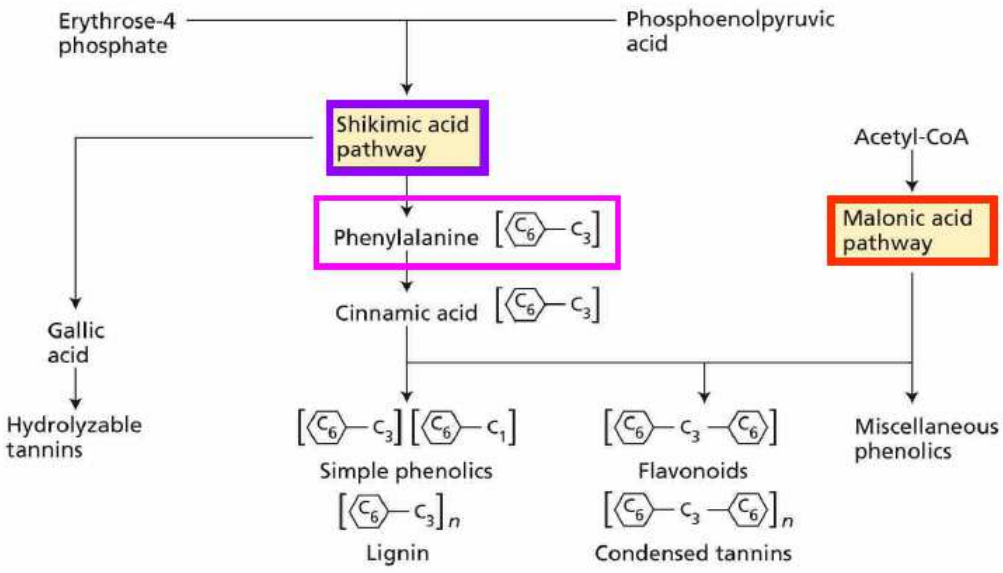


Fig. 4 Biosynthetic relationship among stress-induced phenolic compounds (adapted from Taiz and Zeiger 2008).

1.4.2) Functions of phenolic compounds

Phenolic compounds are involved in many functions in plants, for instance, they can play a role in the protection against microorganisms and herbivores (Demkura *et al.* 2009), in allelopathic mechanisms (Li *et al.* 2010b), in the attraction of pollinators and fruit dispersers or in the mechanical support of plant tissues (see Taiz and Zeiger 2010 and Treutter 2010 for a revision). Phenols are also involved in the photoprotection of plants since their mono- or poly-aromatic character confers them the ability to absorb UV-B radiation (Krauss *et al.* 1997, Close and McArthur 2002). In addition, the presence of conjugated bonds in the aromatic ring/s gives them the ability to scavenge free radicals, avoiding the posterior reaction of these harmful species with other biomolecules since the phenoxy radical form is less reactive than the original oxidizing species (Rice-Evans *et al.* 1997 and Vermerris and Nicholson 2008 for a general view).

Among phenolic compounds, some phenolic acids, such as caffeic acid, p-coumaric acid, ferulic acid and some hydroxicinnamic acid glycosides, have a greater UV-screening ability than flavonoids, showing increases under enhanced UV-B radiation. However, different studies have shown that these phenolic acids are replaced by flavonoids in cells exposed to high sunlight irradiance, since the latter seem to be better antioxidants (Schreiner *et al.* 2012, Brunetti *et al.* 2013). Among flavonoids, the flavonol glycosides, a class of flavonoids with a ketone group[3], and an attached glycoside molecule on the three-ring carbon skeleton, seem to be the most effective compounds in antioxidant protection against UV-B. Dihydroxy B-ring-substituted flavonol glycosides, such as quercetin and luteolin glycoside derivatives, have a greater antioxidant capacity but not a greater ability to absorb UV wavelengths than their monohydroxy B-ring-substituted counterparts, apigenin and kaempferol glycoside derivatives (Fig. 5). Another type of flavonoids, anthocyanins (Fig. 3), were also reported to increase in response to higher levels of UV-B radiation (Brunetti *et al.* 2013).

[3] Organic structure with a carbonyl group (C=O) bounded to two other carbon atoms.

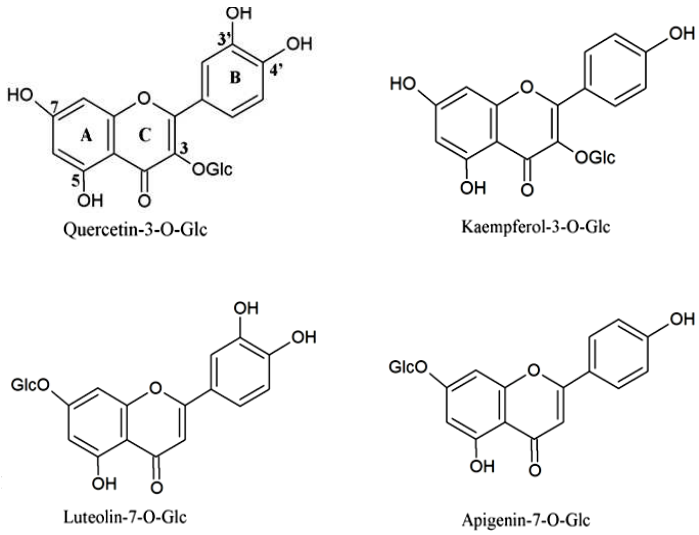


Fig. 5 Chemical structure of mono- (apigenin and kaempferol) and di-hydroxy B-ring-substituted (quercetin and luteolin) flavonoid glucosides (adapted from Brunetti et al. 2013).

1.4.3) Phenolic compounds as biomarkers of UV-B levels

The increase in leaf phenols is a response described in many species to cope with exposure to increased doses of UV-B (Julkunen-Tiitto *et al.* 2005, Caldwell *et al.* 2007). Because of this, it has recently been suggested the possible use of fossil plant material to reconstruct past UV-B levels through the analyses of their content of phenolic compounds (Rozema *et al.* 2009). Since sporopollenin from pollen and cutin from cuticles are highly resistant biopolymers and are well preserved in the geological record (see Rozema *et al.* 2009 for a review), phenols in these structures have been proposed as possible biomarkers of historical levels of UV-B radiation. Rozema *et al.* (2001) found higher levels of ferulic and *p*-coumaric acid in pollen grains of *Vicia faba* grown under enhanced UV-B compared to those grown without UV-B.

Furthermore, since there is a negative relationship between the amount of stratospheric ozone and UV-B levels (Bais *et al.* 2007), reconstructing past UV-B levels can also help to elucidate historical and pre-historical changes in stratospheric

ozone. The record of stratospheric ozone goes back only few decades, the first instrumental measure was in 1920 (Switzerland). On the other hand, the world-wide monitoring of UV-B levels is even shorter, it started around 1990 (Otero *et al.* 2009).

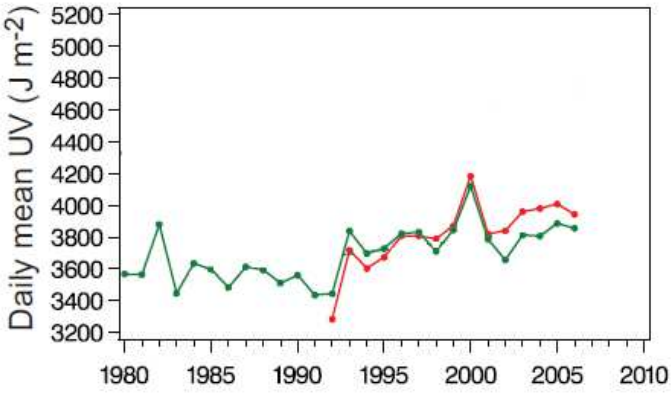


Fig. 6 Summertime (May-August) mean daily erythemal UV doses from ground-based UV measurements (red) and ground-based reconstructions (green) in Thessaloniki (41N, 23E) (adapted from WMO, 2011).

1.5) Focusing on Mediterranean region

For the Mediterranean Basin, which is located in mid-latitudes, models predict higher erythemal UV (UV_E) fluxes, especially in summer and autumn (Fig. 1), mainly due to a decrease in mean cloudiness (McKenzie *et al.* 2011). Decreased cloudiness in the Mediterranean region is linked to the predicted drier conditions for the coming decades in this area (Intergovernmental Panel of Climate Change, IPCC 2012) (Fig. 7). Therefore, although some regions of the Earth are expected to experience reductions in UV-B radiation and increases in precipitation, climatic models predict for the Mediterranean region higher UV-B radiation levels (McKenzie *et al.* 2011) and decreased water availability (IPCC 2012).

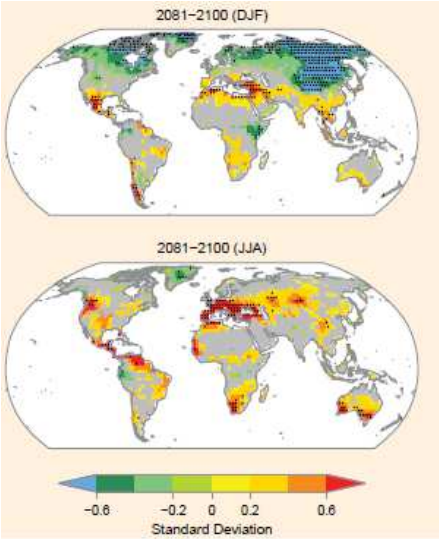


Fig. 7 Projected seasonal changes in consecutive dry days (days with precipitation <1 mm) for the end of 21st century. The maps show the differences between the predicted seasonal averages for 2081-2100 minus the average of 1980-1999. Differences are expressed in units of standard deviations (from IPCC 2012).

In spite of the predicted increase of UV radiation affecting the Mediterranean region in the near future, most studies investigating the effects of UV radiation on plants have been performed with high latitude species, with few studies using Mediterranean plants, particularly woody ones (Björn *et al.* 1997 and Paoletti 2005).

One of the most consistent responses to UV-B radiation reported for Mediterranean woody species is an increase in leaf and cuticle thickness (Manetas *et al.* 1997, Drilias *et al.* 1997, Grammatikopoulos *et al.* 1998). In contrast, increases in UACs in response to enhanced UV-B levels have been found only in a few species, such as *Laurus nobilis*, *Ceratonia siliqua* and *Vitis vinifera* (Grammatikopoulos *et al.* 1998, Núñez-Olivera *et al.* 2006). At the physiological level, Núñez-Olivera *et al.* (2006) reported a de-epoxidation of the V-cycle and a degradation of chlorophylls in response to UV-B increases in two species of *Vitis*. Moreover, while for most of the Mediterranean species studied, different UV-B levels were not associated with a change in photosynthetic rates (for a revision see Paolletti 2005), in some species, such as *Erica fairii* (Musil and Wand 1993) and *Ligustrum vulgare* (Guidi *et al.* 2011), a decrease in photosynthetic rates was induced by enhanced UV-B levels. Such a species-specific effect highlights the importance of doing studies using a range of Mediterranean species.

Considering the published literature, Mediterranean species seem to be more tolerant to UV-B radiation than species from high latitudes (Chartzoulakis and Psarras 2005,

Guidi *et al.* 2011). This has been attributed to the natural adaptations of Mediterranean woody species to high irradiation and drought, such as leaf sclerophylly, which would confer them resistance to UV-B radiation (see Paoletti 2005 for a revision, Bussotti 2008). Conversely, exposure to UV-B can also modulate plant vulnerability to other biotic or abiotic factors (Alexieva *et al.* 2001) due to the activation of similar protective mechanisms. Thus, plant exposure to a stress factor can improve plant resistance to another stress factor, a phenomenon known as cross-tolerance (Hideg *et al.* 2003, Stratmann 2003). For instance, it has been suggested that exposure to UV-B could improve plant resistance to drought. Studies done using Mediterranean pines found a positive effect of UV-B radiation on growth when they were subjected to water stress (Manetas *et al.* 1997). Nevertheless, this beneficial effect seems to be species-specific, since additive negative effects on growth under both enhanced UV-B and water stress have been reported in *Nerium oleander* (Björn *et al.* 1997) and *Phlomis fruticosa* (Nikolopoulos *et al.* 1995). Conversely, Nogués and Baker (2000) did not find any interaction of enhanced UV-B and drought effects on the photosynthesis of three Mediterranean species (*Olea europea* L., *Rosmarinus officinalis* L. and *Lavandula stoechas* L.). Therefore, more studies are needed to elucidate how the interaction between UV-B radiation and low water availability affect Mediterranean plant species.

1.6) Objectives

The aim of this thesis was to investigate the effects of UV-B radiation on woody Mediterranean plant species and its interaction with low water availability. This knowledge will help to predict how expected increases in UV-B levels under drier conditions might affect Mediterranean plant species in the coming decades.

In this sense, the specific objectives of this thesis were:

1) To study the photoprotective responses of woody Mediterranean species to UV-B radiation and the interaction with low water availability, as well as the effects of

these two abiotic factors on plant performance and, ultimately, on plant growth. In particular, the aims were to:

a) Determine whether morphological and biochemical characteristics of schlerophyllous leaves increase plant tolerance to UV-B radiation.

b) Investigate the importance of phenolic compounds and chloroplastic pigments in the photoprotection of the photosystems against UV-B radiation.

c) Elucidate whether low water availability can modulate plant responses to UV-B radiation and whether above-ambient UV-B levels can produce seedlings that are more tolerant to drought.

2) To investigate whether seasonal and altitudinal changes in the content of phenolic compounds of leaves and cuticles of *Buxus sempervirens* follow natural changes in UV-B radiation levels. Ultimately, a more specific aim was to assess whether there was a phenolic compound in leaves or foliar cuticles of *Buxus sempervirens* that could be used as a biomarker of the natural variations in UV-B radiation.

1.7) Thesis structure

This thesis is organized in seven sections. A general introduction is followed by three chapters which deal with the three experiments conducted to approach the main objectives of the thesis **(Chapters II, III and IV)**. Then, there is an integrated discussion of all the results obtained in these three experiments followed by the main conclusions. And finally, there are a chapter with all the references used and the last chapter with the annexes.

The Chapter II titled “Interactive effects of UV radiation and water availability on seedlings of six woody Mediterranean species” and the Chapter III titled “Effects of enhanced UV radiation and water availability on performance, biomass production

and photoprotective mechanisms in *Laurus nobilis* seedlings" present data regarding the first objective of the thesis commented in the above section.

In Chapter II, results from a greenhouse experiment using seedlings of six woody Mediterranean species (three mesophytes and three xerophytes) grown under different levels of UV (with UV-A+UV-B; with UV-A; without UV radiation) and water availability (watered to field capacity and with reduced water) are presented. Morphological, biochemical and physiological data obtained after growing plants for 6 months under the different treatments are discussed.

Chapter III describes the effects of enhanced UV-B radiation and its interaction with low water availability on the physiological performance, production of biomass, and photoprotective mechanisms of 1-year-old *Laurus nobilis* seedlings. Among photoprotective mechanisms, we analyzed the leaf content of the xanthophyll cycle pigments and phenolic compounds of *L. nobilis* grown outdoor under three UV radiation levels (enhanced UV-A, enhanced UV-A+UV-B and ambient UV) and two irrigation conditions (watered to field capacity and with reduced water). While the first presented experiment was conducted in a greenhouse, this one was performed outdoors in order to grow plants under more realistic PAR and UV-A/UV-B ratios.

Finally, Chapter IV deals with the second objective. In this chapter, the seasonal and altitudinal variation in the leaf and cuticle content of phenolic compounds of *Buxus sempervirens* is presented. Leaves of *B. sempervirens* were sampled every 3 months throughout a year (in June, September, December and March) along an altitudinal gradient (from 441 to 1750 m). A UV-B-exclusion experiment was done at the sites with the lowest and highest altitudes to elucidate whether the detected seasonal and altitudinal changes in the content of phenolic compounds of *B. sempervirens* could be attributed to the natural differences in UV-B radiation levels. The possible use of specific phenolic compounds as biomarkers of ambient UV-B levels is also assessed and discussed in this chapter.

Chapter II. Interactive effects of UV radiation and water availability on seedlings of six woody Mediterranean species

The content of this chapter has been published in Physiologia Plantarum as: Bernal M, Llorens L, Badosa J, Verdaguer D. 2013. Interactive effects of UV radiation and water availability on seedlings of six woody Mediterranean species.

Physiologia Plantarum 147: 234-247

Introduction

During the past decades, there has been considerable concern over the effects that the rise in UV-B radiation as a result of the reduction of stratospheric ozone, might have on organisms, and, in particular, on plants. It is well known that UV-B radiation can cause photo-oxidative stress to plants affecting their physiological activity and morphology (Passaglia *et al.* 2009). However, plants have developed different mechanisms to protect themselves from this type of radiation (see Hollósy 2002 for a revision). The first barrier to UV-B radiation is leaf surface reflectance or absorption by leaf hairs (Skaltsa *et al.* 1994). A second barrier is the reduction of UV-B transmittance into the internal leaf tissues due to the absorption by secondary metabolites (mainly phenols) localized in the cuticle (Krauss *et al.* 1997) and/or in the vacuoles of the epidermal cells (Kolb *et al.* 2001, Frohnmeyer and Staiger 2003). Since UV-B is not always totally reflected or attenuated by the cuticle and the epidermal cells causing reactive oxygen species formation, phenolic compounds such as flavonoids (ROS scavengers) and carotenoids (singlet oxygen quenchers) can act as photoprotectants to maintain the function of the photosystem II (Zhishen *et al.* 1999). In addition to biochemical protection, morphological and anatomical changes can also be important in plant defense against UV-B radiation (Frohnmeyer and Staiger 2003). For instance, it is widely accepted that increases in leaf thickness (Jansen 2002), as well as decreases in leaf expansion and plant height (Nogués *et al.* 1998) can also prevent or attenuate damaging UV-B effects by reducing the amount of this radiation that reaches the photosynthetic apparatus.

Plant responses to changes in UV-B radiation will also depend upon concomitant environmental factors, such as UV-A and photosynthetically active radiation (PAR) levels. Several studies have reported that UV-A radiation can activate specific photorepair mechanisms (see Krizek 2004 for a review) and stimulate the synthesis of phenolic compounds, such as flavonoids, which strongly absorb UV-B radiation. PAR can also have a protective effect against UV-B radiation

by increasing leaf thickness and the concentration of flavonoids and other phenolic compounds that act as ultraviolet screens (Krizek 2004). Simultaneous stresses, such as drought, pathogens and nutrient deficit, can also modulate plant responses to UV-B radiation (Paoletti 2005, Caldwell *et al.* 2007). In fact, water availability has been considered one of the most important factors affecting UV-B responses in plants (UNEP 2008), with some studies suggesting that plants could benefit from cross-tolerance when drought and UV-B are applied together (e.g. Yang *et al.* 2005, Poulson *et al.* 2006).

Hence, the aim of the study was to improve our current knowledge of the interactive effects between drought and UV-B radiation on plant species, as well as the underlying functional relationships between them. In particular, we have focused on Mediterranean plant species, which are thought to be adapted to a combination of oxidative stress factors due to the fact that, in Mediterranean ecosystems, plants are exposed to high fluxes of photosynthetically active and UV radiation together with periods of low soil water availability. Indeed, it has been suggested that natural adaptations of Mediterranean plants to excess light and water stress, such as high concentrations of phenolic compounds and a thick cuticle and epidermis, might afford protection against UV-B radiation (Paolleti 2005). In addition, since UV-B induces xeromorphic characteristics, this type of radiation might, in turn, enhance plant resistance to drought stress (Caldwell *et al.* 2007). Induction of stomatal closure by UV-B radiation (Nogués *et al.* 1999) has been suggested to improve the water relationships of leaves under drought conditions, which might lead to increased growth (Poulson *et al.* 2006, Feng *et al.* 2007). Manetas *et al.* (1997) reported that UV-B radiation increased cuticle thickness and epicuticular waxes as well as restricted stomatal opening in *Pinus pinea* L. avoiding excessive water loss from leaves and, therefore, improving leaf relative water content. However, other studies did not find any significant interaction between the effects of UV-B and drought on water relations, photosynthetic performance and/or growth of Mediterranean plant species (Nogués and Baker 2000, Kyparissis *et al.* 2001).

Taking into account that climatic models predict for the Mediterranean Region an intensification of summer drought (the main limiting factor of Mediterranean plant

growth) in the coming decades (IPCC 2007), possibly affecting the amount of UV-B (but also UV-A) radiation reaching ecosystems through changes in cloudiness (Giorgi *et al.* 2004, Bais *et al*. 2007), it is essential to improve our knowledge about how UV radiation, drought and the interaction of these two factors, might affect Mediterranean plant species. Elucidation of these effects and mechanisms will help us to understand the potential impact of future changes in UV radiation and water availability on Mediterranean plant performance. Moreover, elucidating plant responses to UV radiation and drought should also help us to devise strategies for improving plant tolerance to abiotic factors in species of economic interest. Consequently, the aim of the present work was to study the effects of UV radiation (UV-B and UV-A) and its interaction with water availability on several morphological, biochemical and physiological traits of six Mediterranean plant species, three of them considered to be xerophytes (*Daphne gnidium* L., *Pistacia lentiscus* L. and *Phillyrea angustifolia* L.) and the other three considered to be mesophytes (*Ilex aquifolium* L., *Laurus nobilis* L. and *Rosa sempervirens* L.). The comparison between xerophytes and mesophytes will allow us to investigate if plant sensitivity to UV radiation is affected by adaptations to habitats with different water availability. To achieve our goal we studied seedlings of these Mediterranean species grown in a glasshouse under three UV conditions (without UV, with UV-A and with UV-A+UV-B) and two irrigation levels (high and low water availability).

Materials and methods

Plant species and growth conditions

Four hundred and sixty-eight one-year-old seedlings from six Mediterranean plant species (78 seedlings per species), three of them considered to be xerophytes: *Pistacia lentiscus, Daphne gnidium* and *Phillyrea angustifolia*, and the other three being mesophytes: *Rosa sempervirens, Laurus nobilis* and *Ilex aquifolium*, were potted in 2 L (5 cm side x 20 cm depth) pots with 530 g (650 g for *Daphne gnidium*

and *Pistacia lentiscus*) of a mixture containing composted bark of pine and Sphagnum peat (1:1). The growing media was fertilized with osmocote (4 Kg m^{-3}), basal dressing (1 Kg m^{-3}) and dolomite (4 Kg m^{-3}) to avoid nutritional deficiencies during the experiment. The pots were placed in a glasshouse at the University of Girona, Catalonia (NE Spain) (41º58' N, 2º49' E) under controlled minimum temperatures (14 ºC) and irrigation. The roof and walls of the glasshouse stopped, on average, 70% (considering all the day) or 60% (from 10 to 14 h, solar time) of outdoor PAR radiation and approximately 90% of outdoor UV-A radiation. UV-B radiation was completely absent inside the glasshouse.

UV radiation treatment started 27 May 2008, after three weeks of seedling acclimation to the environmental conditions of the glasshouse, whereas the reduction in the water supplied to half of the plants (irrigation treatment) started 23 July 2008. Sampling was conducted at the beginning of October, although relative water content (RWC) was also measured at the beginning of July (more than one month after the UV treatment started and three weeks before the start of the irrigation treatment). All leaves collected were always fully expanded, light-exposed and grown under the different UV conditions.

UV radiation treatment

Three UV radiation conditions (Fig. 8) were applied to seedlings of the six studied species from 27 May to 10 October 2008:

- UV-A+UV-B exposure: Plants were exposed to both UV-A and UV-B radiation. UV radiation (UV-A and UV-B) was supplied with five 1.2 m long 40 W fluorescent lamps (TL 40 W/12 RS, with a peak at 313 nm; Phillips, Spain) mounted in metal frames suspended above the plants. Erythemally weighted UV doses (UV_E) (McKinlay and Diffey 1987), measured at the top of the plant canopies with an UVS-E-T radiometer (Kipp and Zonen, Spain), were in KJ m^{-2} d^{-1}: 1.47 ± 0.06 in June, 1.79 ± 0.05 in July, 1.80 ± 0.06 in August, 2.12 ± 0.05 in September and 2.18 ± 0.07 in October. On average, these UV_E doses were ≈ 3.5, 2.4, 2.0 and 1.2 times lower than outdoor doses for June, July, August and September, respectively, while the UV_E dose in

October was ≈ 1.4 times higher than outdoors (see Verdaguer *et al.* 2012 for more information). Ultraviolet radiation was applied daily centered on solar noon for 3.5-4.5 h (depending on the month). Fluorescent lamps were wrapped with cellulose diacetate foil (Ultraphan URT, 0.1 mm; Digefra GmbH, Munich, Germany) to remove any radiation below 295 nm (UV-C radiation). Cellulose diacetate films were pre-burned for 3 h before being applied to the lamps and they were changed every 36 h of use to avoid the effects of plastic photodegradation.

- UV-A exposure: Plants were exposed only to UV-A radiation in order to control the effects of UV-A on plants exposed to both types of radiation, UV-A and UV-B. Ultraviolet radiation was supplied as in the UV-A+UV-B plot but fluorescent lamps were wrapped with polyester (Mylar D, 0.13 mm thick; PSG Group, England) instead of cellulose diacetate film to stop UV-B and UV-C radiation (transmittance >320 nm). These filters were also changed after every 36 h of use.

- No UV exposure (UV-0): Plants were grown without UV radiation. This plot had the same fluorescent lamps as the others, but they were always turned off.

Location of the plots (each plot measuring 1.15 x 2.0 m) was interchanged every two weeks to minimize site effects. In addition, within each plot, plant positions were switched every week in order to minimize microenvironmental and border effects. Plots were separated by means of curtains of Ultraphan URUV (0.95 mm thick, Digefra, Germany), which excluded all radiation below 395 nm.

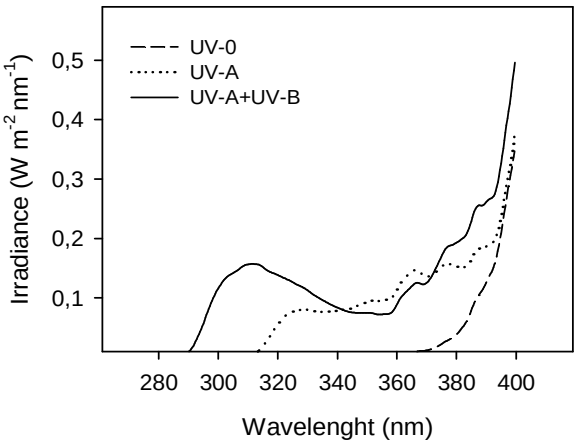


Fig. 8 Spectral irradiance under the three UV conditions applied to plants (UV-0, UV-A and UV-A+UV-B) measured during 15 minutes around noon in October.

All plots were covered by 1.2 x 2.0 m sheets of transparent filters (placed over the fluorescent lamps) that allowed the whole solar spectrum of PAR to penetrate (with 95% transmittance). In the case of UV-A+UV-B and UV-A plots, these filters were made of methacrylate (transmittance > 280 nm) with 75% of transmittance at 320 nm and 90% at 400 nm, allowing, thus, the transmission of the UV-A radiation present in the greenhouse. In the UV-0 plot, the filter was made of polycarbonate (Lermont Plastics, Barcelona, Spain) in order to block ambient UV-A radiation (transmittance > 380 nm).

Irrigation treatment

On 23 July 2008, i.e. almost two months after the UV treatment started, we reduced the water supply to half of the plants (randomly chosen) of each UV plot. From this date and until 15 August 2008, half of the plants of each UV plot were watered to saturation as previously (well-watered plants, WW), whereas the other half (low-watered plants, LW) received 50% of the water applied to well-watered plants. From 15 August 2008 until the end of the experiment (10 October 2008), LW plants received 33% of the water applied to WW ones.

Leaf relative water content and leaf morphological traits

One leaf from four individual plants per species and treatment was sampled at the beginning of July and October to determine its relative water content (RWC, %) and leaf mass per area (LMA, mg cm^{-2}). After collection, leaves were weighed immediately (FW) and scanned (Epson Perfection 1250, Japan). Leaves were then re-hydrated until their turgid weight (TW) was reached by leaving them in distilled water for 48 h in darkness. After the determination of TW, leaves were oven-dried at 70 ºC for 72 h and weighed again to obtain dry weight (DW). The RWC was, determined as (FW-DW/TW-DW) x 100. Leaf area was measured by means of an image processing program (Scion Image, Scion Corporation, USA), with LMA being obtained as the quotient between leaf dry weight and leaf area for each sample.

Quantification of leaf chlorophylls and carotenoids

Samples were obtained from five plants per species and treatment in October. For each plant, a total of four or five discs of 0.64 cm^2 were taken from two leaves, except for *D. gnidium* and *P. angustifolia*, in which case three halves of three leaves were taken. Leaf samples were dropped in liquid nitrogen immediately after sampling and stored at -80 ºC until the analyses. Then, chlorophylls and carotenoids were extracted for each plant by grinding the sample into powder with 80 % acetone. The extract was subsequently filtered (AP2001300, Millipore, Ireland), filled up to 10 ml with 80 % acetone and kept in the darkness at -20 ºC for 24 h. Concentrations of chlorophyll *a* and *b* and carotenoids in the leaf extracts were estimated spectrophotometrically (Thermo, Genesys 6, USA) using Porra (2002) equations for chlorophylls and the Lichtenthaler and Wellburn (1983) equation for carotenoids.

Quantification of leaf total phenolic content

One leaf from four plants per species and treatment was sampled in October. Total phenolic content of each leaf was determined following the method described by Rozema *et al.* (2006). For each leaf, 10 mg of dried material was ground into powder with 2.5 ml of 50% methanol. The extract was shaken for 1 h and subsequently centrifuged for 5 min at 2500 rpm. Fifty µl of the extract was mixed with 3.5 ml of distilled water and 250 µl of Folin Ciocalteu reagent (Panreac, Spain) was added; 8 min later 750 µl of Na_2CO_3 (20%) was added. Absorbance was measured after 2 h at 760 nm with a spectrophotometer (Thermo, Genesys 6, USA). Leaf total phenolic content was calculated from the gallic acid standard curve, prepared from 50 µl of gallic acid standard solution (40, 80, 150, 200, 400, 600, 800 and 1000 mg L^{-1}), and expressed as mg of gallic acid equivalents per g of dry weight, as well as per cm^2 (using LMA for unit conversion).

Quantification of leaf UV-B-absorbing compounds (UACs)

Ultraviolet-B-absorbing compounds were analyzed from one leaf taken from four different plants per species and treatment in October based on the method used by

Ruhland and Day (1996). UV-B-absorbing compounds were extracted grinding 10 mg of dried leaves with 5 ml of acidified methanol ($MeOH:H_2O:HCl$ (90:1:1)). After heating the extract 10 min at 60 ºC and leaving it to cool for 15 min at room temperature, the solution was centrifuged at 3000 rpm for 10 min. Finally, absorbance at 300 nm was measured and results were expressed as absorbance at 300 nm per g of dry weight as well as per cm^2 (using LMA for unit conversion).

Determination of leaf stomatal index (SI)

The stomatal density (SD) and epidermal cells (EC) of the four species *L. nobilis* and *R. sempervirens* (mesophytes) and *P. lentiscus* and *D. gnidium* (xerophytes) were determined. In October, portions of the abaxial side of one leaf from four plants per species and treatment were coated with clear nail polish and covered with adhesive tape. After 30 s, the adhesive tape together with the nail polish were removed and placed on a slide. The quantification of epidermal cells and stomata was done in three different fields of view per sample at 40x magnification using an Optika B-350 microscope (Optika, Ponteranica, Italy) equipped with a 10 MP resolution Canon EOS 400D digital camera (Canon, Tokyo, Japan). Stomatal index (SI) was calculated as 100 x SD/(SD+EC).

Leaf chlorophyll fluorescence

Components of chlorophyll fluorescence were quantified using a portable modulated fluorometer PAM-2100 (Heinz Walz GmbH, Effeltrich, Germany). Measurements were done in October using six plants per species and treatment at midday (10-14 h, solar time). After a dark-adaptation period of at least 30 min, we obtained minimum and maximum dark-adapted fluorescence (F_0, F_m) and F_v/F_m, where $F_v=F_m-F_0$. F_v/F_m has been used as a measure of the potential (or maximum) photochemical efficiency of PSII.

The actual photochemical efficiency of PSII in the light-adapted state was estimated as: $\Delta F/F_m' = (F_m'-F)/F_m'$, where F is the steady-state fluorescence yield under the given environmental conditions, and F_m' is the maximum level of fluorescence obtained during a saturating flash of light (when all the PSII traps are

closed) under the same environmental conditions. From this index, the apparent electron transport rate (ETR) was calculated as:

$$ETR = \Delta F/F_m' \times PAR \times 0.84 \times 0.5$$

where PAR was the incident photosynthetically active radiation (expressed in μmol m^{-2} s^{-1}), 0.84 was the assumed coefficient of absorption of the leaves, and 0.5 was the assumed distribution of absorbed energy between the two photosystems (Galmés *et al.* 2007).

Plant biomass

To determine the effects of treatments (UV and irrigation) on above- and below-ground plant biomass production, five plants per treatment for four species, two xerophytes (*P. angustifolia* and *P. lentiscus*) and two mesophytes (*L. nobilis* and *I. aquifolium*), were harvested at the end of the experiment (October). Above- and below-ground plant biomass were obtained after drying the plant material in an oven at 70 ºC for 72 h.

To account for initial differences in plant biomass among plots, we estimated the biomass for each treated plant at the beginning of the experiment using allometric equations relating the basal diameter and/or length of the main stem (measured with a digital caliper and a ruler, respectively) with the above- and below-ground, as well as total, plant biomass. These allometric equations were obtained by multiple stepwise regression analyses using 10 plants per species showing the same size range as the treated plants at the beginning of the experiment. Statistical analyses confirmed that there were no significant initial differences in plant biomass among the different treatments (data not shown).

Statistical analyses

Ultraviolet, irrigation and species effects on the parameters measured were tested by means of three-way analyses of variance (ANOVA) with Bonferroni pairwise comparisons. Significant differences were assumed at $p \leq 0.05$. Figures 9-11 show

the means for the different variables per species and UV treatment, except: 1) when the interaction among the three studied factors (species, UV and irrigation) was significant in the ANOVA (Table I), in which case we represent the means per species, UV and irrigation conditions (Figs. 12 and 13), or 2) when the only significant interaction was between our two treatments, in which case we show the means per UV and irrigation treatment, pooling all the species (Fig. 14).

Results

General effects of UV radiation and irrigation treatments

At the beginning of July, i.e. more than one month after the start of the UV treatment, the overall leaf RWC of UV-A-treated plants of the six studied species (76.7 ± 1.7%) was significantly higher (p<0.001) than the leaf RWC of plants grown under UV-A+UV-B or without UV radiation (71.0 ± 2.1% and 65.0 ± 1.8%, respectively; Fig. 9).

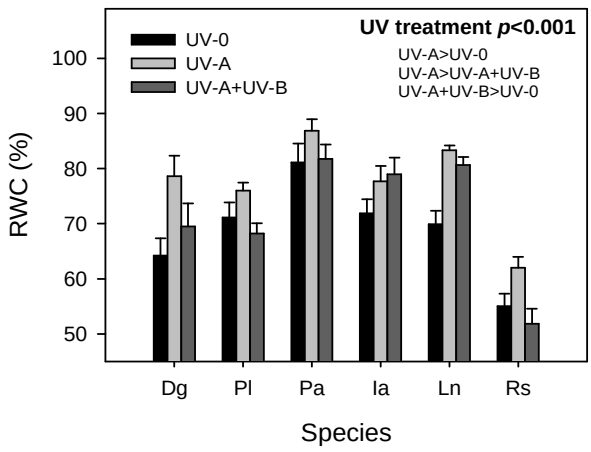


Fig. 9 Leaf relative water content (RWC) for each one of the six studied species under the three UV conditions in July (values are means ± S.E. N = 4). Species names abbreviations: Dg = Daphne gnidium, Pl = Pistacia lentiscus, Pa = Phillyrea angustifolia, Ia = Ilex aquifolium, Ln = Laurus nobilis and Rs = Rosa sempervirens*. UV treatment abbreviations: UV-0 = plants grown without UV, UV-A = plants grown exposed to UV-A, UV-A+UV-B = plants grown exposed to UV-A and UV-B. Since there was a significant effect of the UV treatment on the leaf RWC (p < 0.001), overall significant differences between UV conditions after Bonferroni pairwise comparisons are depicted.*

In turn, plants grown under both types of UV radiation showed, in general, significantly higher leaf RWC than those grown in a UV-free environment. However, these differences were not observed in October (Table I), when plants had a better leaf water status than in July, probably due to milder temperatures inside the glasshouse and/or a possible acclimation response.

At the end of the experiment (October), overall LMA values of the studied species grown exposed to UV-A+UV-B radiation were significantly higher compared to those of plants grown without UV (Table I, Fig. 10), although leaf area was not modified significantly by UV exposition (Table I). Plants exposed to UV-A+UV-B also had significantly greater leaf chlorophyll *a+b* and carotenoid content on an area basis (Table I), and a greater carotenoids/chlorophyll *a+b* ratio (Table I, Fig. 10), compared to plants exposed to UV-A alone. When chlorophylls and carotenoids contents were expressed on a dry weight basis, differences between UV-A+UV-B- and UV-A-treated plants were only significant in the case of carotenoids (Table I). Interestingly, our UV treatment did not change significantly the leaf chlorophyll a/b ratio of the studied species (Table I).

As expected, plants subjected to the low irrigation treatment had lower leaf RWC and smaller leaves (about 5% and 13%, respectively) than well-watered plants (Table I). Drier conditions also significantly reduced the overall chlorophyll a/b ratio of leaves, increased the leaf chlorophyll and carotenoid content per leaf dry weight and decreased the above-ground biomass and total biomass (although these last effects were dependent on the species and the UV treatment, Table I). Neither treatments nor their interactions significantly modified leaf stomatal index, maximum photochemical efficiency of PSII (Fv/Fm) or the shoot/root ratio of the species studied (Table I).

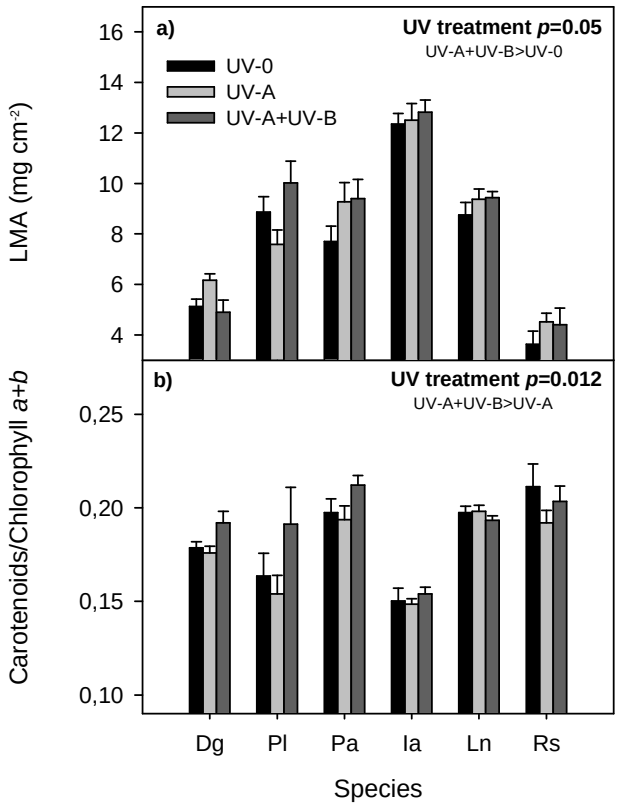


Fig. 10 (a) Leaf mass per area (LMA) and (b) leaf carotenoids/chlorophyll a+b ratio for each one of the six studied species under the three UV conditions in October (values are means ± S.E. N = 8 and N = 10, respectively). Abbreviations of species names and UV conditions as in Fig. 9. Since there was a significant effect of the UV treatment on both variables, overall significant differences between UV conditions after Bonferroni pairwise comparisons are depicted in each case.

Table I *Overall means ± S.E. measured in October for the six (four in the case of biomass variables) studied species grown under the three different UV conditions (UV-0, UV-A and UV-A+UV-B) and the two irrigation levels (WW= well-watered plants, LW=low-watered plants). Statistical significance (p-values) of the effects of our UV and irrigation treatments (UV and I, respectively) on the studied variables is also shown. Different letters and values in boldface indicate statistical differences (p-values ≤ 0.05), ns = non-significant effects. For species factor p-value was lower than 0.001 in all the parameters. Fv/Fm and ETR values were taken at midday. Abbreviations are: Above-gr = above-ground, Below-gr = below-ground, Car = carotenoids, Chl = chlorophylls, ETR = apparent electron transport rate, LMA = leaf mass per area, RWC = relative water content, UAC=UV-B-absorbing compounds.*

	UV treatment			Irrigation treatment		*Treatment effects (p-val)*		Interactions (*p*-val)			
	UV-0	**UV-A**	**UV-A+UV-B**	**WW**	**LW**	**UV**	**I**	**UV x I**	**UV x SP**	**I x SP**	**UV x SP x I**
RWC (%)	78.8±1.5	78.3±1.9	76.8±1.9	**80.1±1.2**	**75.8±1.6**	ns	**0.004**	ns	ns	ns	ns
Leaf area (cm^2)	8.8±1.1	8.6±1.0	7.8±1.0	**9.0±0.9**	**7.8±0.8**	ns	**0.015**	ns	ns	ns	ns
LMA (mg cm^{-2})	**7.7±0.4a**	**8.2±0.4ab**	**8.5±0.5b**	8.1±0.4	8.2±0.4	**0.05**	ns	ns	ns	ns	ns
Stomatal Index (%)	13.1±0.9	12.5±0.6	12.0±0.9	13.2±0.6	11.9±0.8	ns	n s	ns	ns	ns	ns
Chl *a+b* (µg cm^{-2})	**46.8±1.8ab**	**43.1±1.6a**	**48.2±2.1b**	45.2±1.2	46.9±1.8	**0.006**	ns	ns	ns	**<0.001**	ns
Chl *a+b* (mg g DW^{-1})	**5.95±0.32a**	**5.20±0.19b**	**5.55±0.22ab**	**5.35±0.15**	**5.78±0.25**	**0.003**	**0.015**	**0.045**	**0.026**	**<0.001**	**<0.001**
Chl *a*/*b*	3.24±0.06	3.24±0.05	3.31±0.05	**3.32±0.05**	**3.21±0.04**	ns	**0.020**	ns	ns	n.s	ns
Car (µg cm^{-2})	**8.4±0.3b**	**7.6±0.3a**	**9.0±0.3b**	8.2±0.2	8.5±0.3	**<0.001**	ns	ns	ns	**<0.001**	ns
Car (mg g DW^{-1})	**1.10±0.07a**	**0.92±0.04b**	**1.06±0.05a**	**0.99±0.35**	**1.07± 0.05**	**<0.001**	**0.030**	ns	**0.002**	**0.002**	**<0.001**
Car/chl *a+b*	**0.18±0.004ab**	**0.18±0.003a**	**0.19±0.004b**	0.18±0.003	0.18±0.004	**0.012**	ns	ns	ns	ns	ns
Phenols (mg cm^{-2})	0.67±0.08	0.66±0.07	0.59±0.06	0.63±0.06	0.65±0.06	ns	ns	ns	**<0.001**	ns	ns
Phenols (mg g DW^{-1})	87.1±10.1	78.9±8.7	77.9±9.0	79.7±7.3	82.8±7.8	ns	ns	ns	**0.008**	ns	ns
UAC (A_{300} cm^{-2})	**13.14±2.02a**	**13.55±2.01a**	**11.54±1.46b**	12.77±1.47	12.72±1.54	**0.004**	ns	ns	**<0.001**	ns	ns
UAC (A_{300} mg DW^{-1})	**1.7±0.3a**	**1.6±0.2ab**	**1.5±0.2b**	1.6±0.2	1.6±0.2	**0.023**	ns	ns	**<0.001**	ns	ns
Fv/Fm	0.75±0.006	0.76±0.009	0.75±0.009	0.76±0.007	0.75±0 .006	ns	ns	ns	ns	ns	ns
ETR (µmol m^{-2} s^{-1})	**66.1±3.7a**	**81.8±3.5b**	**73.7±3.5ab**	73.1±2.9	74.6±3.0	**0.001**	ns	ns	ns	ns	**0.045**
Below-gr biomass (g)	4.5±0.3	5.0±0.3	5.0 ±0.3	4.6±0.2	5.0±0.3	ns	ns	**0.042**	ns	ns	ns
Above-gr biomass (g)	13.9±1.1	14.6±0.8	13.6±0.7	**14.9±0.8**	**13.2±0.6**	ns	**0.044**	**0.045**	ns	ns	**0.011**
Total biomass (g)	18.4±1.3	19.6±1.0	18.6±1.0	**20.0±1.0**	**17.8±0.7**	ns	**0.046**	**0.034**	ns	ns	**0.019**
Shoot/root ratio	3.2±0.2	3.1±0.2	3.0±0.2	3.1±0.1	3.1±0.1	ns	ns	ns	ns	ns	ns

Interactive effects between the UV treatment and the species and/or the irrigation treatment

Ultraviolet effects on the leaf total content of phenols and UV-B-absorbing compounds (UACs) differed among species (Table I, Fig. 11). Indeed while UV exposure (UV-A and UV-A+UV-B in the case of phenols and UV-A+UV-B in the case of UACs) reduced the leaf amount of these compounds in *P. lentiscus* (Fig. 11), UV increased the leaf content (per area) of phenols and UACs in *L. nobilis* and *I. aquifolium* (Fig. 11). When values of UACs were expressed per dry weight (Fig. 11), the leaf content of these compounds in *D. gnidium* and *R. sempervirens* also showed contrasting responses to our UV treatment, with UV-A+UV-B not affecting or decreasing, respectively, the leaf content of UACs in relation to growth without UV. In the rest of species, the leaf amount of phenols or UACs was not significantly affected by UV.

The effects of UV radiation on the leaf apparent electron transport rates (ETR) differed among species, but they were also dependent on the water supply (as indicated by the significant triple interaction found among these factors, Table I). For two of the species, *P. lentiscus* and *D. gnidium*, significant effects of the UV treatment on leaf ETR values were apparent (Fig. 12).

In the case of *P. lentiscus* (Fig. 12), UV-A exposure increased leaf ETR compared to plants grown without UV (96.07 ± 6.77 *vs* 74.15 ± 6.29 μmol m^{-2} s^{-1}, respectively). However, the effect of UV treatment was dependent on the water supply (p = 0.003), since, when analyzed separately, only low-watered plants of *P. lentiscus* showed significant responses to UV, with plants exposed to UV-A and UV-A+UV-B showing significantly higher leaf ETR values than those grown in a UV radiation-free environment (Fig. 12). In the case of *D. gnidium* (Fig. 12), plants exposed to UV-A alone showed significantly higher leaf ETR than those grown under both types of UV radiation (66.03 ± 7.34 *vs* 37.31 ± 5.95 μmol m^{-2} s^{-1}, respectively) regardless of the watering treatment.

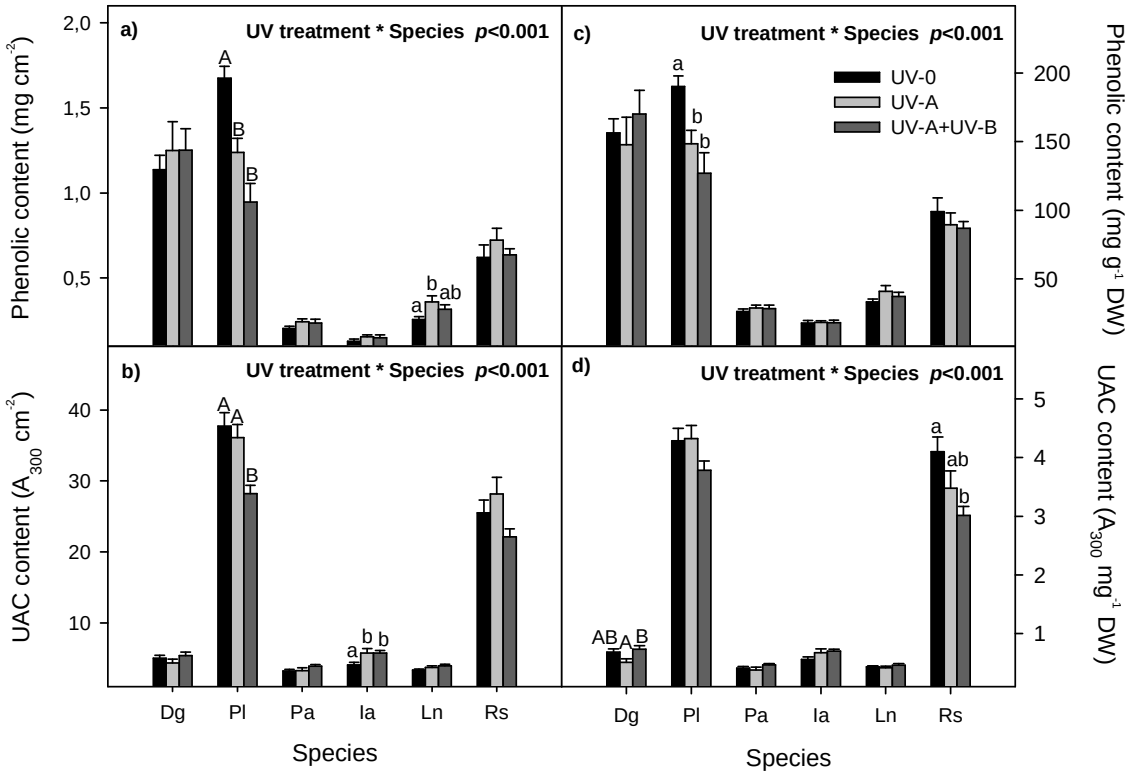


Fig. 11 Leaf content of phenols and UV-B-absorbing compounds (UACs) expressed per area (a, b) and per leaf dry weight (c, d) for each one of the six studied species grown under the three UV conditions in October (values are means ± S.E. N = 8). Abbreviations of species names and UV conditions as in Fig. 9. Since there was a significant interaction between the effects of the UV treatment and the species on these variables, UV treatment effects were analyzed within each species. Differences among UV conditions for each species are indicated with different letters when significant.

Ultraviolet effects on the above-ground plant biomass production were also dependent on species and water supply (see column UV x SP x I in Table I), with the xerophytic species (*P. lentiscus* and *P. angustifolia*) (Fig. 13) being more sensitive to treatments than the mesophytic ones (*I. aquifolium* and *L. nobilis*) (Fig. 13). Indeed, while well-watered plants of *P. lentiscus* produced significantly less above-ground biomass when exposed to UV-A+UV-B than when grown in a UV radiation-free environment, we did not detect significant differences due to UV exposure among low-watered plants of this species (Fig. 13). In the case of *P. angustifolia*, UV radiation did not significantly affect the production of above-ground biomass, but low irrigation reduced it (Fig. 13).

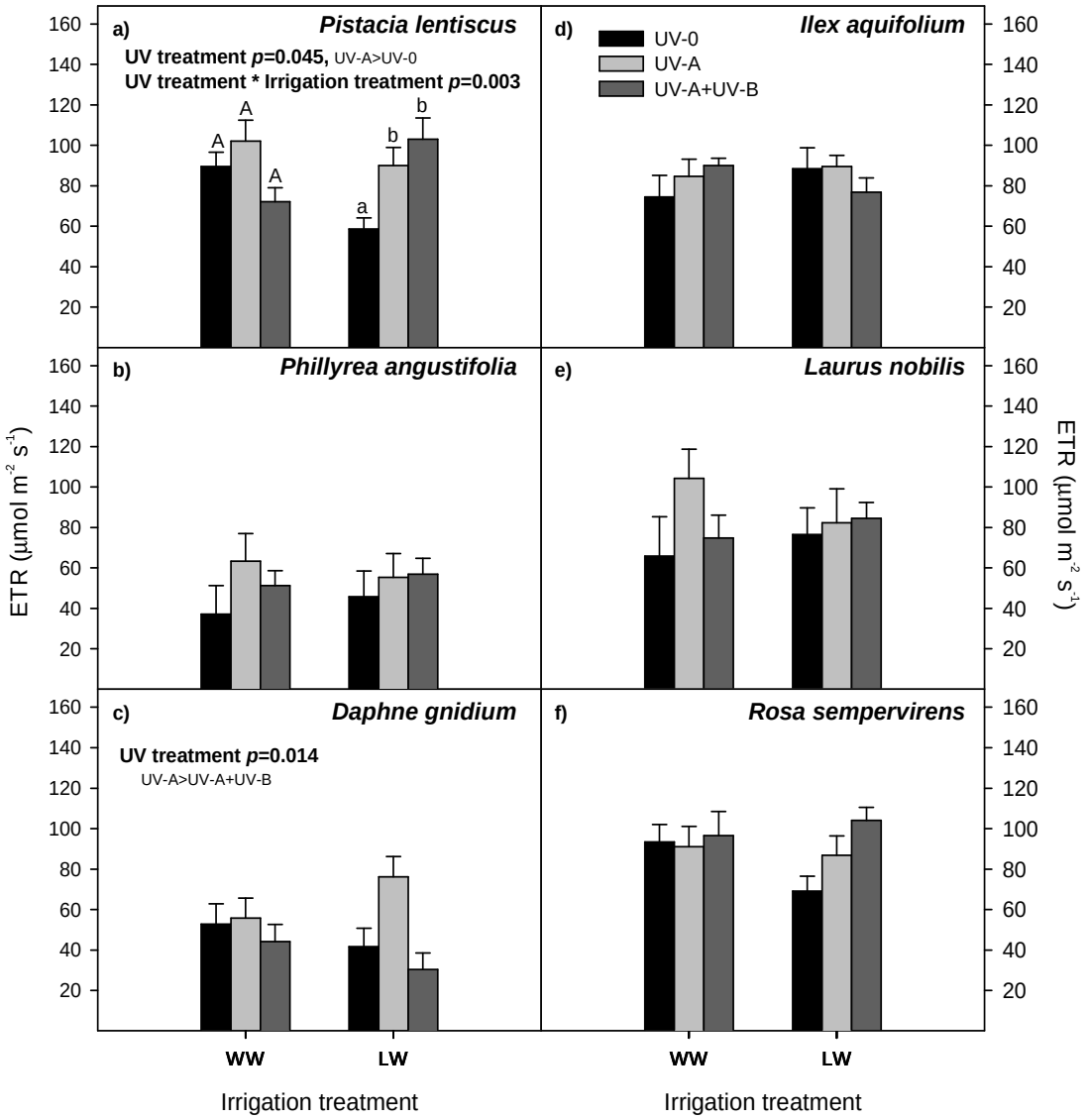


Fig. 12 Leaf apparent electron transport rates (ETR) at midday in October for well- (WW) and low-watered (LW) plants of each one of the six studied species under the three UV conditions (values are means ± S.E. N = 6). UV treatment abbreviations as in Fig. 9. Overall significant differences between UV conditions after Bonferroni pairwise comparisons are depicted when the effect of the UV treatment was significant. Since, in the case of P. lentiscus*, there was a significant interaction (p = 0.003) between the effects of the two treatments (UV and irrigation), we analyzed the UV effects within each irrigation level, with different letters indicating significant differences among UV conditions.*

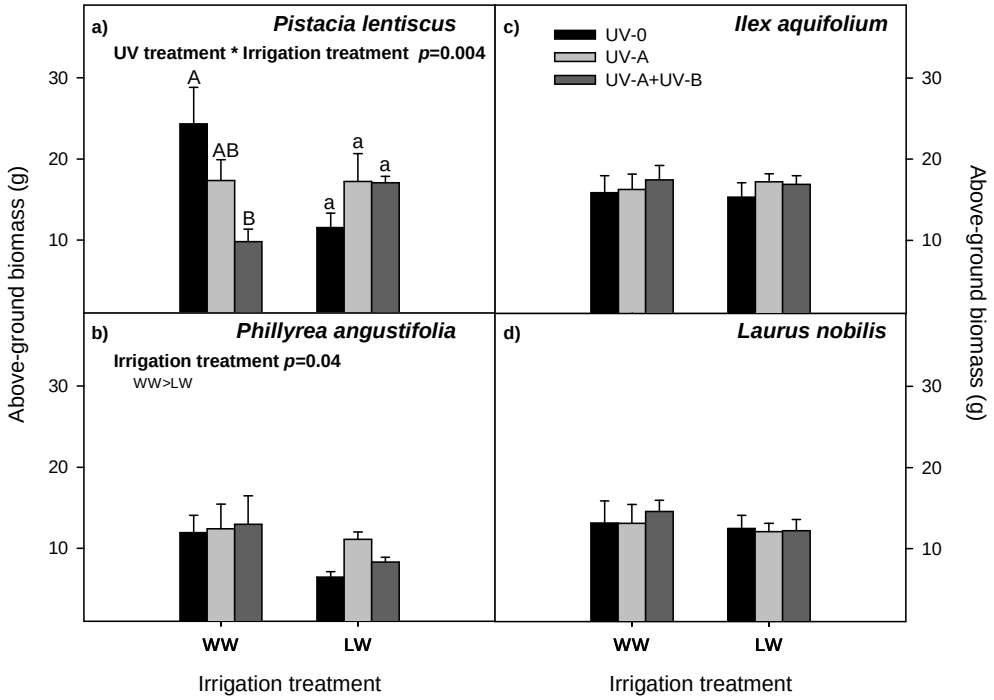


Fig. 13 Above-ground biomass for well- (WW) and low-watered (LW) plants of the xerophytic species Pistacia lentiscus *(a) and* Phillyrea angustifolia *(b) and the mesophytic species* Ilex aquifolium *(c) and* Laurus nobilis *(d) under the three UV conditions in October (values are means ± S.E. N = 5). Overall significant differences between irrigation levels are depicted when the effect of the irrigation treatment was significant. In the case of* P. lentiscus*, since there was a significant interaction (p = 0.004) between the effects of the two treatments (UV and irrigation), we analyzed the UV effects within each irrigation level, with different letters indicating significant differences among UV conditions.*

Regarding below-ground biomass production, statistical analyses revealed that the effect of UV treatment on this variable was only dependent on the water supplied to the plants (Table I). While UV radiation did not significantly modify the overall below-ground biomass production of well-watered plants of the studied species, low-watered plants exposed to UV-A and UV-A+UV-B showed, in general, significantly higher root biomass production compared to those grown without UV (Fig. 14). Even though all species showed the same tendency when grown under water shortage, differences in root biomass between plants exposed to UV and those grown in a UV radiation-free environment were only significant in the case of *P. angustifolia* when statistical analyses were done for each species (Table II). In contrast to the pattern found for low-watered plants, well-watered plants of *P. lentiscus* showed a significant reduction in root growth in response to UV-A+UV-B

exposure (Table II). UV effects on total plant biomass production followed the same patterns for above-ground biomass (Table I).

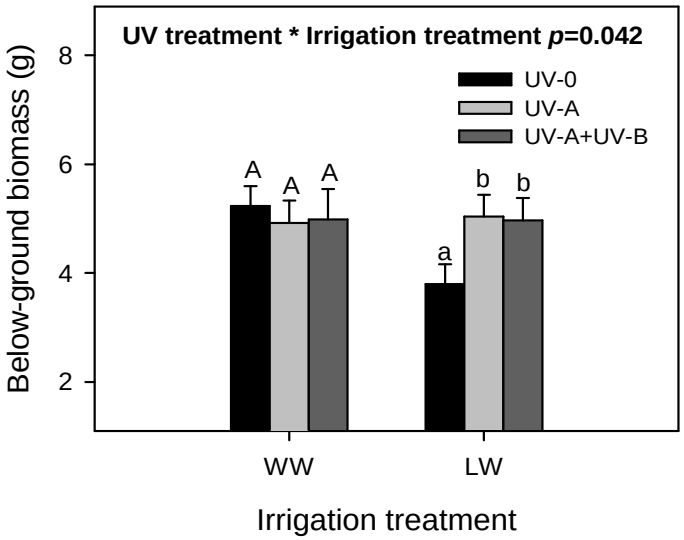


Fig. 14 Below-ground biomass for well- (WW) and low-watered (LW) plants under the three UV conditions in October (values are overall means for Pistacia lentiscus, Phillyrea angustifolia, Ilex aquifolium and Laurus nobilis ± *S.E. N = 20). Since there was a significant interaction (p = 0.042) between the effects of the two treatments (UV and irrigation), we analyzed the effect of the UV treatment within each irrigation level, with different letters indicating significant differences among UV conditions.*

	Well-watered plants (means ± S.E)			*p*-val	Low-watered plants (means ± S.E)			*p*-val
	UV-0	**UV-A**	**UV-A+UV-B**		**UV-0**	**UV-A**	**UV-A+UV-B**	
Pl	**5.47±1.15a**	**3.91±0.49ab**	**2.30±0.41b**	**0.04**	2.50±0.32	4.13±0.68	3.55±0.40	ns
Pa	4.50±0.48	4.13±0.89	3.45±0.56	ns	**2.11±0.18a**	**3.30±0.35b**	**3.25±0.33b**	**0.02**
Ia	5.09±0.57	5.02±0.46	6.08±0.34	ns	4.56±0.36	5.69±0.43	5.77±0.45	ns
Ln	5.17±0.65	5.83±0.94	7.47±1.10	ns	5.73±0.54	6.46±0.99	6.91±0.79	ns

Table II *October overall means ± S.E. for the below-ground biomass (g) of well- and low-watered plants of* P. lentiscus (Pl), P. angustifolia (Pa), I. aquifolium (Ia) and L. nobilis (Ln) *grown under three UV conditions (UV-0, UV-A and UV-A+UV-B). Different letters and values in boldface indicate statistical differences (p ≤ 0.05).*

Discussion

Leaf morphological changes have been considered to be of special relevance in plant defense against harmful effects of UV-B radiation on photosynthetic apparatus. Hence, it is not surprising that species exposed to both types of UV radiation, UV-A and UV-B, showed significantly higher overall LMA values than those grown without UV radiation (Table I, Fig. 10). Taking into account that leaf area was not significantly affected by UV exposure (Table I), an increase in LMA might be due to an increase in leaf thickness, as shown in a parallel study using the same experimental design and species (Verdaguer *et al*. 2012), although we cannot exclude the possibility of a change in leaf density (Witkowski and Lamont 1991). It is generally accepted that an increase in leaf thickness, and consequently in LMA, is an adaptative response to UV-B radiation, since a longer radiation path within the leaf attenuates the penetration of this harmful radiation into the mesophyll protecting the photosynthetic apparatus (Wand 1995).

Plants grown under both UV-A and UV-B radiation also showed higher overall leaf carotenoids/chlorophyll a+b ratios than plants grown exposed to UV-A alone (Table I, Fig. 10), which would suggest a UV-B-induced increase in this ratio. This is in accordance with previous experiments showing approximately 7% higher carotenoids/chlorophyll a+b ratios in plants grown under ambient (or near-ambient) UV-B radiation levels than in those exposed to reduced levels of this radiation (Hunt and Neil 1999). Increased leaf carotenoids/chlorophyll a+b ratios have been related to increased protection against photo-oxidation, since it is well known that some carotenoids (such as β-carotene and zeaxanthin) are involved in the photoprotection of the photosynthetic apparatus (Demmig-Adams *et al.* 1996, Solovchenko and Merzlyak 2008). Although increases in this ratio have been associated with decreases in photosynthetic light-use efficiency (Filella *et al.* 2009), we only found decreases in the leaf ETR values upon exposure to UV-B radiation in *D. gnidium* (Fig. 12).

Regarding other photoprotective variables, such as the leaf content of phenols and UV-B-absorbing compounds (UACs), we did not find UV-induced changes in the amount of these compounds in most of the studied species (Table I, Fig. 11). This suggests that, in the species tested, the biosynthesis of these compounds might be regulated by other factors, such as for example developmental stage, herbivory or temperature, rather than UV radiation, which is in agreement with previous studies for other Mediterranean species (reviewed in Paoletti 2005). Thus, the function of these compounds in protection against UV radiation remains unclear for Mediterranean species. Nevertheless, we cannot exclude the possibilities that in our experiment the UV radiation levels applied were too low to induce an increase in the synthesis of these compounds or that exposure to UV radiation might have differentially affected the proportions of the different phenols or UACs without changing their overall content.

Despite the lack of a general response, we detected some UV-induced changes in the leaf content of phenols and/or UACs of some species (Fig. 11). Among the changes found, the most consistent response was the reduction in the leaf content of phenols (expressed both per leaf area and per leaf dry weight) and UACs (per area) of *P. lentiscus* plants exposed to UV-A and UV-A+UV-B radiation in the case of phenols and to UV-A+UV-B radiation in the case of UACs in relation to plants grown without UV radiation. Romani *et al.* (2002) reported that leaves of *P. lentiscus* have a high total amount of polyphenols, which, in addition to protecting plant cells from the detrimental effects of short-wave radiation (UV-B and UV-A), can also counteract the negative effects of reactive oxygen species (free radicals) on cell metabolism (Romani *et al*. 2002, Baratto *et al*. 2003). Taking this into consideration, lower leaf content of phenols in *P. lentiscus* exposed to UV radiation would suggest lower photo-oxidative stress, which would be in agreement with higher leaf photochemical efficiency in response to UV radiation; however, UV only increased the leaf ETR values of *P. lentiscus* plants grown under a low water supply (Fig. 12).

Interestingly, while UV radiation exposure did not modify the above-ground (and total) plant biomass production in three of the four species studied, it inhibited the above-ground growth in well-watered plants of *P. lentiscus*, but not in low-watered ones (Fig. 13). The UV-induced inhibition of growth measured in well-watered plants of *P.*

lentiscus is in agreement with the results of the majority of studies using plants grown under conditions of UV exclusion or attenuation (e.g. Kadur *et al*. 2007, Tsormpatsidis *et al*. 2010). However, our results show that UV radiation effects on the above-ground plant biomass production can be different when plants are grown under conditions of water shortage. Indeed, in our experiment, exposure to UV radiation tended to abolish the drought-induced reduction in growth experienced by plants of *P. lentiscus* grown without UV radiation (Fig. 13), which is in agreement with higher leaf ETR values measured in UV-exposed plants under water shortage (Fig. 12). Since we did not find differences between above-ground biomass or leaf ETR values for low-watered *P. lentiscus* plants grown exposed to UV-A radiation alone and those grown under UV-A+UV-B, our results suggest that the UV-induced beneficial effect found under water deficit was mainly due to plant exposure to UV-A radiation. Other studies have also shown increased tolerance to drought in response to UV-A+UV-B radiation (e.g. Manetas *et al.* 1997, Poulson *et al*. 2006, Feng *et al.* 2007), although, in these studies, UV-A and UV-B radiation was enhanced above ambient levels and the beneficial effect was attributed to UV-B radiation.

Regarding below-ground biomass, the effect of UV radiation was only dependent on the water supplied to plants (Table I). Indeed, while UV radiation did not affect the overall below-ground growth of well-watered plants, low-watered plants exposed to UV-A and UV-A+UV-B showed, in general, a higher root biomass than those grown without UV (Fig. 14). Despite all the species showed this pattern, the effect was only significant for low-watered plants of *P. angustifolia* when the analyses were done for each species separately (Table II). Previous studies investigating the effect of UV radiation on below-ground growth have usually reported increases in biomass production in response to UV exclusion, which is in agreement with the results found for well-watered plants of *P. lentiscus* (Table II). This suggests that ambient levels of UV radiation inhibit root growth (Zaller *et al.* 2002, Rinnan *et al.* 2005). However, most of these studies have been conducted on high-latitude plant species growing under high water availability. Among the few studies investigating the interactive effects between UV-B radiation and drought on below-ground biomass production, some studies have found no significant interactive effects between these two factors on root growth (Nogués and Baker 2000), others have concluded that drought stress counteracted the UV-B-induced inhibition in root growth experienced

by well-watered plants (Nogués *et al*. 1998, Feng *et al*. 2007). However, in contrast to these studies, our results point to a beneficial effect of UV-A radiation, not UV-B, under conditions of water shortage, since we did not detect significant differences in the below-ground biomass of low-watered plants grown exposed to UV-A radiation and those grown exposed to UV-A+UV-B (Fig. 14).

The biological effects of UV-A radiation on plant photosynthetic performance and growth have received less attention than the effects of UV-B radiation. In general, UV-A supplementation studies have found an UV-A-induced inhibition of photosystem II activity (Vass *et al.* 2002, Unal *et al.* 2009). However, Sullivan *et al*. (2003) found a species-specific response of leaf ETR to enhanced UV-A radiation, since UV-A supplementation increased leaf ETR in maple, but it reduced or did not affect it in sweet gum and tulip poplar, respectively. We are not aware of studies investigating the effects of UV-A radiation on water-stressed plants, and hence, to our knowledge, this is the first study suggesting a beneficial effect of exposure to UV-A radiation on below-ground plant growth (Fig. 14). Unfortunately, results do not allow to provide a conclusive explanation about how UV-A radiation can benefit root growth in low-watered plants. We can only speculate that this might be related with a UV-A-induced increase in water use efficiency (WUE, the ratio of assimilation to transpiration) under low water availability, based on the significantly higher values of leaf RWC in summer, a stressful period with high temperatures, and of leaf ETR in autumn (overall means of ETR in μmol m^{-2} s^{-1} ± S.E. for low-watered plants of the six studied species pooled together: UV-A = 80.07 ± 4.60; UV-A+UV-B = 75.94 ± 5.34; UV-0 = 63.35 ± 4.72; p = 0.011) in plants grown exposed to UV-A and UV-A+UV-B compared to those grown without UV radiation. Accordingly, in a parallel study using four (*D. gnidium*, *P. lentiscus*, *I. aquifolium* and *L. nobilis*) of the six species studied, we detected that plant growth under UV-A radiation tended to increase leaf WUE of low-watered plants, although this effect was not found when plants were grown under both types of UV radiation (overall means in μmol CO_2 mol^{-1} H_2O ± S.E. for low-watered plants of the four studied species pooled together: UV-A = 137.49 ± 11.52; UV-A+UV-B = 102.72 ± 19.44; UV-0 = 101.27 ± 10.33; Llusia *et al*. 2012 for more information). Hence, more studies are needed to disentangle this question. Despite the observed effects of our UV treatment on the leaf ETR and plant biomass, neither UV-A nor UV-A+UV-B exposure affected the leaf potential photochemical efficiency

(Fv/Fm) of plants at midday (Table I). The absence of UV effects on Fv/Fm agrees with previous literature (Paoletti 2005, Kadur *et al*. 2007) and is in accordance with the idea that Mediterranean plant species have efficient protective mechanisms against UV radiation (see reviews of Paoletti 2005 and Caldwell *et al.* 2007). In the present study, the increases in LMA and leaf carotenoids/chlorophyll *a+b* ratio found in response to UV-A+UV-B exposure (Table I) might have been the main responses for avoiding UV-B radiation damage at the doses supplied, since other photoprotective factors, such as the leaf content of UACs and phenolic compounds, did not show a general response to the UV treatment. Plants of the studied species grown under a low water supply showed, in general, lower values of leaf RWC, leaf area, leaf chlorophyll *a/b* ratio and biomass than well-watered ones. Nevertheless, the reduction in water availability did not affect significantly their overall leaf photochemical efficiency at midday (Table I). In agreement with previous studies showing a remarkable resistance of the photosynthetic apparatus to dehydration (e.g. Genty *et al.* 1987, Havaux 1992, Llorens *et al*. 2003, Prieto *et al*. 2009). We did not find different responses to the UV treatment between mesophytes and xerophytes, despite a species-specific response to UV for several of the variables measured. The similarity in the responses between these two functional groups of species might be caused by the similar range of leaf sclerophylly of the species chosen as mesophytes and xerophytes (LMA range from 4.69 to 12.81 mg cm^{-2} and from 5.86 to 11.04 mg cm^{-2} in mesophytes and xerophytes, respectively), suggesting that the degree of leaf sclerophylly might play a more important role in the adaptation of Mediterranean plant species to UV radiation than habitat characteristics (see also Verdaguer *et al.* 2012).

In conclusion, our study showed that, in comparison with plants grown without UV radiation, exposition of plants to UV-A radiation under low water availability had beneficial effects on the production of below-ground biomass of the species studied (particularly *P. angustifolia*), as well as on leaf photosynthetic activity (measured as leaf ETR) and above-ground biomass production of *P. lentiscus*, while UV-B radiation only increased photoprotective mechanisms, such as LMA and the leaf carotenoids/chlorophyll *a+b* ratio. Results also show a species-specific response to UV radiation for some of the variables measured, although differences do not seem to be related with the xerophytic or mesophytic character of the species.

Chapter III. Effects of enhanced UV radiation and water availability on photosynthetic performance, biomass production and photoprotective mechanisms in *Laurus nobilis seedlings.*

The content of this chapter is in preparation to be submitted as:

Bernal M, Verdaguer D, Badosa J, Abadía A, Llusia J, Peñuelas J, Núñez-Olivera E, Llorens L. Effects of enhanced UV radiation and water availability on photosynthetic performance, biomass production and photoprotective mechanisms in *Laurus nobilis* seedlings.

Introduction

Levels of UV-B radiation reaching Earth's surface have increased in recent decades due to the depletion of the ozone layer which is not expected to recover until 2025-2040 at mid-latitudes (World Meteorological Organization 2010). Furthermore, the expected decreases in the mean cloudiness of the Mediterranean Basin (Giorgi *et al*. 2004) will likely expose plants of terrestrial ecosystems in this region to higher fluxes of UV-B and also UV-A radiation in the near future. In addition to higher levels of UV radiation, Mediterranean plants are expected to experience longer dry periods and thus, decreases in the availability of water in the forthcoming decades (IPCC 2012).

Previous studies have found a positive interactive effect between drought and UV-B radiation on plant physiological performance and growth (see Caldwell *et al*. 2007 for a review), a phenomenon known as cross-tolerance (Stratmann 2003). For example, the exposure to enhanced levels of UV-B (Duan *et al*. 2008, Sangtarash *et al*. 2009b) or UV-A+UV-B (Poulson *et al*. 2002) radiation can partially alleviates negative effects of drought on biomass production, although this effect has not been found in all species studied (Nogués and Baker 2000, Turtola *et al*. 2005, Ballaré *et al*. 2011). However, very little data is available on the interactive effects of drought and UV radiation on the growth of Mediterranean plants, and even less data is available for woody evergreen sclerophyll plants adapted to drought (Paoletti 2005, Nogués and Baker 2000, Kyparissis *et al.* 2001, Manetas *et al.* 1997). Sclerophylls are widely distributed in the Mediterranean region and their stiff, hard and coriaceous leaves prevent them from cell collapse and damage under water shortage (Micco and Aronne 2012). Moreover, it has been suggested that the more sclerophyll leaves are, the more tolerant to UV radiation become (Verdaguer *et al.* 2012). Hence, sclerophyll Mediterranean species could be more resistant to the higher levels of UV radiation and drought expected in the

future than soft-leaved species without the need to display a wide battery of morphological and physiological strategies.

Among the most common plant strategies to avoid the damaging effects of UV-B radiation are those related to the prevention of penetration of UV-B radiation into the leaf, either by developing thicker, hairier, or waxier leaves (Barnes *et al*. 2005, Jansen 2002), or by increasing the content of UV-B-absorbing compounds, mainly phenols (Julkunen-Tiitto *et al*. 2005). The main groups of phenols related to the protection of plants from UV-B radiation are hydroxycinnamic acids and flavonoids. Hydroxycinnamic acids are compounds that predominantly screen UV radiation (Kolb *et al*. 2001). Among flavonoids, quercetin and kaempferol derivatives have been associated with the absorption of UV-B radiation to prevent it from reaching the photosynthetic machinery and with scavenging of reactive oxygen species (ROS) (Krauss *et al*. 1997, Rice-Evans *et al*. 1997, see Vermerris and Nicholson 2008 for a revision). Increases in phenols have also been associated with other environmental stresses, such as drought (Redha *et al*. 2012), although the effect of water deficit on phenolic content appears to be species-specific (see Treutter 2006 for a review). Little is known about the interactive effects of UV-B radiation and low water availability on the accumulation of phenolic compounds in plants with the few studies done to date reporting inconsistent results. Balakumar *et al.* (1993) and Nogués *et al*. (1998) suggested a synergistic effect of UV-B radiation and drought on the accumulation of foliar flavonoids and phenols, respectively, while Turtola *et al*. (2005) found that the accumulation of phenols induced by UV-B radiation was lower when plants were stressed by drought. In contrast, Alexieva *et al.* 2001 and Sangtarash *et al*. (2009a, b) found no interactive effect between UV-B radiation and drought on total foliar phenols. Moreover, there is no information on the combined effect of UV-B radiation and drought on the leaf phenol content of sclerophyll plants.

In conditions of high irradiance or water shortage (which can limit CO_2 fixation), an excess of absorbed light by plants can cause photo-oxidative damage (Foyer *et al*. 1994). Plants have thus developed different photoprotective mechanisms, some involving changes in foliar pools of chlorophylls and carotenoids to counteract these stressful conditions. A

reduction in the foliar content of chlorophyll has been considered to be an effective mechanism to avoid photoinhibition because lower levels of light absorption by chlorophyll translate to a lower potential for leaves to generate ROS (Munné-Bosch and Alegre 2000). Foliar carotenoids are part of the antenna complex, but some, such as β-carotene, can also act as effective antioxidants or others, such as the xanthophyll cycle pigments, can contribute to the dissipation of excess light energy as heat.

In terrestrial plants, two xanthophyll cycles have been associated with the thermal dissipation of excess light: the ubiquitous violaxanthin cycle (V-cycle), also known simply as the xanthophyll cycle because it was the first to be discovered (Demmig-Adams *et al*. 1996), and the lutein-epoxide cycle (Lx-cycle), which seems to be specific for some species (Bungard *et al*. 1999, García-Plazaola *et al*. 2002, Llorens *et al*. 2002). In the V-cycle, zeaxanthin (Z), the most effective xanthophyll in the dissipation of excess energy as heat (Demmig-Adams *et al*. 1996, García-Plazaola *et al*. 2007), is formed by de-epoxidation of violaxanthin (V) via the intermediate antheraxanthin (A). The de-epoxidation state of the V-cycle (DEPS) has been reported to increase under enhanced UV-B radiation in leaves of *Fagus sylvatica* (Šprtová *et al*. 2003, Láposi *et al*. 2009) or to decrease under UV exclusion in leaves of *Vitis vinifera* (Núñez-Olivera *et al*. 2006) and in pine needles (Martz *et al*. 2007). Other studies have failed to find any effect of ambient UV-B radiation, compared to the absence of UV-B radiation on the V-cycle de-epoxidation of some species, such as pea or spruce (Bolink *et al*. 2001, Kirchgebner *et al*. 2003). The de-epoxidation of lutein epoxide to lutein in the Lx-cycle has also been associated with the thermal dissipation of energy in some species (Llorens *et al*. 2002, García-Plazaola *et al.* 2007), but, to our knowledge, it has never been investigated in relation to changes in UV-B radiation. Besides, it is neither known whether UV-B effects on V- and Lx-cycles could be influenced by water availability in sclerophyll species.

The aim of this study was to examine the effect of an increase in erythemally-weighted doses of UV radiation (UV_E) in combination with two levels of water availability (high and low) on growth, foliar gas exchange, and water relations in seedlings of the evergreen sclerophyll Mediterranean species

Laurus nobilis L. (laurel). We also investigated the effects of these two factors on the foliar content of chloroplastic pigments (especially those belonging to the xanthophyll cycles) and of specific phenolic compounds. *Laurus nobilis* is an evergreen tree or shrub native to the southern Mediterranean region of great ecological and economic interest, which has been widely studied due to its pharmaceutical and alimentary properties. Moroever, its adaptability to drought (Arena *et al*. 2008, Maatallah *et al*. 2010) and high levels of light (Fiorini *et al*. 1998, Kang *et al*. 2002, Esteban *et al*. 2007 and 2008) is well known.

Materials and methods

Plant material and experimental design

One-year-old seedlings of *L. nobilis* with a root ball were planted in 2 L pots (5 cm wide x 20 cm deep) with 530 g of a mixture containing composted pine bark and *Sphagnum* peat (1:1 by volume). The growth medium was fertilised with Osmocote (4 kg m^{-3}), basal dressing (1 kg m^{-3}), and dolomite (4 kg m^{-3}) to avoid nutritional deficiencies during the experiment. Seedlings were grown under controlled irrigation and supplemented with UV radiation in an outdoor setting to obtain more realistic and balanced ratios of UV radiation/PAR (photosynthetically active radiation). Seedlings were distributed in nine plots built with 130 x 120 cm metallic frames equipped with four fluorescent lamps mounted overhead. These nine plots were organised into three blocks, with each block having one plot with the following UV radiation conditions: ambient UV radiation, enhanced UV-A radiation, and enhanced UV-A+UV-B radiation. Within each plot, two irrigation regimes were applied (see below). Each combination of UV radiation and irrigation was, thus, replicated three times in a randomised complete block design.

The experiment was conducted in an experimental field (Can Vilallonga) in the vicinity of Cassà de la Selva (Girona, northeastern Iberian Peninsula,

41º53' N, 2º52' E) from May 29, 2009 to January 21, 2010. Before the UV treatment began, the plants were allowed to acclimate to the environmental conditions of the site for 2 weeks. On May 25, immediately prior to starting the experiment, we measured the length and basal diameter (at 2 cm above the cotyledonary node) of the main stem of four plants from each UV radiation and irrigation treatment. Statistical analysis indicated that initial differences in these parameters among plants in the different UV radiation and irrigation treatments were not significant (data not shown). Unless otherwise noted, all samples were collected at midday in September 2009 from randomly chosen seedlings.

UV-radiation treatment

The three UV-radiation treatments (Table III) were:

- Enhanced UV-A+UV-B: UV radiation (UV-A+UV-B) was supplied with four 40 W fluorescent lamps 1.2 m in length (TL 40W/12 RS, with a peak at 313 nm; Phillips, Spain) mounted in metal frames (1.3 x 1.2 m). On average, UV supplementation enhanced erythemally-weighted doses of UV radiation (UV_E, McKinlay and Diffey 1987) 23% above ambient UV_E levels. UV_E doses (ambient UV_E plus supplemented UV_E) received by the plants are presented in Table III, with increased doses in relation to control plants ranging from 19 to 38%, depending on the month. Ultraviolet radiation was applied daily for 2.5-3.5 h (depending on the month) centered at solar noon. The fluorescent lamps were wrapped with cellulose diacetate foil (Ultraphan URT, 0.1 mm, Digefra GmbH, Munich, Germany) to remove wavelengths <280 nm (UV-C radiation). Cellulose diacetate films were pre-burned for 3 h before being applied to the lamps and were replaced after 36 h of use to avoid the effects of plastic photodegradation.

- Enhanced UV-A: This treatment served as a control for the UV-A+UV-B treatment. Ultraviolet radiation was supplied as described in the

UV-A+UV-B plots, but the lamps were wrapped with polyester instead of cellulose diacetate films (Mylar D, 0.13 mm; PSG Group, England) to block both UV-B and UV-C radiation (transmittance >320 nm).

- Ambient UV: Seedlings in these plots received only solar UV radiation. The plots were equipped with wooden frames to ensure that plants were exposed to the same conditions of shading as the plants in the other two radiation conditions.

Within each plot, the plants were rotated every week to minimise microenvironmental and border effects. UV_E was measured at the top of plant canopies with a UVS-E-T radiometer (Kipp and Zonen, The Netherlands). To prevent UV radiation contamination among plots, two 120 x 30 cm clear sheets of polycarbonate (no transmission below 400 nm) were fastened along the two sides of the metal frames parallel to the fluorescent lamps.

Month	Rainfall ($L\ m^{-2}$)	UV_E ($kJ\ m^{-2}\ day^{-1}$) in ambient UV plots	UV_E ($kJ\ m^{-2}\ day^{-1}$) in enhanced UV-A+UV-B plots	PAR ($kJ\ m^{-2}\ day^{-1}$)
June	11	4.191 ± 0.156	4.976 ± 0.175	9874 ± 427
July	4.8	3.875 ± 0.230	4.658 ± 0.249	7910 ± 644
August	14.6	2.759 ± 0.081	3.510 ± 0.094	6828 ± 369
September	43.2	2.086 ± 0.081	2.603 ± 0.103	5041 ± 253
October	73.7	1.724 ± 0.082	2.261 ± 0.104	4524 ± 268
November	21.5	0.934 ± 0.057	1.263 ± 0.074	2364 ± 198
December	18.4	0.398 ± 0.057	0.525 ± 0.072	458 ± 173
January	71.1	0.494 ± 0.046	0.601 ± 0.048	1427 ± 235

Table III *Total monthly rainfall in Cassà de la Selva, monthly average UV_E doses ($kJ\ m^{-2}\ day^{-1}$) in ambient UV and enhanced UV-A+UV-B plots, and monthly average photosynthetically active radiation (PAR) doses ($kJ\ m^{-2}\ day^{-1}$) ± S.E.*

Irrigation treatment

Plants received water from rainfall and from a controlled drip-irrigation system, which was programmed according to the specific irrigation treatment and monthly rainfall (Table III). Two irrigation regimes were applied after June 11: within each plot, half of the seedlings (chosen randomly) were irrigated to field capacity (well-watered plants, WW), which ranged from 0.2 to 1.34 L per day depending on the precipitation, and the other half (low-watered plants, LW) received approximately 38% and 25% less water from June to September and from October to December, respectively, than WW plants.

Biomass production and foliar morphological traits

Above- and below-ground biomass were determined for four seedlings from each plot and irrigation treatment (three were harvested in September and one in January). The leaves, stems, and roots were separated and oven-dried at 70 ºC for 72 h.

One fully expanded leaf from four individual plants per plot and irrigation treatment was sampled to determine its thickness and leaf mass per area (LMA, mg cm^{-2}). Foliar thickness was measured with a portable micrometer (mod. 4000DIG, Baxlo, Spain). The leaves were then scanned (Epson perfection 1250, USA), and their areas determined using an image-analysis programme (ImageTool, University of Texas Health Science Center, USA). The leaves were oven-dried at 70 ºC for 72 h to assess their dry mass (DM). The LMA were calculated as the quotient between foliar DM and foliar area for each leaf.

Relative water content

The relative water content (RWC, %) was measured in the same leaves used to analyse morphological traits. After collection, leaves were weighed to obtain their fresh mass (FM) and then they were stored in darkness with distilled water for 48 h to determine their turgid mass (TM). The DM was subsequently

measured (as described above), and the RWC was calculated as: (FM-DM/TM-DM) x 100.

Gas-exchange measurements

Foliar gas exchange was measured in one plant per plot and irrigation treatment, using one young, fully expanded leaf located at the top of the canopy. Measurements were taken using a gas-exchange system (CI-340 Hand-Held Photosynthesis System, CID, Inc., Camas, WA 98607 USA) as described in Llusia *et al*. (2012) but applying 1000 µmol m^{-2} s^{-1} PAR.

Chlorophyll fluorescence

The components of foliar chlorophyll fluorescence were quantified using a PAM-2100 portable modulated fluorometer (Heinz Walz GmbH, Effeltrich, Germany) for four seedlings per plot and irrigation treatment at predawn and midday, using one exposed and fully developed leaf per seedling. Minimum (F_o) and maximum (F_m) dark-adapted fluorescence was determined and used to calculate the potential photochemical efficiency of photosystem II as F_v/F_m, where F_v is variable fluorescence and $F_v = F_m-F_o$. The actual photochemical efficiency of photosystem II in the light-adapted state was also calculated ($\Delta F/F_m' = (F_m'-F)/F_m'$), where F is the steady-state fluorescence yield under the given environmental conditions and F_m' is the maximum level of fluorescence obtained during a saturating flash of light. The apparent electron transport rate (ETR) was then calculated as $ETR = \Delta F/F_m' \times PAR \times 0.84 \times 0.5$, where PAR was the incident photosynthetically active radiation (expressed in µmol m^{-2} s^{-1}), 0.84 was the assumed coefficient of absorption of the leaves, and 0.5 was the assumed distribution of absorbed energy between the two photosystems (Galmés *et al.* 2007). The non-photochemical quenching coefficient (NPQ) was determined as $(F_m-F_m')/F_m'$ from the predawn and midday measurements.

Photosynthetic pigments

At least three foliar discs of 0.64 cm^2, one from each of three plants per plot and irrigation treatment, were sampled at predawn and midday, immediately frozen in liquid nitrogen, and stored at -80 ºC until analysis. Photosynthetic pigments were extracted with 1 mL of acetone in the presence of liquid nitrogen and ascorbate by grinding the foliar discs in a mortar. Four mL of acetone were then added, and the mixture was stored at -20 ºC, as previously described (Abadía and Abadía 1993). Pigment extracts were thawed on ice, filtered through a 0.45-µm filter, and analyzed by high-performance liquid chromatography (HPLC) following the method described by Larbi *et al*. (2004). Two phases were pumped instead of three: phase A [acetonitrile:methanol 7/1 (v/v)] was pumped for 3.4 min, and phase B [acetronitrile:methanol:water:ethyl acetate 7/0.96/0.04/8 (v/v/v/v)] was pumped for 4.6 min. Triethylamine was added to each phase to obtain a final concentration of 0.7% in the mixture. The injection volume was 20 µL, and the analysis time for each sample was 15 min, including the equilibration time, which consisted of flushing phase A for 5 min at the beginning. Results were expressed in µg of pigment per g of foliar DM using LMA conversion.

Total phenols

One fully expanded leaf from four plants per plot and irrigation treatment was sampled. From each leaf, 10 mg of dried material was ground to a powder and mixed with 2.5 mL of 50% methanol. The extract was then shaken for 1 h and subsequently centrifuged for 5 min at 800 g. Total phenolic content was determined following the method described in Rozema *et al*. (2006). A fraction of the extract (50 µL) was mixed with 3.5 mL of distilled water and 250 µL of Folin-Ciocalteu reagent (Merck). After 8 min, 750 µL of Na_2CO_3 (20%) was added. Absorbance was measured 2 h later at 760 nm with a spectrophotometer (U-2000, Hitachi, USA). The total phenolic content of leaves was calculated from the standard curve for gallic acid, prepared from 50 µL of a

standard solution of gallic acid (40, 80, 150, 200, 400, 600, 800, and 1000 mg L^{-1}) and expressed as mg of gallic acid equivalents per g of DM.

UV-absorbing compounds (UACs)

Methanol-soluble, methanol-insoluble, alkali-extractable, and cell-wall UV-absorbing compounds were analyzed for four plants per plot and irrigation treatment. Three foliar discs of 0.64 cm^2 were collected from each plant, frozen in liquid nitrogen, and stored at -80 ºC until analysis. Total foliar content of UACs and foliar concentrations of several phenolic compounds were measured, respectively, by spectrophotometry (Perkin-Elmer, Wilton, CT) and HPLC (Agilent HP1100 HPLC system, Agilent Technologies, Palo Alto, CA). The extraction and analytical methods were as reported in Fabón *et al.* (2010). Briefly, after grinding the plant material in a Tissue Lyser (Qiagen, Hilden, Germany), 5 ml of methanol:water:7 M HCl (70:29:1) was added and the mixture stored for at least 20 h at 4 ºC in the dark. The extract was centrifuged at 6000 g for 15 min at 10 ºC, and the supernatant was removed and used for the determination of methanol-soluble UACs (mainly located in the vacuoles). The pellet was stored at -80 ºC and later used for the determination of the methanol-insoluble UACs (mainly located in the cell wall).

For the determination of methanol-soluble phenols by HPLC, 250 µL of the supernatant were filtered (0.22 µm) and pumped into the HPLC. The remainder of the supernatant was used to determine total methanol-soluble UACs spectrophotometrically in arbitrary units, as the area under the absorbance curve in the intervals 280-315 nm and 280-400 nm ($UAC_{280-315}$ and $UAC_{280-400}$) per unit of DM. For the methanol-insoluble UACs, the pellet remaining from the methanol extraction was hydrolysed with 2 mL of 1 M NaOH, and the mixture was heated at 80 ºC for 3 h. One mL of 5.6 N HCl was added, and the UACs were extracted three times with 2 mL of ethyl acetate. The supernatants from each extraction were collected and evaporated. The residue was dissolved in methanol, and the amount of phenols specific to the cell wall were determined by HPLC, while the total content of UACs was determined spectrophotometrically in the same units as above.

Statistical analyses

The main effects of UV radiation treatment, irrigation treatment, block, and their interactions, on all variables except the foliar gas-exchange measurements, were assessed by three-way analysis of variance (ANOVA). When the overall effect of the UV treatment or the interaction between treatments was significant, we additionally test the effects of the UV treatment within each watering regime using two-way ANOVA, with block and UV radiation treatment as fixed factors. Pairwise comparisons between UV conditions or blocks were analyzed using Duncan's test. For the foliar gas-exchange data, the effects of the UV and irrigation treatments were analyzed using two-way ANOVA, since only one plant per plot and irrigation treatment was sampled. Kolmogorov-Smirnov and Levene's tests were used to test normality and homoscedasticity, respectively, and data was log-transformed when necessary. When normally distributed data did not meet the assumption of homoscedasticity, the Games-Howell post-hoc test was applied. A significance level of $p \leq 0.05$ was used for all statistical tests.

Results

Seedling biomass and leaf morphological traits

Exposure to enhanced levels of UV-A and UV-A+UV-B radiation increased the production of biomass in laurel seedlings. Seedlings grown under these conditions had, respectively, 36% and 41% greater stem biomass and 26% and 30% greater root biomass than control seedlings grown under ambient levels of UV radiation (Table IV). However, when analysis were conducted within each irrigation condition, only low-watered plants, but not well-watered ones, exposed to enhanced UV-A and UV-A+UV-B radiation had greater foliar, stem and root biomass than control plants ($F_{2,36} = 3.90$, $p = 0.03$; $F_{2,36} = 5.56$, $p = 0.01$ and

$F_{2,36}$ = 3.55, p = 0.04, respectively) (Fig. 15). Nevertheless, these plants did not show changes in the root-to-shoot ratio (data not shown).

Leaves from UV-A+UV-B-supplemented plants were about 11% thicker than those of control plants, but neither foliar area nor LMA changed significantly (Table IV). Overall, plant biomass and leaf morphological traits did not differ significantly between the two irrigation conditions (Table IV).

Leaf physiological parameters

A significant interaction between the effects of the UV radiation and irrigation treatments was found for leaf RWC (Table IV). For control plants, well-watered seedlings had a 3.4% higher foliar RWC than did low-watered seedlings ($F_{1,23}$ = 9.01, p < 0.01) (Fig. 16). Besides, the UV radiation treatment did not affect the foliar RWC of well-watered plants, but low-watered seedlings had 2.2% and 2.6% higher foliar RWCs when subjected to enhanced UV-A and UV-A+UV-B radiation, respectively, compared to control seedlings, although the difference was only significant in the UV-A+UV-B-supplemented plants (Fig. 16).

The effect of irrigation treatment on leaf gas exchange parameters differed between UV-conditions, which would explain the interaction found between treatments (Table IV). Plants supplemented with UV-A radiation had higher rates of photosynthesis (A), transpiration (E), and stomatal conductance (g_s) when grown under low-irrigation than when grown under well-irrigation. On the contrary, in control treatment plants grown under low water availability had lower E than well-watered ones (Fig. 17). No changes were observed on leaf gas exchange parameters between well- and low-watered plants supplemented with UV-A+UV-B radiation (Fig. 17). Water-use efficiency (WUE) was significantly higher in low- than in well-watered plants (Table IV), which was basically due to the higher WUE values found for plants supplemented with UV-A and UV-A+UV-B radiation under water shortage (100% and 56%, respectively) (Fig. 17).

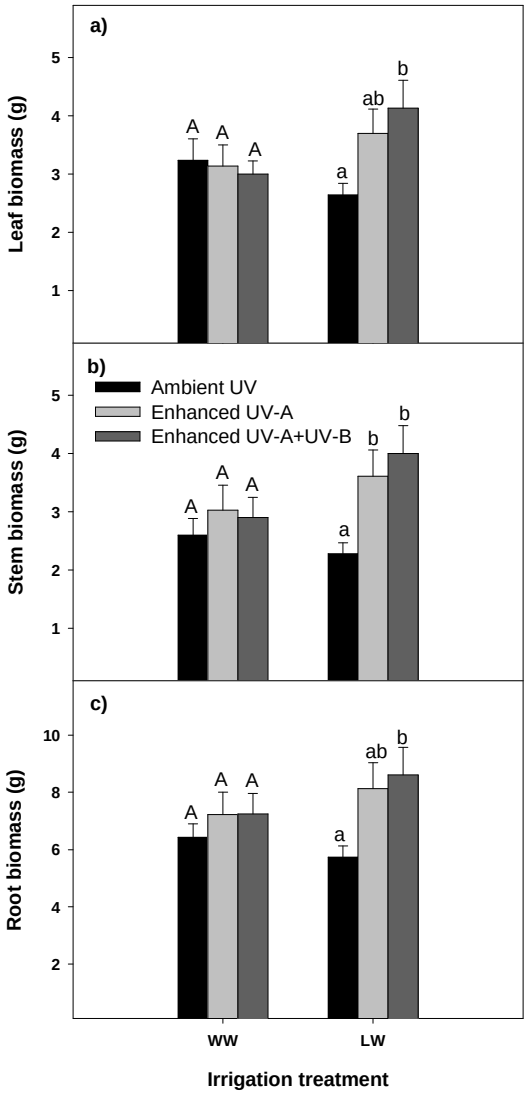


Fig. 15 Leaf, stem, and root biomass for well- (WW) and low-watered (LW) seedlings of Laurus nobilis *grown under three UV conditions. Values are means ± S.E. (N = 12). Different letters indicate statistically significant differences (p ≤ 0.05) among UV conditions within each irrigation level.*

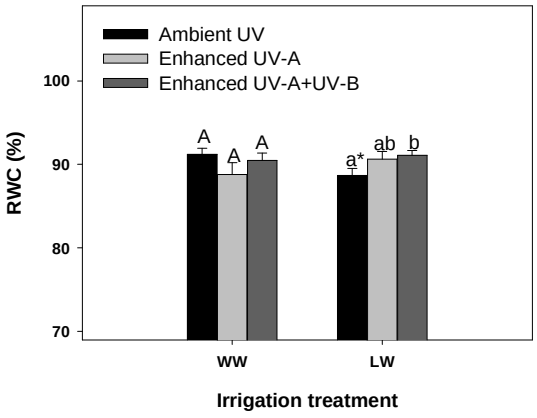


Fig. 16 Foliar RWC for well- (WW) and low-watered (LW) seedlings of Laurus nobilis *grown under three UV radiation conditions. Values are means ± S.E. (N = 12). Different letters indicate statistically significant differences (p ≤ 0.05) among UV conditions within each irrigation level. The asterisk indicates a significant difference between WW and LW plants exposed to ambient UV conditions.*

	UV radiation treatment				Irrigation treatment			Interaction UV*irrigation
	Ambient UV	Enhanced UV-A	Enhanced UV-A+UV-B	*p*- value	WW	LW	*p*-value	*p*-value
Leaf thickness (mm)	**0.40 ± 0.009 a**	**0.42 ± 0.012 ab**	**0.45 ± 0.015 b**	**0.04**	0.42 ± 0.010	0.43 ± 0.011	ns	ns
Leaf area	11.08 ± 0.65	10.14 ± 0.56	11.15 ± 0.69	ns	10.73 ± 0 .62	10.84 ± 0.40	ns	ns
LMA	12.50 ± 0.21	13.12 ± 0.27	13.15 ± 0.41	ns	12.91 ± 0 .30	12.93 ± 0.20	ns	ns
Total biomass (g)	**11.46 ± 0.64 a**	**14.41 ± 1.15 b**	**14.94 ± 1.14 b**	**0.03**	14.28 ± 0.94	12.93 ± 0.73	ns	ns
Stem biomass (g)	**2.44 ± 0.17 a**	**3.32 ± 0.03 b**	**3.45 ± 0.31 b**	**0.01**	3.30 ± 0.25	2.84 ± 0.20	ns	ns
Root biomass (g)	**6.08 ± 0.31 a**	**7.67 ± 0.59 b**	**7.92 ± 0.61 b**	**0.02**	7.49 ± 0.49	6.96 ± 0.38	ns	ns
Leaf biomass (g)	2.94 ± 0.21	3.42 ± 0.28	3.56 ± 0.28	ns	3.49 ± 0.24	3.12 ± 0.18	ns	ns
RWC (%)	89.93 ± 0.62	89.73 ± 0.84	90.79 ± 0.52	ns	90.14 ± 0 .62	90.14 ± 0.48	ns	**0.02**
A (µmol CO_2 m^{-2} s^{-1})	6.11 ± 1.07	6.76 ± 1.87	5.99 ± 0.92	ns	**4.49 ± 0.74**	**7.63 ± 1.15**	**0.05**	**0.05**
E (mmol H_2O m^{-2} s^{-1})	1.31 ± 0.07	1.37 ± 0.11	1.29 ± 0.11	ns	1.31 ± 0.06	1.34 ± 0.09	ns	**0.05**
g_s (mmol H_2O m^{-2} s^{-1})	59.69 ± 6.34	61.06 ± 7.03	53.06 ± 4.13	ns	56.73 ± 4 .64	59.14 ± 5.06	ns	**0.05**
WUE (µmol CO_2 $mmol^{-1}$ H_2O)	0.10 ± 0.02	0.10 ± 0.02	0.11 ± 0.01	ns	**0.09 ± 0.01**	**0.13 ± 0.01**	**0.03**	ns
ETR midday	68.30 ± 9.22	69.13 ± 7.88	71.06 ± 9.33	ns	75.84 ± 7 .17	63.16 ± 7.00	ns	ns
Fv/Fm midday	0.66 ± 0.01	0.66 ± 0.01	0.66 ± 0.01	ns	0.66 ± 0.01	0.65 ± 0.01	ns	ns
NPQ midday	1.72 ± 0.19	1.82 ± 0.21	1.52 ± 0.04	ns	1.78 ± 0.18	1.59 ± 0.06	ns	ns

Table IV *Overall mean ± S.E. for plant biomass and the leaf morphological parameters of* Laurus nobilis *seedlings grown under three different UV radiation conditions (ambient UV, enhanced UV-A, and enhanced UV-A+UV-B; N = 24) and two irrigation levels (WW = well-watered, LW = low-watered; N = 36). Different letters and values in boldface indicate statistical differences (p ≤ 0.05) in pairwise comparisons. LMA = Leaf mass per area; ns = not significant.*

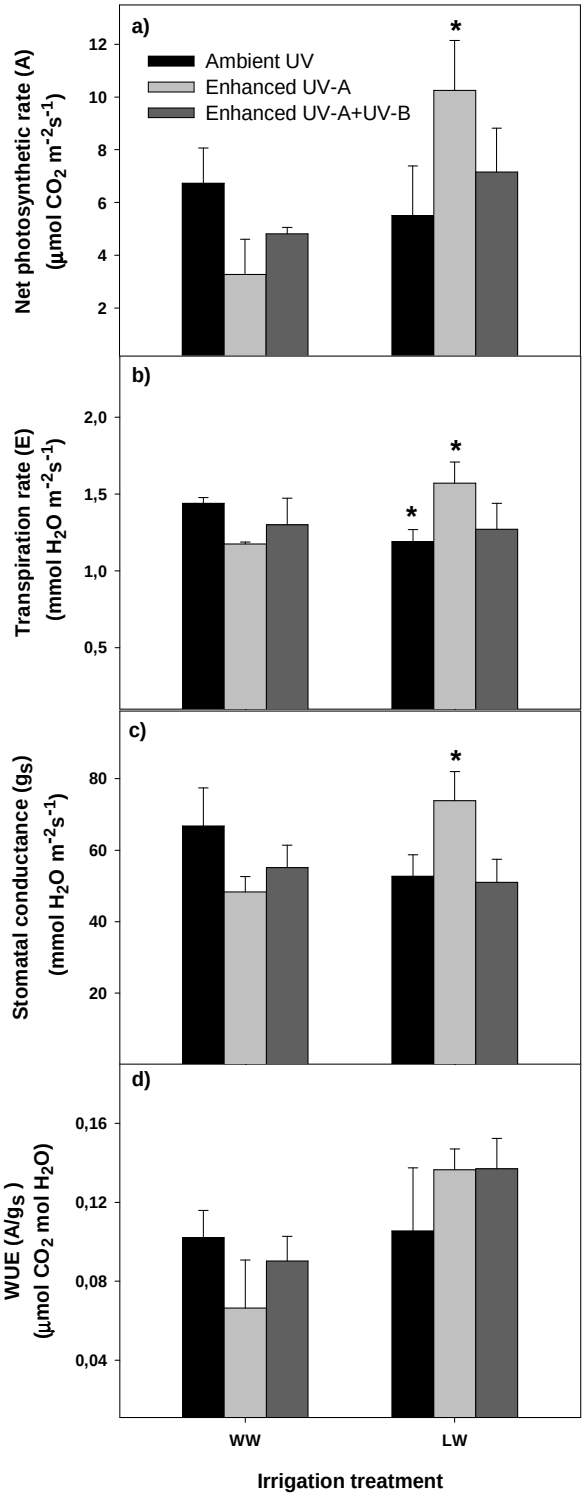


Fig. 17 a) Net photosynthetic rate (A), b) transpiration rate (E), c) stomatal conductance (g_s), and d) water-use efficiency (WUE) of Laurus nobilis *seedlings exposed to different UV and irrigation conditions. Values are means ± S.E. (N = 3). The asterisks indicate statistically significant differences between WW and LW plants grown under the same UV conditions ($p \leq 0.05$). UV conditions were not significantly different within each irrigation level.*

The parameters derived from foliar chlorophyll fluorescence measurements (ETR, F_v/F_m, and NPQ) were unaffected by the treatments, either at predawn or at midday (Table IV). Nevertheless, when the effects of the UV radiation treatment on these parameters were analyzed separately for well- and low-watered seedlings, low-watered plants exposed to higher UV-A radiation fluxes had significantly higher NPQ values at midday than plants grown under ambient UV levels or UV-A+UV-B supplementation (Fig. 18).

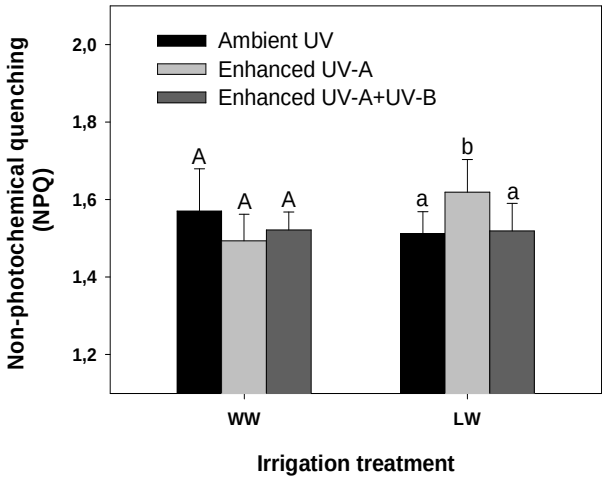


Fig. 18 Non-photochemical quenching (NPQ) for well- (WW) and low-watered (LW) seedlings of Laurus nobilis *under three UV conditions at midday. Values are means ± S.E. (N = 12). Different letters indicate statistically significant differences (p ≤ 0.05) among UV conditions within each irrigation level.*

Leaf photosynthetic pigments

The results consistently indicated that, at predawn and midday, laurel seedlings growing under enhanced UV-A radiation had the lowest foliar content of most of the photosynthetic pigments related to the absorption of light: chlorophyll *a*+*b*, neoxanthin, β-carotene, and violaxanthin (although differences in this last pigment were only significant at midday) (Table V). At predawn, however, UV radiation effects on the foliar content of chlorophylls and β- and α-carotene were dependent on the amount of water supplied to the plants (Table V), since differences were only significant for low-watered seedlings (Fig. 19).

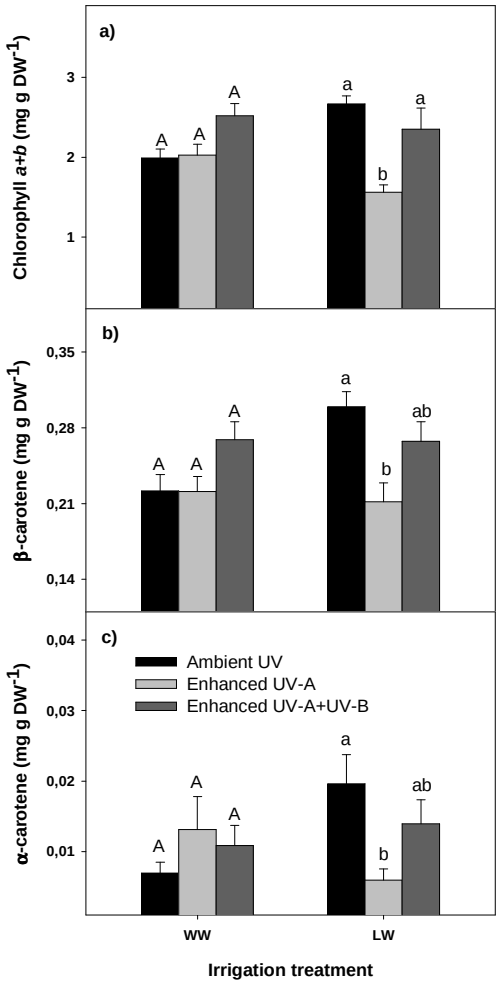


Fig. 19 Predawn foliar content of chlorophyll a+b and α- and β-carotene for well- (WW) and low-watered (LW) seedlings of Laurus nobilis *grown under three UV conditions. Values are means ± S.E. (N = 9). Different letters indicate statistically significant differences (p ≤ 0.05) among UV conditions within each irrigation level.*

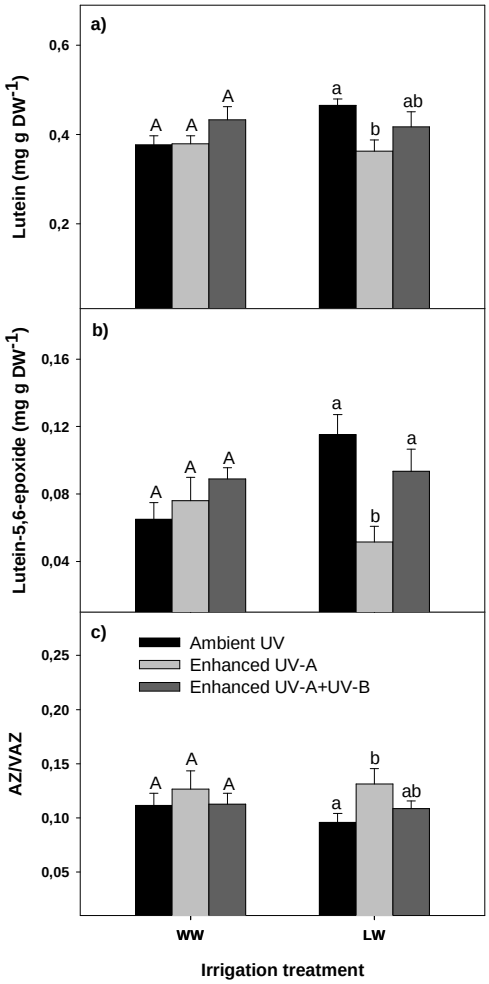


Fig. 20 Predawn foliar content of lutein and lutein-5,6-epoxide and foliar de-epoxidation state of the V-cycle (AZ/VAZ) for well- (WW) and low-watered (LW) seedlings of Laurus nobilis *under three UV conditions. Values are means ± S.E. (N = 9). Different letters indicate statistically significant differences (p ≤ 0.05) among UV conditions within each irrigation level.*

At predawn, UV-A-supplemented leaves had significantly higher zeaxanthin content than ambient UV plants (Table V). Low-watered plants exposed to enhanced UV-A radiation also had the highest de-epoxidation state of the V-cycle (AZ/VAZ) and the lowest foliar content of lutein and lutein-5,6-epoxide (Fig. 20). The total pool of V-cycle pigments (VAZ) at predawn was unaffected by the treatments (Table V). In contrast, a significant interactive effect was

observed between treatments in total foliar content of Lx-cycle pigments (Table V). Under water shortage, UV-A-treated plants had lower total leaf contents of Lx-cycle pigments than those growing under the other two UV conditions (Fig. 20). At midday, plants grown under enhanced UV-A radiation had the highest amount of zeaxanthin, although this difference was only significant in comparison to plants supplemented with UV-A+UV-B radiation (about 150% higher) (Table V). Leaves exposed to UV-A radiation also had a higher AZ/VAZ index compared to UV-A+UV-B-supplemented leaves (Table V).

	UV treatment				Irrigation treatment			UV * irrigation
	Ambient UV	Enhanced UV-A	Enhanced UV-A+UV-B	*p*-val	WW	LW	*p*-val	*p*-value
(A) PREDAWN								
Chlorophyll *a+b* (mg g DM^{-1})	**4.32 ± 0.24 a**	**3.33 ± 0.22 b**	**4.52 ± 0.33 a**	**0.01**	4.04 ± 0.20	4.07 ± 0.30	ns	**0.02**
Chlorophyll *a/b*	3.89 ± 0.06	4.11 ± 0.10	3.88 ± 0.07	ns	3.89 ± 0.06	4.02 ± 0.07	ns	ns
Neoxanthin (mg g DM^{-1})	**0.12 ± 0.005 a**	**0.10 ± 0.01 b**	**0.13 ± 0.01 a**	**<0.01**	0.12 ± 0.01	0.12 ± 0.01	ns	ns
β-carotene (mg g DM^{-1})	**0.28 ± 0.01 a**	**0.23 ± 0.01 b**	**0.29 ± 0.01 a**	**<0.01**	0.26 ± 0.01	0.28 ± 0.01	ns	**0.01**
α-carotene (mg g DM^{-1})	0.01 ± 0.003	0.01 ± 0.003	0.01 ± 0.002	ns	0.01 ± 0. 002	0.01 ± 0.002	ns	**0.02**
Violaxanthin (mg g DM^{-1})	0.26 ± 0.01	0.23 ± 0.01	0.25 ± 0.01	ns	0.24 ± 0.01	0.25 ± 0.01	ns	ns
Anteraxanthin (mg g DM^{-1})	0.02 ± 0.002	0.02 ± 0.002	0.02 ± 0.002	ns	0.02 ± 0. 002	0.02 ± 0.001	ns	ns
Zeaxanthin (mg g DM^{-1})	**0.005 ± 0.001 a**	**0.008 ± 0.001 b**	**0.006 ± 0.001 ab**	**0.05**	0.007 ± 0.001	0.007 ± 0.001	ns	ns
VAZ pool	0.28 ± 0.01	0.26 ± 0.01	0.28 ± 0.01	ns	0.27 ± 0.01	0.28 ± 0.01	ns	ns
AZ/VAZ	0.09 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	ns	0.11 ± 0.01	0.10 ± 0.01	ns	ns
Lutein (mg g DM^{-1})	0.39 ± 0.01	0.34 ± 0.01	0.39 ± 0.02	ns	0.37 ± 0.01	0.38 ± 0.02	ns	ns
Lutein-5,6-epoxide (mg g DM^{-1})	**0.04 ± 0.004 a**	**0.03 ± 0.004 b**	**0.04 ± 0.003 a**	**0.02**	0.03 ± 0.003	0.04 ± 0.004	ns	**<0.01**
Lutein+Lutein-5,6-epoxide (mg g DM^{-1})	**0.43 ± 0.02 a**	**0.37 ± 0.02 b**	**0.43 ± 0.02 a**	**0.03**	0.40 ± 0.01	0.42 ± 0.02	ns	**0.03**
(B) MIDDAY								
Chlorophyll *a+b* (mg g DM^{-1})	**4.16 ± 0.16 a**	**3.50 ± 0.19 b**	**4.42 ± 0.24 a**	**0.01**	4.05 ± 0.20	4.03 ± 0.15	ns	ns
Chlorophyll *a/b*	3.63 ± 0.06	3.68 ± 0.07	3.77 ± 0.09	ns	3.68 ± 0.06	3.71 ± 0.06	ns	ns
Neoxanthin (mg g DM^{-1})	**0.11 ± 0.01 ab**	**0.09 ± 0.01 b**	**0.12 ± 0.01 a**	**0.03**	0.11 ± 0.01	0.11 ± 0.01	ns	ns
β-carotene (mg g DM^{-1})	**0.27 ± 0.01 a**	**0.22 ± 0.01 b**	**0.29 ± 0.01 a**	**<0.01**	0.26 ± 0.01	0.26 ± 0.01	ns	ns
α-carotene (mg g DM^{-1})	0.01 ± 0.003	0.01 ± 0.002	0.02 ± 0.003	ns	0.01 ± 0. 002	0.02 ± 0.003	ns	ns
Violaxanthin (mg g DM^{-1})	**0.19 ± 0.01 a**	**0.15 ± 0.01 b**	**0.22 ± 0.01 a**	**<0.01**	0.18 ± 0.01	0.19 ± 0.01	ns	ns
Anteraxanthin (mg g DM^{-1})	0.04 ± 0.003	0.04 ± 0.004	0.03 ± 0.003	ns	0.03 ± 0. 003	0.03 ± 0.003	ns	ns
Zeaxanthin (mg g DM^{-1})	**0.03 ± 0.01 ab**	**0.05 ± 0.01 a**	**0.02 ± 0.003 b**	**0.03**	0.03 ± 0.01	0.03 ± 0.01	ns	ns
VAZ pool	0.26 ± 0.01	0.24 ± 0.01	0.27 ± 0.01	ns	0.25 ± 0. 01	0.26 ± 0.01	ns	ns
AZ/VAZ	**0.26 ± 0.03ab**	**0.35 ± 0.06 a**	**0.19 ± 0.02 b**	**0.02**	0.28 ± 0.04	0.25 ± 0.03	ns	ns
Lutein (mg g DM^{-1})	0.39 ± 0.01	0.34 ± 0.02	0.39 ± 0.02	ns	0.37 ± 0.02	0.37 ± 0.01	ns	ns
Lutein-5,6-epoxide (mg g DM^{-1})	**0.04 ± 0.005ab**	**0.03 ± 0.004 a**	**0.05 ± 0.004 b**	**0.01**	0.04 ± 0.04	0.04 ± 0.004	ns	ns
Lutein+Lutein-5,6-epoxide (mg g DM^{-1})	**0.43 ± 0.01 a**	**0.37 ± 0.02 b**	**0.43 ± 0.02 ab**	**0.02**	0.41 ± 0.02	0.41 ± 0.01	ns	ns

Table V *Overall means ± S.E. at predawn (A) and midday (B) for the foliar content of photosynthetic pigments of* Laurus nobilis *seedlings grown under three different UV conditions (ambient UV, enhanced UV-A, and enhanced UV-A+UV-B) and two irrigation levels (WW = well-watered and LW = low-watered). The effects (p-values) of the UV and irrigation treatments and the interactions between both factors were tested by three-way ANOVA, with a total of 18 degrees of freedom for the UV treatment and 27 for the irrigation treatment. Different letters and values in boldface indicate statistical differences (p ≤ 0.05) in pairwise comparisons. VAZ = Violaxanthin+Anteraxanthin+Zeaxanthin, AZ = Anteraxanthin+Zeaxanthin; ns = not significant.*

UV-absorbing compounds

Neither the total leaf phenolic content nor the total leaf content of methanol-soluble (mainly located in vacuoles) and methanol-insoluble (from the cell wall) UV-absorbing compounds (as $UAC_{280-400}$) or UV-B-absorbing compounds ($UAC_{280-315}$) varied in response to the treatments (Table VI). Eight methanol-soluble compounds were detected. Two belonged to the quercetin family (quercetin 3-*O*-glucuronide, quercetin-*O*-glycoside) and two to the kaempferol family (cis-trans-kaempferol 3-*O*-glucoside, kaempferol 3,7-*O*-diglucoside) (Table VI). There were also three unidentified compounds, named as C1, C2, and C3, with a highly similar spectra to that of quercetins, which were, thus, considered from the quercetin family. Finally, another unidentified compound, C4, had an spectra with a high similarity to that of kaempferols and it was considered from the kaempferol family (Table VI).

The total leaf content of quercetin and kaempferol decreased when plants were exposed to enhanced UV-A or enhanced UV-A+UV-B radiation compared to control plants, although the kaempferol content only differed significantly between UV-A-supplemented and control plants (Table VI). Among quercetins, the leaf content of quercetin 3-*O*-glucuronide was lower in UV-A- and UV-A+UV-B-supplemented plants than in control ones, while the foliar amount of C1 compound was significantly lower only in UV-A-supplemented plants (Table VI). Leaves exposed to enhanced-UV-A radiation had also smaller amounts of cis-trans-kaempferol 3-*O*-glucoside than control plants (Table VI). Only two cell-wall compounds were detected: *p*-coumaric (a hydroxycinnamic acid) and one kaempferol derivative, but they did not respond significantly to UV radiation treatment (Table VI). Neither the irrigation treatment nor its interaction with the UV radiation treatment significantly affected the leaf content of the methanol-soluble and methanol-insoluble compounds detected.

	UV treatment				Irrigation treatment			Interaction UV * Irrigation
	Ambient UV	**Enhanced UV-A**	**Enhanced UV-A+UV-B**	***p*-val**	**WW**	**LW**	***p*-val**	***p*-value**
Total phenols	69.58 ± 3.55	70.35 ± 4.77	75.07 ± 4.39	ns	72.22 ± 3 .91	71.04 ± 3.02	ns	ns
(A) Methanol-soluble compounds								
$UAC_{280-315}$	0.74 ± 0.03	0.79 ± 0.02	0.75 ± 0.02	ns	0.75 ± 0.02	0.77 ± 0.02	ns	ns
$UAC_{280-400}$	1.73 ± 0.07	1.16 ± 0.03	1.15 ± 0.04	ns	1.19 ± 0.03	1.13 ± 0.03	ns	ns
Quercetins	**30.17 ± 3.81 a**	**20.50 ± 2.35 b**	**22.10 ± 1.89 b**	**0.01**	24.95 ± 2.58	23.65 ± 2.18	ns	ns
Kaempferols	**14.65 ± 1.72 a**	**11.16 ± 1.30 b**	**12.58 ± 1.38 ab**	**0.05**	12.91 ± 1.38	12.73 ± 1.04	ns	ns
Quercetins								
Quercetin 3-*O*-glucuronide	**12.77 ± 1.75 a**	**7.98 ± 0.90b**	**8.86 ± 0.77b**	**0.01**	10.01 ± 1.05	9.78 ± 1.06	ns	ns
Quercetin-*O*-glycoside	7.54 ± 0.86	5.67 ± 0.80	6.25 ± 1.06	ns	6.88 ± 0.8 6	6.11 ± 0.61	ns	ns
C1	**4.69 ± 0.57 a**	**2.61 ± 0.38 b**	**3.21 ± 0.34 ab**	**<0.01**	3.65 ± 0.44	3.37 ± 0.33	ns	ns
C2	3.58 ± 0.73	3.06 ± 0.55	2.58 ± 0.30	ns	2.99 ± 0.48	3.16 ± 0.42	ns	ns
C3	1.59 ± 0.28	1.17 ± 0.15	1.20 ± 0.09	ns	1.41 ± 0.19	1.23 ± 0.12	ns	ns
Kaempferols								
Cis-trans-kaempferol 3-*O*-glucoside	**10.55 ± 1.35 a**	**7.55 ± 0.85 b**	**9.22 ± 1.12 ab**	**0.03**	9.50 ± 1.13	8.75 ± 0.68	ns	ns
Kaempferol 3,7-*O* -diglucoside	0.59 ± 0.07	0.41 ± 0.05	0.46 ± 0.04	ns	0.49 ± 0. 04	0.49 ± 0.05	ns	ns
C4	3.51 ± 0.37	3.20 ± 0.61	2.89 ± 0.29	ns	2.91 ± 0.24	3.49 ± 0.44	ns	ns
(B) Cell-wall compounds								
$UAC_{280-315}$	0.56 ± 0.02	0.58 ± 0.01	0.60 ± 0.01	ns	0.57 ± 0.01	0.59 ± 0.02	ns	ns
$UAC_{280-400}$	1.36 ± 0.06	1.42 ± 0.02	1.45 ± 0.03	ns	1.39 ± 0.02	1.43 ± 0.04	ns	ns
***p*-coumaric**	0.49 ± 0.02	0.43 ± 0.02	0.48 ± 0.01	ns	0.47 ± 0. 02	0.46 ± 0.01	ns	ns
Kaempferol derivative	0.74 ± 0.02	0.67 ± 0.042	0.74 ± 0.03	ns	0.72 ± 0.02	0.71 ± 0.03	ns	ns

Table VI *Overall means ± S.E. of total foliar phenolic content (mg AG equivalents g DM^{-1}), (A) methanol-soluble compounds (mg g DM^{-1}), and (B) methanol-insoluble cell-wall compounds (mg g DM^{-1}) of* Laurus nobilis *seedlings grown under three different UV conditions (ambient UV, enhanced UV-A, and enhanced UV-A+UV-B, N = 24) and two irrigation levels (WW = well-watered, LW = low-watered, N = 36). Different letters and values in boldface indicate statistical differences (p ≤ 0.05) in pairwise comparisons. UAC = UV-absorbing compounds; ns = not significant.*

Discussion

Effects of enhanced UV radiation and low irrigation on plant biomass and leaf gas exchange

Plant biomass accumulation is an integrated parameter used commonly as indicator of sensitivity to stressful conditions (Smith *et al*. 2000). In our experiment, enhanced UV radiation (UV-A and UV-A+UV-B) positively affected the biomass accumulation of *L. nobilis* seedlings, indicating that this sclerophyll species has effective mechanisms to deal with the increased levels of UV radiation. Our results also indicate that this UV-stimulation of growth is basically a response to enhanced UV-A radiation, since no significant differences were found between plants grown under enhanced UV-A radiation and those grown under UV-A+UV-B supplementation. Other studies have reported no effect of UV-B radiation on plant biomass accumulation (de la Rosa *et al*. 2001, Kostina *et al*. 2001, Hakala *et al*. 2002, Zaller *et al*. 2004, Bassman and Robberecht 2006, Wang *et al*. 2008), although a negative effect has also been reported in many studies, mainly on crop species (Yuan *et al*. 2002, Zavala and Ravetta 2002, Feng *et al*. 2003, Gao *et al*. 2003, Zheng *et al*. 2003, Yao *et al*. 2006, Kadur *et al*. 2007, Surabhi *et al*. 2009, Tsormpatsidis *et al*. 2010, Yao and Liu 2009). The effect of UV-A radiation on plant biomass has been studied much less than the effects of UV-B radiation. As it has also been found for UV-B, UV-A radiation appears to have a species-specific effect on plant growth. UV-A radiation negatively affected biomass in the roots of *Quercus robur* (Newsham *et al.* 1999) and total biomass in the cucumber (Krizek *et al*. 1997) but promoted overall growth in the radish (Tezuka *et al*. 1994). In the radish, the stimulation of growth in response to UV-A radiation was associated with an increase in the foliar content of chlorophylls, in the photosynthetic activity, and in the nitrogen metabolism. In the present study, however, plants grown under enhanced UV-A

radiation had lower chlorophyll content in leaves in relation to control plants (Table V).

Despite this general effect of UV-A radiation on growth, the beneficial effects of enhanced UV radiation levels on biomass accumulation were more pronounced in plants receiving limited water (Fig. 15). This suggests a positive interaction between UV radiation and the availability of water, which is in agreement with our previous study using seedlings of six Mediterranean species (Bernal *et al*. 2013). The exposure of plants to enhanced UV radiation may have mitigated the negative impact of low water availability on the water status of plants, since leaf RWC was not reduced in plants grown under UV-A or UV-A+UV-B supplementations but was reduced in the control plants in response to drier conditions (Fig. 16). In *Arabidopsis*, higher leaf water content in response to enhanced UV radiation and drought was attributed to the production of osmolytes and stress-related proteins such as dehydrins (Schmidt *et al*. 2000), an increase in proline content, and a decrease in stomatal conductance (Poulson *et al*. 2006). Nogués *et al*. (1998) also related the improvement in the water status of peas subjected to low water availability and enhanced UV-B radiation to a reduction in stomatal conductance. Furthermore, an increase in the thickness of needle cuticles in *Pinus pinea* in response to enhanced UV-B radiation was associated with reduced loss of water from cuticular transpiration and thus with an amelioration of water deficit in UV-B-irradiated plants (Petropoulou *et al*. 1995, Manetas *et al*. 1997). On the other hand, no effect of enhanced UV-B radiation on water status was reported for the Mediterranean species *Olea europea* L., *Rosmarinus officinalis* L. and *Lavandula stoechas* L. under drought conditions (Nogués and Baker 2000).

In the present study, an increase in leaf RWC in response to enhanced UV radiation under water shortage (Fig. 16) was not associated with a decrease in stomatal conductance (g_s) (Fig. 17). Instead, greater leaf thickness in UV-A+UV-B-irradiated seedlings may at least partially explain their higher foliar RWC, since thicker leaves have been associated with an improvement in water relations (Gitz and Liu-Gitz 2003, Ennajeh *et al*. 2010). As expected for plants with improved water status, UV-A- and UV-A+UV-B-irradiated plants tended to have higher foliar WUE than control plants under low irrigation, although the

differences were not significant (Fig. 17). Increases in foliar WUE in response to above-ambient levels of UV-B radiation have been mainly associated with significant reductions in foliar g_s (Gitz *et al*. 2005, Yang *et al*. 2008, Sangtarash *et al*. 2009a, b, Singh *et al*. 2011). In the present study, higher leaf WUE for plants exposed to enhanced UV-A and UV-A+UV-B radiation under water shortage could be due to the proportionally greater increases in foliar photosynthetic rates (86% and 30%, respectively) than in foliar g_s (Fig. 17). An improvement in WUE and in the water status of UV-irradiated leaves of *L. nobilis* under conditions of low water availability might account for their greater accumulation of biomass.

The role of photosynthetic pigments in the response of plants to enhanced UV radiation and low watering

The xanthophyll V- and Lx-cycles have been associated with thermal dissipation of excess light energy in the antenna of photosystem II (García-Plazaola *et al*. 2007), and, in particular, this has been reported for *L. nobilis* (Esteban *et al*. 2007 and 2008). In the present study, UV-A treatment affected both cycles. At predawn, laurel seedlings grown under enhanced UV-A radiation had higher levels of zeaxanthin than controls (Table V). Under low irrigation, they also had a higher de-epoxidation state of the V-cycle (Fig. 20), which suggests that these plants required more dissipation of light energy in the antenna. Similar results were found at midday, when UV-A-irradiated leaves had higher zeaxanthin content and AZ/VAZ index than UV-A+UV-B-irradiated ones (Table V). Under low irrigation, UV-A-irradiated plants also had the highest values of NPQ (Fig. 18). A lower leaf content of lutein-5,6-epoxide in this seedlings (Fig. 20) is also consistent with a higher thermal dissipation (Bungard *et al*. 1999, García-Plazaola *et al.* 2002, Llorens *et al*. 2002). Both xanthophyll cycles could thus have contributed to the dissipation of excess light energy in the antenna as a response to UV-A-supplementation. This response would also include a reduction in the foliar content of light-absorbing pigments (Table V, Fig.19), since UV-A-irradiated leaves of laurel had the lowest content of chlorophylls, neoxanthin, β-carotene, violaxanthin, and lutein, probably to avoid the

imbalance between light absorption and the energy required for photosynthesis (Munné-Bosch and Alegre 2000). Therefore, despite UV-A radiation being the most effective type of UV radiation for growth stimulation in laurel seedlings under limited water, these plants required different strategies for acclimation, such as the de-epoxidation of the xanthophyll cycles and a reduction in the amount of light-absorbing pigments to prevent cellular damage. Interestingly, when plants were also irradiated with UV-B radiation, these changes were not significant. In the case of the de-epoxidation of the V-cycle, this might be due to a UV-B-induced inhibition of the violaxanthin de-epoxidase, as it has been observed in isolated thylakoids and in intact leaves of pea (Pfündel *et al*. 1992). Yang *et al*. (2007) reported higher levels of violaxanthin and lower levels of zeaxanthin in leaves of wheat plants subjected to UV-B plus UV-A radiation relative to those irradiated only with UV-A radiation. In parallel, they found a stimulation of the components of the cellular antioxidant system, such as superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase, in UV-B+UV-A-supplemented plants. These authors suggested that in UV-B+UV-A-irradiated plants the endogenous antioxidant defence system is enhanced while in UV-A-irradiated ones is enhanced the thermal dissipation of light energy.

Response of specific phenolic compounds to enhanced UV radiation and low irrigation

An increase in foliar UACs, especially phenols, is a response mechanism to contend with UV radiation enhancement (Julkunen-Tiitto *et al*. 2005, Caldwell *et al*. 2007). However, in the present study, the experimental increases in UV radiation did not change the foliar content of UACs or total phenolic compounds in *L. nobilis* seedlings (Table VI), in agreement with previous studies (Bernal *et al*. 2013). A lack of UV-induced effects on the total foliar content of phenolic compounds is common in Mediterranean species, because their adaptations to high light intensities and drought stress allow them to maintain a large constitutive pool of these compounds (see Paoletti 2005 for a review). However, plants grown under increased UV-A radiation, had lower concentrations of

quercetin and kaempferol derivatives in the vacuoles than control plants (Table VI). The presence of similar amounts of these compounds in UV-A+UV-B- and UV-A-supplemented plants indicate that UV-B supplementation did not exert an additional downregulation of the foliar accumulation of these compounds (Table VI). The higher need for energy dissipation in water-limited plants grown under enhanced UV-A radiation would suggest a possible photo-oxidative stress. However, these plants had the lowest levels of quercetins and kaempferols, which are expected to increase under oxidative stress (Pollastri and Tattini 2011). In agreement with our results, Wilson *et al.* (2001) reported a general downregulation of extractable flavonoids mediated by UV-A radiation in *Brassica napus*. Decreases in quercetin glycoside under enhanced UV radiation might be due to a UV-mediated breakdown of quercetin (Falhman and Krol 2009). Nevertheless, the photoprotective mechanisms detected in UV-A-irradiated plants (i.e. a reduction in the leaf amount of light-harvesting pigments and an increase in the dissipation of excess energy as heat) may lower the production of ROS and thus the requirement for antioxidant compounds, which would be consistent with the low levels of β-carotene found in leaves from UV-A-irradiated plants. Moreover, it cannot be disregarded a decrease in the levels of these compounds (due to structural changes) once they have acted as antioxidants. It is evident that *L. nobilis* has the ability to use a range of different mechanisms to cope with enhanced UV-A and UV-A+UV-B radiation.

In conclusion, an interactive effect between UV radiation and drought on biomass production and morphological and physiological traits in seedlings of the sclerophyll *L. nobilis* was found. Specifically, UV radiation increased plant growth and improved leaf water status under water shortage. Results suggest that, when seedlings are water-limited, UV-A supplementation could ameliorate water deficit resulting in higher leaf RWC, stomatal conductance, photosynthetic rates, and growth. Results also suggest that UV-A-supplemented plants when grown under low water availability had an excess of light, because they activated photoprotective mechanisms such as the de-epoxidation of the xanthophyll cycle for thermal dissipation of this energy. Accordingly, these plants had higher NPQ values, which would indicate a greater dissipation of

energy as heat, and lower foliar contents of light-harvesting pigments, which would reduce the absorption of light by the antennae. Additional exposure to UV-B radiation seemed to counteract the effects of UV-A radiation in terms of the photoprotective mechanisms studied without changing the beneficial effects of this radiation on growth. This indicates different responses to UV-A and UV-B radiation. UV-B radiation is likely to have induced photoprotective mechanisms other than those addressed in this study, such as the biosynthesis of enzymes related to the scavenging of oxygen radicals or to the repair of DNA and/or proteins (Hollósy 2002). The research presented here highlights the importance of considering UV-A radiation when investigating the effects of UV-B radiation, because both types of radiation may affect plant performance differently and may thus interact to influence overall plant response.

Chapter IV. Altitudinal and seasonal changes of phenolic compounds in *Buxus sempervirens* leaves and cuticles

The main content of this chapter has been submitted to Plant physiology and biochemistry as: Bernal M, Llorens L, Julkunen-Tiitto R, Badosa J, Verdaguer D. Altitudinal and seasonal changes of phenolic compounds in *Buxus sempervirens* leaves and cuticles.

Introduction

The amount of UV-B radiation reaching the Earth's surface depends on many factors, among them, ozone, clouds, aerosols and surface albedo (Bais *et al.* 2007). Levels of UV-B radiation also change seasonally and altitudinally (McKenzie *et al.* 2001, Pfeifer *et al.* 2006). Indeed, while the highest levels of UV-B in European ecosystems are recorded in summer, especially in June, the lowest are in winter (Seckmeyer *et al.* 2008). In addition, while total irradiance and UV-A radiation increase on average 8 ± 2% and 9 ± 2% per 1000 m of elevation, respectively, UV-B radiation increases 18 ± 2% (Blumthaler *et al.* 1997). Consequently, the altitudinal migration of plant species in response to global warming (Peñuelas *et al.* 2007, Parolo and Rossi 2007) will expose them to higher doses of UV radiation, especially UV-B. Taking into account that climate models predict increases in the UV-B levels for the Mediterranean Basin in summer (McKenzie *et al.* 2011), it is essential to improve our knowledge on how Mediterranean and sub-Mediterranean plant species respond to the altitudinal and seasonal changes in UV-B radiation in order to predict their capacity to cope with future increases in UV-B radiation.

Ultraviolet-B radiation is the most energetic component of sunlight reaching the Earth's surface and can be a photo-oxidative stress factor for plants that affects their physiological activity and morphology (e.g. Stratmann 2003, Caldwell *et al.* 2007). Among plant responses to increases in UV-B radiation, there are changes at the morphological (e.g. increases in leaf thickness and specific leaf weight or decreases in plant height) and biochemical levels (e.g. the synthesis of phenolic compounds) (Paoletti 2005, Caldwell *et al.* 2007). It is well known that a general response to enhanced levels of UV-B radiation is the biosynthesis of phenolic compounds (e.g. Julkunen-Tiitto *et al.* 2005), since they can help filter UV radiation avoiding or minimizing penetration of UV-B into internal tissues (Krauss *et al.* 1997). Phenolic compounds can also act as antioxidants by scavenging the free radicals produced under oxidative stress (e.g. Treutter 2010). Therefore, variations in

the leaf phenolic content of plants have been related to the natural changes in UV-B radiation of the environment in which plants grow. Hence, higher leaf phenolic content is expected with increasing altitudes (Rozema *et al.* 1997, Filella and Peñuelas 1999, Yang *et al.* 2005, Alonso-Amelot *et al.* 2007, González *et al.* 2007, Roblek *et al.* 2008, Murai *et al.* 2009, Wong *et al.* 2009, Sudeshna and Swati 2011, Kaur *et al.* 2012) or in the seasons with the highest UV-B irradiance (Stephanou and Manetas 1997, González *et al.* 2007, Núñez-Olivera *et al.* 2009), although a compound-specific variation has been reported (Solar *et al.* 2006). However, altitudinal or seasonal variations in leaf phenols have also been related to other environmental factors, such as temperature (Bilger *et al.* 2007, Albert *et al.* 2009, Zidorn 2010), edaphic properties (texture and nutritional soil parameters) (Duarte *et al.* 2012) or water availability (Alonso-Amelot *et al.* 2007). Some previous studies have also suggested an ontogenetic effect more than an environmental effect on the total amount of UV-absorbing compounds in Mediterranean (Liakoura *et al.* 2001) or sub-Mediterranean (Sommavilla *et al.* 2012) species. Therefore, one of the goals of our study was to investigate whether the seasonal and altitudinal changes in the leaf content of phenolic compounds in the common box (*Buxus sempervirens* L.) could be attributed to the natural changes in levels of UV-B radiation.

Phenolic compounds belong to a large family of secondary metabolites, which can be involved in many functions ranging from plant growth and development to plant defense. The most common phenolics in nature are the phenylpropanoids, which are derived from phenylalanine (see Vermerris and Nicholson 2008 for a review). Several phenylpropanoids, like cinnamic acids (e.g. *p*-coumaric, ferulic and caffeic acids) and flavonoids, have been proposed as the phenols with the highest absorption in the UV radiation range (Julkunen-Tiitto *et al.* 2005). While cinnamic acids only absorb in the UV radiation range and, thus, their main function seems to be the screening of UV radiation (Agatti and Tattini 2010), flavonoids also absorb in the blue to green region of the visible spectrum. Although the predominant role of flavonoids is photoprotection (Treutter 2006), they have a broad range of functions in plants as, for instance, in flower coloration, plant defense and signaling (Treutter 2010). Flavonoids with a catechol group in the B-ring, such as quercetin and luteolin derivatives, are among the most effective antioxidant compounds and, hence, they

have been reported to increase under supplemental UV-B radiation (Markham *et al.* 1998, Tegelberg and Julkunen-Tiitto 2001, Edreva *et al.* 2005).

Since the leaf cuticle is the first barrier to UV-B radiation, it is expected that plants accumulate there high amounts of UV-B-absorbing compounds (UACs) in response to UV-B exposure. Krauss *et al.* (1997) found that the leaf cuticles of 27 woody species effectively attenuated radiation in the UV-B range of the solar spectrum due to their phenol content, especially hydroxycinnamic acids. Hydroxycinnamic acids are covalently attached to the cutin polymer and/or epicuticular waxes (Riley and Kolattukudy 1975, Liakopoulos *et al.* 2001). Since cutin is a highly resistant biopolymer well preserved in the geological record, the determination of the content of certain UACs, such as hydroxycinnamic acids, in fossil plant cuticles might allow the reconstruction of past levels of UV-B radiation (e.g. Rozema *et al.* 2009, Willis *et al.* 2011). Nevertheless, the use of the cuticle content of certain UACs as biomarkers of solar UV-B fluxes requires prior knowledge of the species-specific responses of these compounds to UV-B radiation because different patterns have been found depending on the species and compounds involved (Kotilainen *et al.* 2010, Neugart *et al.* 2012).

Consequently, the aims of this study were: a) to identify and quantify the phenolic compounds in *Buxus sempervirens* leaves and cuticles, b) to investigate the seasonal and altitudinal changes in these compounds and whether any variations found can be related to the natural fluctuations in UV-B radiation, and c) to elucidate the possible use of specific phenolic compounds of leaf cuticles as bioindicators of ambient UV-B radiation. To achieve these goals we sampled leaves of *B. sempervirens* every three months from June 2010 till March 2011 along an altitudinal gradient (from 441 to 1750 m). Since other abiotic factors, such as temperature, water or nutrient availability usually change with elevation and can influence plant responses (Zidorn 2010), we conducted a UV-exclusion experiment in two of the four localities selected (the lowest and the highest) to help us to discern whether any differences found along the seasonal and/or altitudinal UV-B gradient could be attributed to UV-B radiation.

We focused our study on *B. sempervirens* because it is an evergreen schlerophyllous shrub widely distributed in the sub-Mediterranean region that can be

found along a wide elevational gradient (from 400 to 2000 m). In addition to its ecological relevance, *B. sempervirens* is a species of economical importance since it is used as an ornamental plant, its wood has been historically commercialized, and leaf extracts have also been traditionally important due to their medicinal properties (Orhan *et al.* 2012). To our knowledge, this is the first study where leaf and cuticle levels of specific phenols have been quantified seasonally in a species growing along an altitudinal gradient in combination with an experimental approach.

Materials and methods

Study site, target species and sampling regime

Leaves of *Buxus sempervirens* L. were collected every three months from June 2010 to March 2011 at four localities distributed along an altitudinal gradient in the Girona province (Northeast of Spain). The sampling sites selected were: Llémana (441 m above the sea level; 42°03' N, 2°39' E), Campdevànol (792 m a.s.l.; 42°14' N, 2°10' E), Taga (1319 m a.s.l.; 42°15' N, 2°14' E) and Tosses (1750 m a.s.l.; 42°20' N, 2°04' E) (see map in Annex I). The vegetation at all the sites is an open south-east orientated montane scrub with *B. sempervirens* being the dominant species. Soils at all the localities were calcareous. For each site, we chose six individual plants of *B. sempervirens* (separated by 2-10 m from each other) and marked them to allow re-sampling. Samplings were performed at the end of June, September and December 2010, and at the beginning of March 2011. All the sampled leaves had developed during 2010, were fully expanded and belonged to the south part of the plant canopy, being totally sun exposed. In June, due to the altitudinal delay in leaf ontogeny, leaves from 1319 m and 1750 m were sampled one and three weeks later respectively, than those of the lowest localities. On the rest of the seasons we did not have this temporal variability because, as all the sampled leaves had been already grown, the sampling days were more or less consecutively.

Measurements for the month of the study were collected from nine nearby meteorological stations belonging to the Catalan Meteorological Service (SMC, www.meteo.cat) to estimate the monthly mean temperature of each sampling site (Table VII). Stations were selected based on their altitude (between 150 and 2000 m a.s.l.) and their proximity to the study sites (maximum distance: 25 km) (see Annex I). Mean values of temperature for each sampling site were estimated monthly using the equations obtained by means of linear regressions between altitude and temperature. UV-B radiation received at each site assuming clear sky conditions (Table VII) were estimated using the SMARTS2 model (Gueymard 1995); as inputs, daily total ozone column estimations (Ahmad *et al.* 2003), and climatic aerosol optical depth (using the method from Badosa *et al.* 2005) were considered. Monthly mean values from the computed daily UV-B radiation were calculated for each one of the sampling months and sites (Table VII).

UV-exclusion at the lowest and the highest localities of the altitudinal gradient

Fig. 21 Experimental set-up in one of the studied plants of B. sempervirens *at Llémana (441 m a.s.l.).*

At the lowest and the highest localities (Vall de Llémana and Tosses), a UV exclusion treatment with three different UV conditions was established in five of the six selected plants using plastic films that filter different parts of the UV spectrum. Nine branches (three branches per UV condition) from each plant were surrounded with wire frames supporting different filters (20 x 20 cm in size), leaving the back and front sides open for ventilation (Fig. 21). The three UV radiation conditions were: UV-B exclusion (the filter used was Mylar D, 0.13 mm; PSG Group, England), UV-A+UV-B exclusion (the filter used was Ultraphan URUV 0.95 mm thick; Digefra, Germany)

and Control, in which case the filter allowed the penetration of both, UV-A and UV-B radiation (Teflon, 0.05 mm thick; Nowofol GmbH&Co, Germany). Hereafter, this different conditions are named as UV-A, UV-0 and UV-A+UV-B, respectively. The frames with the filters were placed on the plants from mid-July 2010 until mid-March 2011 at the south-facing side of each shrub. All of the plastic films were replaced in mid-October 2010 to avoid filter degradation effects. Leaves from the different UV conditions were sampled in September 2010 and March 2011. In order to account for any effects of the experimental structure, we also sampled leaves from outside the treated branches but on the same plants.

	Air temperature (ºC)				UV-B (kJ m^{-2})			
	June	Sept.	December	March	June	Sept.	December	March
Llém	18.3 ± 0.5	17.6 ± 0.4	4.6 ± 1.2	8.6 ± 0.3	37.6 ± 0 .5	23.4 ± 0.6	4.9 ± 0.3	16.7 ± 0.5
Camp	16.1 ± 0.6	15.7 ± 0.5	3.2 ± 1.5	6.8 ± 0.3	39.1 ± 0 .5	24.5 ± 0.6	4.8 ± 0.2	17.2 ± 0.6
Taga	12.9 ± 0.7	12.7 ± 0.7	1.0 ± 2.0	3.9 ± 0.4	41.0 ± 0 .5	25.6 ± 0.7	5.0 ± 0.2	17.9 ± 0.6
Toss	10.3 ± 0.9	10.4 ± 0.8	-0.8 ± 2.4	1.6 ± 0.5	42.7 ± 0.6	26.5 ± 0.7	5.1 ± 0.2	18.7 ± 0.7

Table VII *Mean air temperature and daily UV-B radiation doses for the months and the localities studied (Llem = Llémana, Camp = Campdevànol, Taga = Taga and Toss = Tosses) assuming clear sky conditions.*

Determination of leaf relative water content and leaf morphological traits

At each sampling date in June, September and December 2010 and March 2011, one leaf was removed from three non-treated branches from each one of the six plants per locality. At the highest and the lowest localities, from each one of the five plants with the experimental set-up, we also sampled three leaves per UV condition (one leaf from each branch) in September 2010 and March 2011. Leaf thickness was measured at three different points on each leaf using a micrometer (mod. 4000 DIG, Baxlo, Spain). To estimate the leaf relative water content (RWC, %), fresh leaves were weighed (FW) and subsequently re-hydrated until they reached their turgid weight (TW) by leaving them in distilled water for 48 h in darkness. After weighing re-hydrated leaves, they were oven-dried at 70 ºC for 72 h and reweighed to obtain the dry weight (DW). Leaf RWC (%) was calculated as (FW-DW/TW-DW) x 100. To determine the leaf mass per area (LMA, mg cm^{-2}), the perimeter of each leaf was

drawn after recollection and, once the images were scanned, the leaf area was measured by means of an image processing program (Image tool, University of Texas Health Science Center, USA). Leaf mass area was calculated for each leaf as the quotient between leaf dry weight and leaf area.

Chemical analysis and identification of leaf phenolic compounds

In June, September, December and March, we sampled, with a cork-borer, six discs of 6 mm of diameter (avoiding the central vein) per plant from five of the six plants per altitude. The six discs were obtained from two leaves per branch from three non-treated branches. At the highest and the lowest localities, in September and March, two leaf discs were taken from each one of the three different branches per UV condition and plant. Discs were immediately frozen in liquid N_2 and, once in the lab, they were stored at -80 °C until analyzed.

For chemical analysis, leaf discs from the same plant or from the same UV condition within each plant were pooled (approximately 20 mg of fresh weight) and homogenized with a Precellys homogenizer (Bertin Technologies, Bretoneux, France) with 0.6 ml cold methanol (HPLC grade) for 20 s. After homogenization, samples were left in an ice bath (at 4 °C) for 15 min and then centrifuged (13000 rpm for 3 min; Centrifuge 5415R, Hamburg, Germany). The supernatant was decanted into a 6 ml glass tube, and the pellet was homogenized three times more in 0.5 ml of methanol and then left in an ice bath for 5 min. The methanol of combined extracts was dried in a Rotavapor (Concentrator Plus, Hamburg, Germany) and dried samples stored at 4 °C until analysis. The samples were dissolved in MilliQ-water:methanol (1:1) and phenols were analyzed by high performance liquid chromatography (HPLC).

The HPLC system used for the analysis (Agilent 1100 Series HPLC, Germany) consisted of a quaternary solvent delivery system, an autosampler and a photodiode array detector coupled with an HP data system and a PC. Auto-injected samples of 10 µL in volume were separated on a HP Hypersil ODS column (4.6 x 60 mm, 3 µm particles), with the column temperature being 28 °C. The eluent flow was 2 mL min^{-1} of the elution solvents A (aqueous 1.5% tetrahydrofuran and 0.25% orthophosphoric acid) and B (MeOH) and the elution gradient was set as described

in Julkunen-Tiitto and Sorsa (2001). Runs were monitored at 220, 270, 320 and 360 nm. Quantification was based on commercial standards: gallic acid (Aldrich, Steinheim, Germany) for the digalloylglucose and pentagalloylglucose; chlorogenic acid (3-caffeoylquinic acid) (Aldrich, Steinheim, Germany) for the caffeic acid, cinnamic acid derivatives and chlorogenic acid; isorhamnetin 3-glucoside (Extrasynthese, Genay Cedex, France) for quercetine derivatives, isorhamnetin-dirhamoside+Kaempferol 3-glucoside and isorhamentin-rhamnoside; salicin (Aldrich, Steinheim, Germany) for neolignan and the unknown, and luteolin (Roth, Lauterbourg, Germany) for luteolin derivative. The identification of compounds was based on comparisons of retention times, the spectral characteristics and UHPLC-qtof-MS. UHPLC-DAD (Model 1200 Agilent Technologies) JETSTREAM/QTOFMS (Model 6340 Agilent Technologies) was equipped with a 2.1 x 60 mm, 1.7 µm C_{18} column (Agilent Technologies). Solvent A was 1.5% tetrahydrofurane and 0.25% acetic acid in HPLC quality water and the solvent B was 100% methanol. A gradient run was as follows: from 0 to 1.5 min, B 0%; from 1.5 to 3 min, 0 to 15% B; from 3 to 6 min, 10 to 30% B; from 6 to 12 min, 30 to 50% B; from 12 to 20 min, 50 to 100%; and from 20 to 22 min, 100 to 0% B. The flow rate was 0.4 mL min^{-1} and the injection volume 0.2 µL. The UHPLC injector and oven temperature was 22 and 30 ^{o}C, respectively. The mass spectra were acquired at positive and negative ion mode depending on the compounds and the mass range was from 100 to 3000 *m/z*. The temperature of the drying gas and sheath gas was 350 ^{o}C, and flow rates 12 L min^{-1} and 11 L min^{-1}, respectively. The following parameters were used: nebulizer pressure 35 psi, capillary voltage 3500 V, nozzle voltage 1000 V, fragmentor voltage 80 V, skimmer voltage 65 V and octopole voltage 750 V. The reference mass *m/z* 922.0098 was used for accurate mass measurements.

Chemical analysis and identification of leaf cuticle phenolic compounds

At each sampling date (June, September and December 2010 and March 2011), two leaves per branch from three non-treated branches of each one of the six selected plants per altitude were sampled for cuticle isolation, and extraction and subsequent HPLC analyses of phenolic compounds. In September 2010 and March 2011 at the highest and the lowest localities, two leaves from each of the three UV-treated

branches per plant (five plants per locality) were also sampled to analyze UV radiation treatment effect on the content of phenolic compounds in leaf cuticles. Leaves were immediately frozen in liquid N_2 and stored at -80 °C until cuticle isolation.

Cuticle isolation

From each leaf, discs of 6 or 8 mm diameter (depending on leaf size) were taken and the cuticles isolated following the procedure described by Schönherr and Riederer (1986). After marking the abaxial side with a permanent marker, leaf discs were placed in a 15 mL falcon tube with an aqueous mixture of 2% (v/v) cellulose (SIGMA C2730), 2% (v/v) pectinase (SIGMA P2611), 0.96% citric acid (1M) at pH 3 and 0.1% sodium azide (1M NaN_3) and kept at room temperature with agitation for 72 h. The water solution in the falcon tube was changed after 36 h in order to accelerate the enzymatic digestion process. NaN_3 was added to prevent growth of microorganisms. After 72 h of enzyme incubation, isolated cuticles were recovered, washed with distilled water and left with borate buffer (pH 9) 0.02 M during 24 h to remove cellular debris. Then, they were washed three times with deionized water, air-dried at room temperature, and cuticles from the adaxial surface of leaves (the non-marked ones) were collected in aluminum bags, and stored at 4 °C until phenolic extraction.

Phenolic extraction, analyses and identification

Cuticle discs from the same plant or from the same UV condition within each plant were pooled (total dry weight varied from 3 to 7 mg) and their phenolic compounds were extracted and analyzed following the procedure outlined above for leaves. The injection volume for cuticle samples was 60 µL and running time was extended to 70 min (A+B both 50% from 50 to 70 min). Quantification of phenols was based on the commercial standards: isorhamnetin-3-glucoside (Extrasynthese, Genay Cedex, France) for quercetin and rhamnetin derivatives; protochatechuic acid (Phytolab, Vestenbergsgreuth, Germany) for protochateuic acid; eryodictiol 7-glucoside (Phytolab, Vestenbergsgreuth, Germany) for eryodictiol; and apigenin

(4.5,7-trihydroxyflavone) (Phytolab, Vestenbergsgreuth, Germany) for apigenin and apigenin+luteolin.

Statistical analyses

For non-treated leaf samples seasonal patterns of variables measured were analyzed by means of repeated measures analysis of variance (ANOVAR) with altitude as the between-subjects factor. Tukey pairwise post-hoc comparisons were used to identify significant differences among altitudes. The effect of altitude within each sampling date was analyzed by means of one-way analyses of variance (ANOVA). When data did not meet the assumption of homoscedasticity, we used the Dunnet T3 post-hoc test for non-homogeneous data. Principal component analyses (PCA) were done separately for each sampling date using the leaf or cuticle content of the different phenolic compounds. All PCAs were done without rotation. Contribution of the different phenols to the new factors created by the PCA was considered significant when factor loadings were greater than or equal to 0.7. Component scores (the values of individual cases for the new factors) obtained by means of each PCA were used to ordinate data for the different altitudes studied. To analyze the effects of our UV-exclusion experiment on the variables measured two-way ANOVA was used for each sampling date (September 2010 and March 2011) with treatment and altitude as factors.

Results

Leaf relative water content and morphological traits

On average, the leaf RWC of plants was 79.54 ± 0.64%, with no significant differences among altitudes or sampling dates (Fig. 22) or among UV radiation conditions (Table VIII). Regarding leaf morphological traits, in June, the leaf mass per area (LMA) at the two lowest localities (441 and 792 m) was higher than at the

two highest ones (1319 and 1750 m), basically due to a greater leaf thickness (Fig. 22). Conversely, leaves from the lowest locality showed the lowest LMA in December and the lowest leaf thickness at the last sampling date (March 2011) (Fig. 22). Leaf area tended to be greater at the two lowest localities, although differences were only significant in September and March (Fig. 22). Neither leaf RWC, thickness, area nor LMA were significantly affected by the UV radiation treatment applied (Table VIII).

Parameters	Altitude (m)	September			March		
		UV-0	UV-A	UV-A+UV-B	UV-0	UV-A	UV-A+UV-B
RWC (%)	441	78.5 ± 3.0	76.4 ± 0.8	74.5 ± 3.1	77.5 ± 3.8	85.1 ± 6.0	74.0 ± 2.4
	1750	84.0 ± 1.8	83.3 ± 1.8	83.7 ± 1.8	72.0 ± 4.3	66.3 ± 3.7	75.0 ± 2.7
LMA (mg cm^{-2})	441	36.3 ± 2.2	36.8 ± 1.5	38.7 ± 0.7	49.3 ± 1.7	50.9 ± 4.0	53.2 ± 2.2
	1750	34.8 ± 1.2	37.4 ± 1.9	35.9 ± 1.1	53.5 ± 1.6	54.4 ± 1.5	52.6 ± 1.6
Leaf thickness (mm)	441	0.52 ± 0.04	0.48 ± 0.02	0.50 ± 0.04	0.42 ± 0.01	0.42 ± 0.02	0.44 ± 0.02
	1750	0.52 ± 0.02	0.58 ± 0.04	0.50 ± 0.02	0.49 ± 0.02	0.48 ± 0.03	0.47 ± 0.01
Leaf area (cm^2)	441	2.1 ± 0.2	2.2 ± 0.3	2.2 ± 0.2	1.6 ± 0.2	1.5 ± 0.3	1.5 ± 0.2
	1750	1.9 ± 0.1	2.1 ± 0.2	2.0 ± 0.1	1.3 ± 0.1	1.3 ± 0.2	1.4 ± 0.1

Table VIII *Leaf RWC and morphological traits of UV-treated leaves of* B. sempervirens *for the two altitudes studied in September 2010 and March 2011. Values are means ± S.E. (N = 5).*

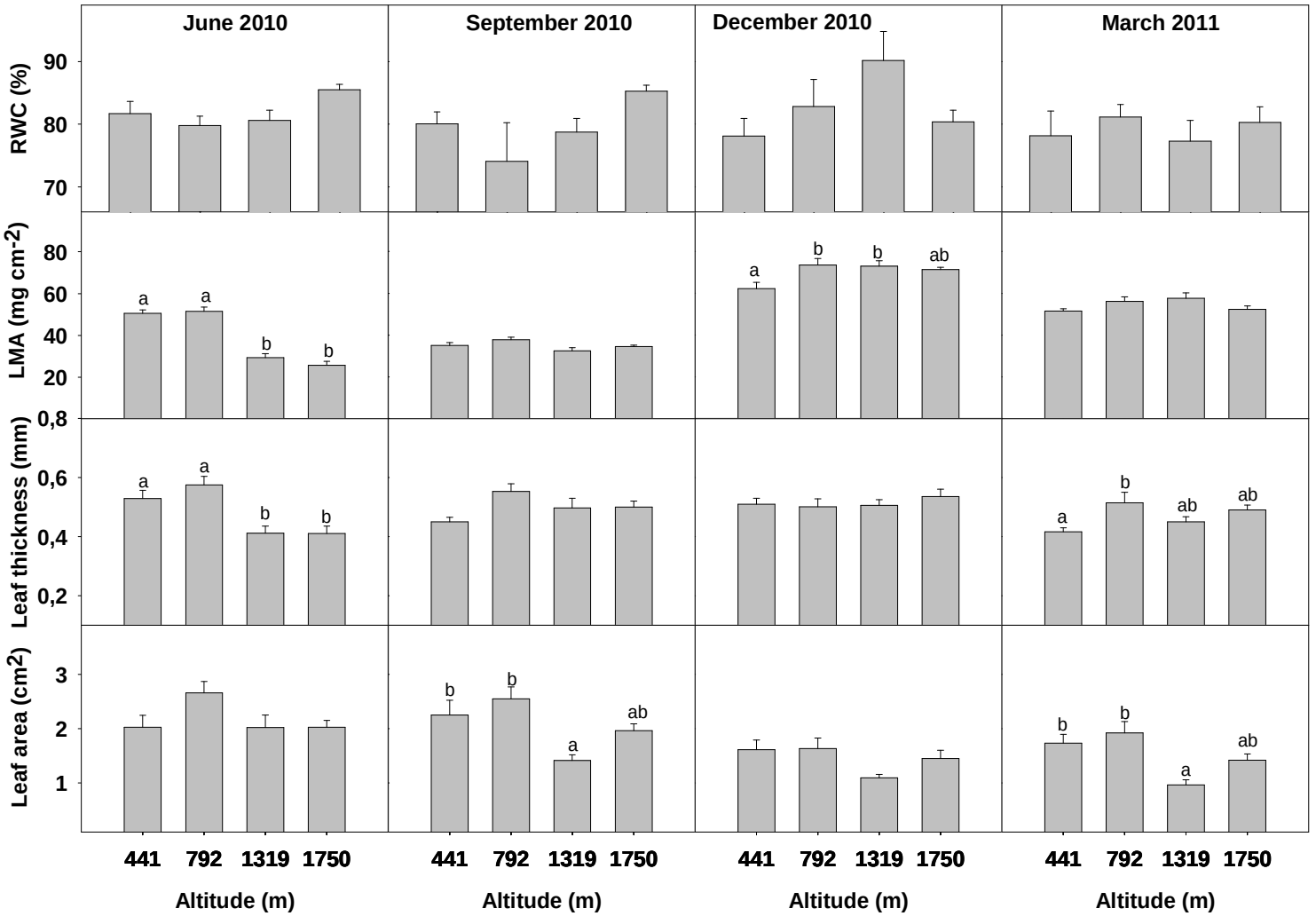


Fig. 22 Mean values ± S.E. (N = 6 plants) of relative water content (RWC), mass per area (LMA), thickness and area of non-treated leaves of B. sempervirens *for each sampling date and altitude. The four studied localities were: Llémana (441 m), Campdevànol (792 m), Taga (1319 m) and Tosses (1750 m).*

Phenolic compounds in leaves

A total of 19 phenolic compounds were detected in *Buxus sempervirens* leaves, which we grouped into four classes: flavonoids, gallotannins, neolignans and phenolic acids (Annex II). Six flavonoids were detected (Table IX): two quercetins (quercetin 3-arabinoside and quercetin-aglycon), one kaempferol (kaempferol 3-glucoside), one luteolin derivative and two flavonoids that have been tentatively identified by qtof-MS analysis as glycosylated flavonols with rhamnose (isorhamnetin-dirhamnoside and isorhamnetin-rhamnoside). Since peaks corresponding to isorhamnetin-dirhamnoside and the kaempferol 3-glucoside were overlapping, they were quantified together using isorhamnetin 3-glucoside as a standard. For gallotannins, we found only two derivatives, digalloylglucose and pentagalloylglucose, the first one being the most abundant (Table IX). We also detected two neolignans, although one of them could not be quantified due to its low amount (Fig. 23). Among the eight phenolic acids identified, six of them were derivatives of cinnamic acid while the other two were chlorogenic acid and a derivative of caffeic acid (Table X). Finally, another phenolic compound was detected and quantified, which could not be identified and is referred hereafter as 'unknown' (Table IX).

Seasonal effects

In *B. sempervirens* leaves, the total content of phenolic compounds (the sum of all the phenols quantified) was significantly lower in June (48.44 ± 2.37 mg g DW^{-1}) than in September (67.46 ± 3.05 mg g DW^{-1}) ($F_{1,5}$ = 18.52, p = 0.001), but it did not change significantly among the other sampling dates (Fig. 23). This difference between June and September was basically due to the increase in the leaf content of the unknown compound ($F_{1,5}$ = 31.21, p < 0.001, Table IX). The overall leaf content of gallotannins also increased throughout the study period (Fig. 23), with a marked increase in digalloylglucose derivative between December and March ($F_{1,5}$ = 59.99, p < 0.001, Table IX). Foliar contents of flavonoids and phenolic acids were similar between June and September, but some flavonoids (such as quercetin 3-arabinoside and isorhamnetin-rhamnoside) and the neolignan were more abundant in the first

than in the second sampling date ($F_{1,5}$ = 31.87, p < 0.001, Fig. 23). Moreover, as total phenols, the overall leaf content of cinnamic acid derivative 2 was lower in June than in September ($F_{1,5}$ = 12.29, p = 0.003, Table X). The caffeic acid derivative increased from June to March (being the means ± S.E. in mg g DW^{-1} with the four altitudes pooled: June: 0.70 ± 0.13, September: 1.06 ± 0.27, December: 1.33 ± 0.29 and March: 1.43 ± 0.31), while the greatest overall amount of chlorogenic acid was found in September (Table X).

Altitudinal effects

Even though the leaf total content of phenolic compounds did not show a consistent response to the altitudinal gradient (Fig. 23), the leaf content of flavonoids was higher at the highest locality than at the two lower ones in June (Fig. 23). More specifically, in this first sampling, quercetin 3-arabinoside, quercetin-aglycon and isorhamnetin-rhamnoside showed significant increases with altitude (Table IX). The leaf content of quercetin 3-arabinoside also increased significantly with altitude in March (Table IX). For total amounts of gallotannins, differences among altitudes were also significant in June, when leaves from the two lowest altitudes showed the lowest amounts of these compounds, although differences were only significant in relation to leaves from 1319 m (Fig. 23). The overall leaf content of phenolic acids also increased with altitude ($F_{3,5}$ = 6.06, p = 0.006), particularly in June and December (Fig. 23). Among phenolic acids, the altitudinal gradient was significantly related to the leaf content of caffeic acid derivative ($F_{3,5}$ = 8.59, p = 0.001) and cinnamic acid derivative 1 ($F_{3,5}$ = 6.10, p = 0.006). In June, the leaf content of cinnamic acid derivatives 3 and 4, as well as the total leaf content in cinnamic acids, also increased significantly along the altitudinal gradient (Table X). The leaf content of neolignan also increase with altitude ($F_{3,5}$ = 7.80, p = 0.002).

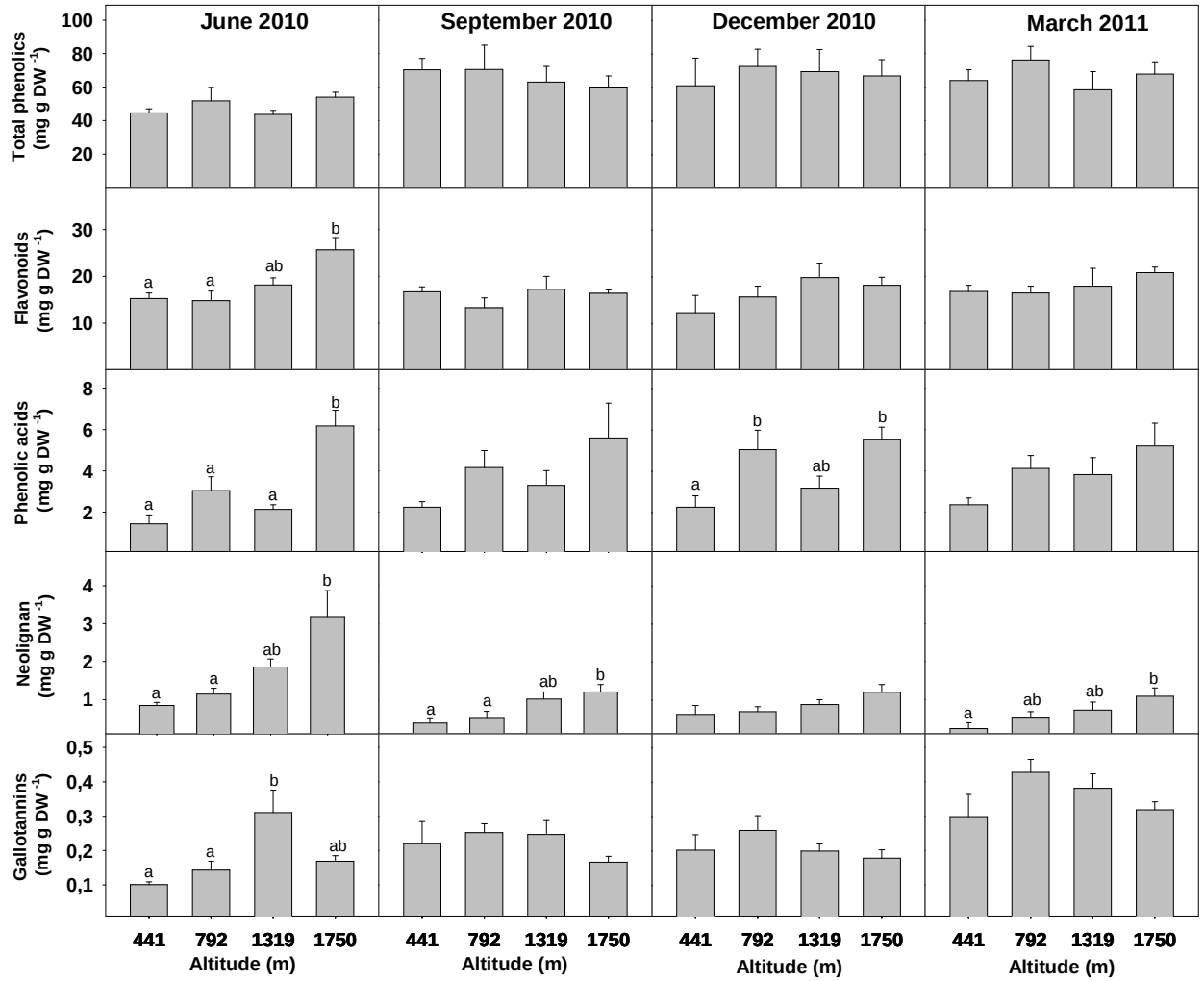


Fig. 23 Leaf content (mg g DW^{-1}) of total phenolic compounds, flavonoids, phenolic acids, neolignan and gallotannins in B. sempervirens *for each sampling date and altitude (values are means ± S.E. of five plants). Different letters indicate significant differences ($p \leq 0.05$) among altitudes within each sampling date.*

	Altitude (m)	Flavonoids					Unknown	Gallotannins	
		Quercetin 3-arabinoside	Quercetin-aglycon	Isorhamnetin-dirhamnoside+kaempferol 3-glucoside	isorhamnetin-rhamnoside	Luteolin der.		Digalloylglucose	Pentagalloylglucose
June	441	**0.43 ± 0.12 a**	**0.25 ± 0.11 a**	5.68 ± 0.80	**8.88 ± 0.68 a**	0.02 ± 0.02	26.87 ± 2.32	0.09 ± 0.01	**0.01 ± 0.006 a**
	792	**0.66 ± 0.22 a**	**0.28 ± 0.08 a**	3.70 ± 0.27	**10.18 ± 1.53 a**	0.00 ± 0.00	32.60 ± 6.70	0.11 ± 0.03	**0.03 ± 0.008 a**
	1319	**1.40 ± 0.46 ab**	**0.63 ± 0.15 ab**	3.52 ± 0.54	**12.57 ± 1.04 ab**	0.00 ± 0.00	21.20 ± 1.55	0.21 ± 0.07	**0.10 ± 0.008 b**
	1750	**2.49 ± 0.66 b**	**0.76 ± 0.11 b**	5.78 ± 0.73	**16.61 ± 1.87 b**	0.00 ± 0.00	18.76 ± 1.97	0.15 ± 0.02	**0.02 ± 0.02 a**
September	441	0.58 ± 0.24	0.24 ± 0.08	6.58 ± 0.75	8.90 ± 0.62	**0.41 ± 0.05 ab**	50.65 ± 7.25	0.17 ± 0.05	0.05 ± 0.02
	792	0.42 ± 0.15	0.11 ± 0.05	5.29 ± 0.61	7.26 ± 1.52	**0.21 ± 0.03 a**	52.22 ± 12.99	0.22 ± 0.02	0.03 ± 0.01
	1319	1.07 ± 0.23	0.19 ± 0.06	5.84 ± 1.35	9.42 ± 1.13	**0.73 ± 0.12 b**	41.10 ± 8.09	0.21 ± 0.03	0.03 ± 0.01
	1750	0.89 ± 0.10	0.20 ± 0.03	6.64 ± 0.43	8.50 ± 0.51	**0.15 ± 0.05 a**	36.74 ± 6.07	0.15 ± 0.01	0.02 ± 0.01
December	441	0.75 ± 0.29	0.22 ± 0.07	4.28 ± 1.17	6.40 ± 2.11	**0.58 ± 0.14 ab**	45.48 ± 12.67	0.16 ± 0.04	**0.04 ± 0.01 b**
	792	0.96 ± 0.33	0.13 ± 0.06	5.76 ± 0.45	8.09 ± 1.56	**0.61 ± 0.07 ab**	50.79 ± 8.41	0.22 ± 0.04	**0.04 ± 0.003 b**
	1319	1.76 ± 0.42	0.35 ± 0.05	5.91 ± 1.64	10.78 ± 1.49	**0.94 ± 0.15 b**	45.16 ± 10.40	0.17 ± 0.02	**0.03 ± 0.01 ab**
	1750	1.41 ± 0.20	0.24 ± 0.10	6.92 ± 0.56	9.03 ± 1.003	**0.45 ± 0.07 a**	41.68 ± 8.99	0.17 ± 0.03	**0.01 ± 0.004 a**
March	441	**0.68 ± 0.17 a**	0.27 ± 0.07	6.71 ± 1.09	8.73 ± 0.54	**0.38 ± 0.07 a**	44.21 ± 6.54	0.25 ± 0.05	0.06 ± 0.02
	792	**0.95 ± 0.19 ab**	0.21 ± 0.03	5.92 ± 0.63	8.81 ± 1.00	**0.56 ± 0.05 ab**	54.63 ± 7,36	0.39 ± 0.04	0.03 ± 0.004
	1319	**1.53 ± 0.41 ab**	0.28 ± 0.08	5.11 ± 1.25	10.16 ± 2.22	**0.79 ± 0.15 b**	35.66 ± 7.69	0.33 ± 0.03	0.05 ± 0.02
	1750	**1.99 ± 0.25 b**	0.31 ± 0.06	7.34 ± 0.54	10.62 ± 0.87	**0.50 ± 0.07 ab**	40.30 ± 6.88	0.30 ± 0.02	0.01 ± 0.01

Table IX *Mean content (mg g DW^{-1}) of the flavonoids and gallotannins identified and the unknown in leaves of* B. sempervirens*. Values are means ± S.E. of five plants for each sampling date and altitude. Different letters and values in boldface indicate statistical differences ($p \leq 0.05$).*

	Altitude (m)	Caffeic acid der.	Chlorogenic acid	Cinnamic acid der. (1)	Cinnamic acid der. (2)	Cinnamic acid der. (3)	Cinnamic acid der. (4)	Cinnamic acid der. (5)	Cinnamic acid der. (6)	Total of Cinnamic acid der.
June	441	**0.16 ± 0.10 a**	0.76 ± 0.27	**0.12 ± 0.04 a**	0.14 ± 0.04	**0.02 ± 0.02 a**	**0.04 ± 0.03 a**	0.21 ± 0.03	0.00 ± 0.00	**0.53 ± 0.10 a**
	792	**1.03 ± 0.30 b**	1.07 ± 0.26	**0.25 ± 0.02 ab**	0.16 ± 0.06	**0.23 ± 0.11 a**	**0.12 ± 0.04 a**	0.18 ± 0.02	0.00 ± 0.00	**0.95 ± 0.19 a**
	1319	**0.44 ± 0.13 ab**	0.28 ± 0.07	**0.35 ± 0.05 ab**	0.02 ± 0.02	**0.58 ± 0.15 a**	**0.22 ± 0.06 ab**	0.26 ± 0.10	0.00 ± 0.00	**1.43 ± 0.16 ab**
	1750	**1.20 ± 0.23 b**	0.74 ± 0.15	**0.80 ± 0.27 b**	0.41 ± 0.11	**2.12 ± 0.34 b**	**0.45 ± 0.04 b**	0.25 ± 0.09	0.20 ± 0.14	**4.23 ± 0.67 b**
September	441	**0.16 ± 0.07 a**	1.01 ± 0.13	0.12 ± 0.01	0.21 ± 0.03	0.38 ± 0.24	0.13 ± 0.04	0.18 ± 0.04	0.05 ± 0.03	1.07 ± 0.19
	792	**1.47 ± 0.39 ab**	1.77 ± 0.36	0.12 ± 0.01	0.32 ± 0.05	0.16 ± 0.05	0.14 ± 0.01	0.13 ± 0.03	0.07 ± 0.03	0.93 ± 0.10
	1319	**0.56 ± 0.12 a**	1.51 ± 0.57	0.17 ± 0.03	0.28 ± 0.06	0.41 ± 0.12	0.12 ± 0.03	0.15 ± 0.05	0.11 ± 0.03	1.23 ± 0.14
	1750	**2.06 ± 0.81 b**	2.11 ± 0.49	0.28 ± 0.14	0.44 ± 0.13	0.24 ± 0.10	0.17 ± 0.05	0.25 ± 0.07	0.03 ± 0.03	1.41 ± 0.44
December	441	**0.07 ± 0.03 a**	0.74 ± 0.18	0.08 ± 0.01	0.20 ± 0.06	0.86 ± 0.52	0.03 ± 0.02	0.21 ± 0.06	0.05 ± 0.03	1.43 ± 0.54
	792	**2.08 ± 0.64 bc**	1.81 ± 0.38	0.10 ± 0.01	0.37 ± 0.10	0.32 ± 0.09	0.11 ± 0.03	0.15 ± 0.03	0.08 ± 0.03	1.13 ± 0.12
	1319	**0.60 ± 0.24 ab**	1.06 ± 0.35	0.11 ± 0.01	0.28 ± 0.06	0.83 ± 0.24	0.08 ± 0.03	0.13 ± 0.04	0.09 ± 0.03	1.51 ± 0.18
	1750	**2.58 ± 0.37 c**	1.65 ± 0.10	0.19 ± 0.08	0.45 ± 0.16	0.33 ± 0.12	0.11 ± 0.03	0.11 ± 0.03	0.11 ± 0.04	1.30 ± 0.26
March	441	**0.15 ± 0.07 a**	0.94 ± 0.15	**0.09 ± 0.01 ab**	0.20 ± 0.03	0.62 ± 0.25	**0.12 ± 0.04 b**	0.19 ± 0.05	0.06 ± 0.02	1.28 ± 0.20
	792	**1.50 ± 0.45 ab**	1.71 ± 0.26	**0.07 ± 0.01 a**	0.21 ± 0.04	0.32 ± 0.06	**0.12 ± 0.02 b**	0.10 ± 0.03	0.08 ± 0.03	0.92 ± 0.08
	1319	**1.22 ± 0.39 a**	1.33 ± 0.47	**0.11 ± 0.01 ab**	0.26 ± 0.07	0.69 ± 0.07	**0.00 ± 0.00 a**	0.11 ± 0.03	0.10 ± 0.03	1.26 ± 0.10
	1750	**2.87 ± 0.76 b**	1.31 ± 0.26	**0.15 ± 0.03 b**	0.33 ± 0.08	0.24 ± 0.05	**0.03 ± 0.02 ab**	0.15 ± 0.07	0.12 ± 0.05	1.03 ± 0.17

Table X *Mean content (mg g DW^{-1}) of the phenolic acids identified in leaves of* B. sempervirens*. Values are means ± S.E. of five plants for each sampling date and altitude. Different letters and values in boldface indicate statistical differences ($p \leq 0.05$).*

Principal component analyses for leaf phenolic compounds

The two new factors obtained from the PCAs derived for each sampling date explained the 52%, 47%, 45% and 43% of the variance for June, September, December and March, respectively. Factor loadings are shown in Table XI. They represent the correlation of the original variable with the new PC factors (i.e. the extent to which an originally measured item contributes to the underlying PC factor; Tausz *et al.* 1998). PC1 separated the lowest and the highest altitudes in June and March, while PC2 segregated them in December (Fig. 24). Taking into account the factor loadings for the different variables, these values indicate that in June leaves from the highest altitude had greater amounts of cinnamic acid derivative 1, 3 and 4, quercetin 3-arabinoside, quercetin-aglycon and isorhamnetin-rhamnoside than leaves from the lowest altitude. In contrast, leaves from the highest altitude were characterised by a higher content of caffeic acid derivative in December and cinnamic acid derivative 2 and pentagalloylglucose in March.

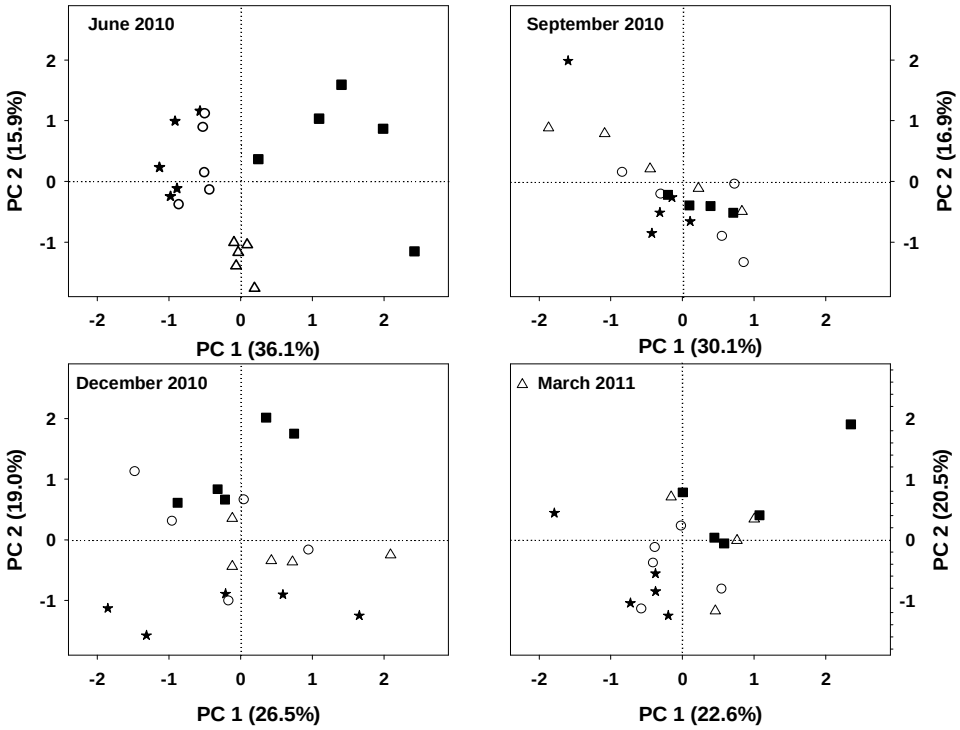


Fig. 24 Ordination by principal component analysis of the entire leaf content of phenolic compounds of B. sempervirens *from four different altitudes (★ 441 m, ◇ 792 m, △ 1319 m, ■ 1750 m) for each sampling date.*

Phenolic compound	June		September		December		March	
	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2
Caffeic acid der.	.539	.292	**.779**	.439	-.216	**.893**	.656	.260
Chlorogenic acid	-.121	.699	**.864**	.155	-.362	.654	.580	-.153
Cinnamic acid der. (1)	**.815**	-.078	.600	.655	.171	.522	.539	.568
Cinnamic acid der. (2)	.533	**.779**	**.892**	.369	-.097	.627	**.708**	.312
Cinnamic acid der. (3)	**.963**	.118	-.253	.651	.685	-.298	-.543	.246
Cinnamic acid der. (4)	**.886**	.024	.637	-.029	-.401	.487	-.243	-.631
Cinnamic acid der. (5)	.154	-.316	.585	.384	-.062	-.412	.491	.034
Cinnamic acid der. (6)	.645	.102	-.047	-.281	.271	.224	.423	.037
Unknown	-.475	.006	-.411	-.295	.681	.006	-.466	.279
Quercetin-3-arabinoside	**.818**	-.345	-.467	.642	**.727**	.203	.272	**.814**
Quercetin-aglycon	**.730**	-.251	-.337	.248	**.719**	.171	-.224	.643
Isorhamnetin-dirhamnoside+Kaempferol-3-glucoside	.197	.566	-.399	.148	**.703**	.405	-.316	.220
Isorhamnetin-rhamnoside	**.854**	-.126	-.540	.503	**.883**	.284	-.210	**.819**
Luteolin der.	-.209	-.027	**-.750**	.236	.626	-.221	-.235	.548
Digalloylglucose	.216	-.174	-.445	.404	.495	-.143	.041	.391
Pentagalloylglucose	.097	**-.914**	-.115	.624	.078	-.503	**-.721**	.432
Neolignan	.659	.010	.256	.043	.394	.386	.673	.087

Table XI *Principal component solution from PCA of the identified phenolic compounds of leaves of* B. sempervirens *for each sampling date (N = 20 in June and December 2010 and N = 24 in September 2010 and March 2011). Contribution of variables to each axis was considered significant when factor loadings ≥ 0.70 (marked in bold).*

UV treatment effects

The UV-exclusion experiment significantly affected the leaf content of luteolin derivative of *B. sempervirens* in September at 441 m ($F_{2,5}$ = 5.76, p = 0.018), since leaves grown without UV (UV-0) showed significantly lower amounts of this flavonoid than those exposed to UV-A and UV-B radiation (Fig. 25). The rest of the compounds detected were not affected by UV-exclusion.

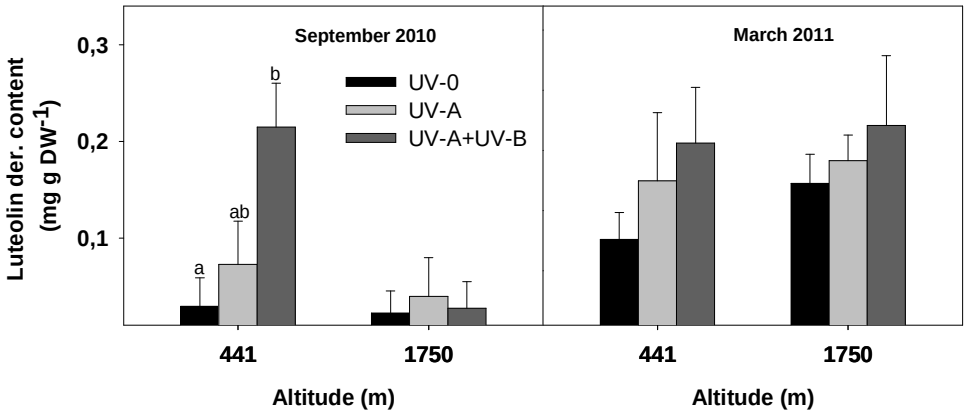


Fig. 25 Mean leaf content of luteolin derivative in B. sempervirens *leaves under the different UV-exclusion conditions in September 2010 and March 2011 at the lowest (Llémana, 441 m) and the highest (Tosses, 1750 m) localities. Values are means ± S.E. for 20 plants (5 plants per locality and sampling date). Different letters indicate significant differences (p ≤ 0.05) among altitudes within each sampling date.*

Cuticle content of phenolic compounds

Eight phenolic compounds (seven flavonoids and one phenolic acid, a protochatechuic acid derivative) were identified in the cuticles of *B. sempervirens* (Annex I). The amount of protochatechuic acid derivative was so low that it could not be accurately quantified.

Seasonal effects

In June, we did not detect any phenolic compound in the cuticles of *B. sempervirens* leaves. The total flavonoid content of cuticles increased significantly about the 71% between September and December sampling dates ($F_{1,6}$ = 52.62, p < 0.001, Fig. 26), with the cuticle content of rhamnetin derivative 1, quercetin derivative 2, apigenin derivative+luteolin derivative showing a maximum in December (Fig. 26). In contrast, the maximum cuticle content of eriodictyol, rhamnetin derivative 2, quercetin derivative 1 and apigenin was found in March 2011 (Fig. 26).

Altitudinal effects

The total amount of flavonoids in the cuticles of *B. sempervirens* leaves decreased with altitude ($F_{1,6}$ = 3.877, p = 0.004), since for all of the compounds detected, except rhamnetin derivative 1, post-hoc analyses revealed overall lower amounts at the highest altitude (1750 m) than at the lowest one (441 m) (Fig. 26). The overall total flavonoid content at the lowest altitude was 13.8 mg g DW^{-1} while at the highest one was only 3.9 mg g DW^{-1}, with cuticle samples from mid-altitudes showing intermediate values.

UV radiation treatment effects

No effects of the UV treatment applied were found on the phenolic compounds identified in cuticles.

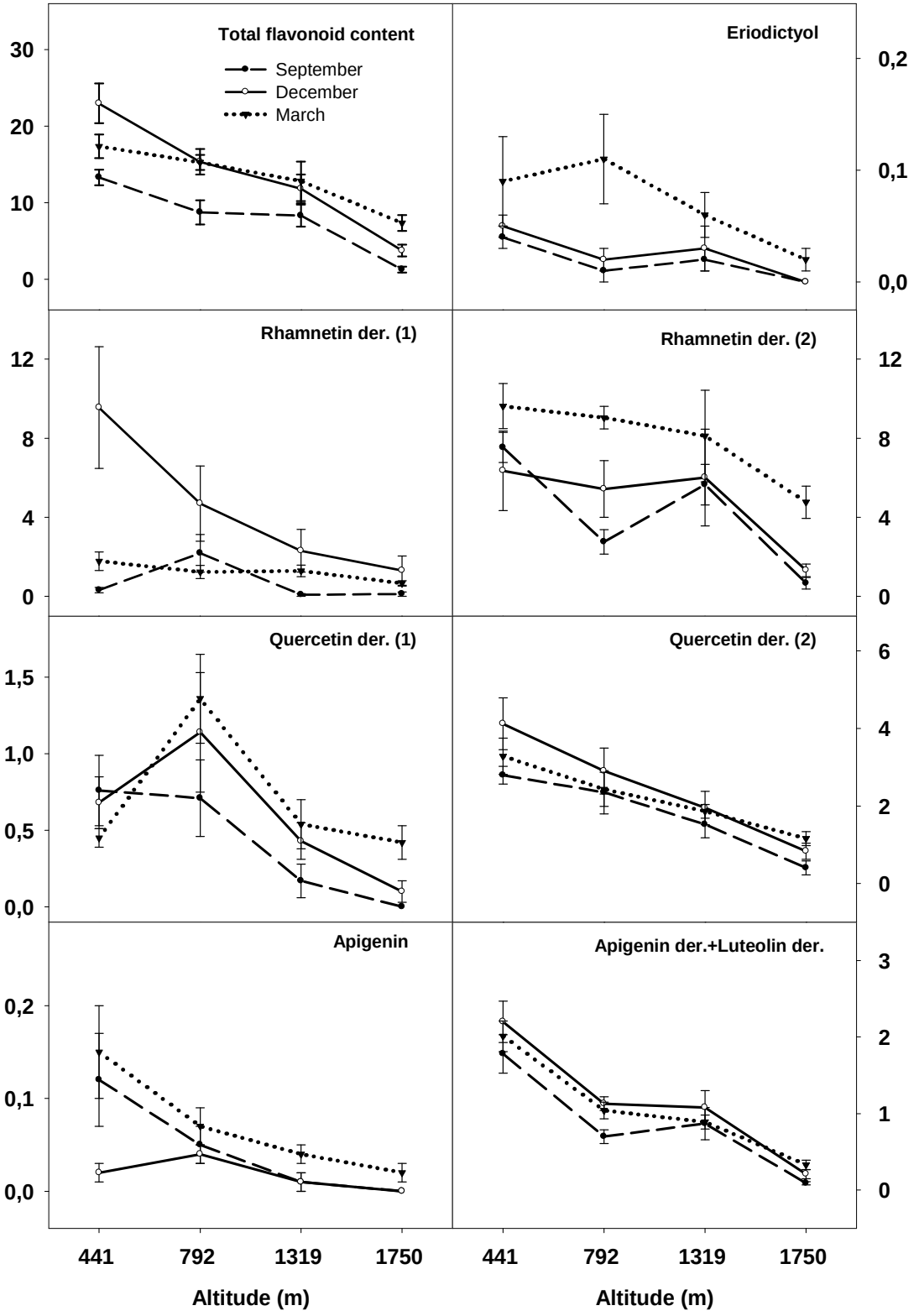


Fig. 26 Total content (mg g DW^{-1}) of flavonoids, as well as of each one of the identified flavonoids, per altitude and sampling date in cuticles of B. sempervirens *leaves (N = 6, except at 1319 m where N = 5).*

Principal component analyses on leaf cuticle phenolic compounds

The two PC factors explained 75%, 75% and 66% of the variance in the data set for September, December and March, respectively. PC1 separated the lowest and the highest altitudes in all the sampling dates (Fig. 27). Factor loadings for the different variables shown in Table XII indicate that cuticles of *B. sempervirens* from the lowest altitude had greater amounts of phenolic compounds than those from the highest altitude, particularly eriodictyol, the two quercetin derivatives, rhamnetin derivative 2, and apigenin derivative+luteolin derivative in September, eriodictyol, quercetin derivative 2 and apigenin derivative+luteolin derivative in December and quercetin derivative 2, rhamnetin derivative 2, apigenin and apigenin derivative+luteolin derivative in March.

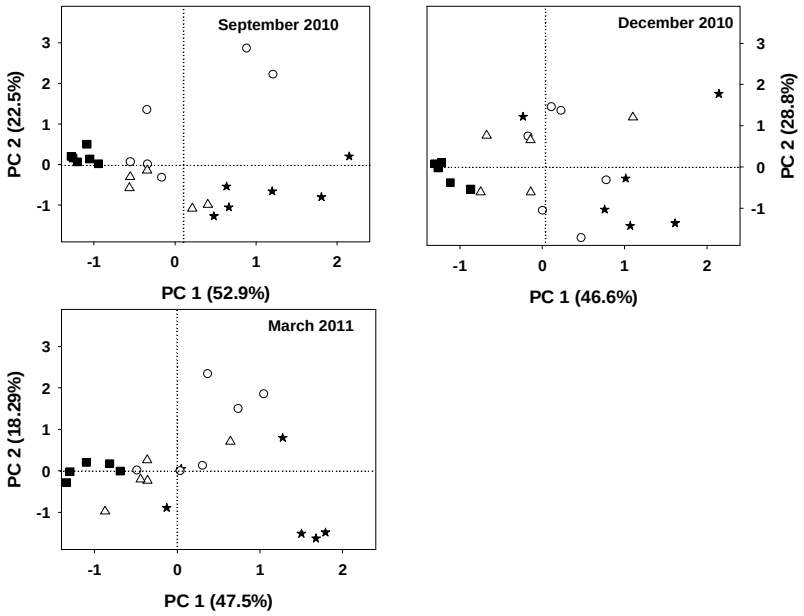


Fig. 27 Ordination by principal component analysis of the cuticle content of phenolic compounds in leaves of B. sempervirens *from four different altitudes (★ 441 m, ◇ 792 m, △ 1319 m, ■ 1750 m) for September and December 2010 and March 2011 data.*

Phenolic compound	September		December		March	
	PC1	PC2	PC1	PC2	PC1	PC2
Eriodictyol	**0.761**	-0.214	**0.802**	0.011	0.510	0.585
Quercetin derivative 1	**0.820**	0.491	0.492	-0.509	0.385	**0.758**
Quercetin derivative 2	**0.835**	0.102	**0.922**	-0.111	**0.706**	-0.306
Rhamnetin derivative 1	0.374	**0.864**	0.546	**-0.774**	0.699	-0.381
Rhamnetin derivative 2	**0.751**	-0.556	0.447	**0.745**	**0.748**	0.247
Apigenin	0.699	0.149	0.404	**0.755**	**0.820**	-0.161
Apigenin derivative+ Luteolin derivative	**0.753**	-0.445	**0.930**	0.139	**0.835**	-0.192

Table XII *Principal component solution from PCA of the phenolic compounds identified in cuticles of* B. sempervirens *leaves for September and December 2010 and March 2011 (N = 24 in September and December and N = 23 in March 2011). Contribution of variables to each axis was considered significant when factor loadings ≥ 0.70 (marked in bold).*

Discussion

In leaves of *Buxus sempervirens* L., neither the total amount of phenols nor flavonoids showed a consistent response to the seasonal or altitudinal UV-B gradient throughout the study period. Indeed, contrary to our expectation based on the highest UV-B radiation levels in June, *B. sempervirens* leaves showed a 36% lower amount of phenols in this month than in September (Fig. 23). In addition, while UV-B radiation decreased from September to March, the leaf content of phenols or flavonoids did not show any response to this decrease. Similarly, for the altitudinal gradient, we did not find significant differences among altitudes in the total leaf content of phenols or flavonoids (except in June in the case of flavonoids). Our UV-exclusion treatments mirrored these results, since they did not affect the total amount of phenols or flavonoids in leaves from *B. sempervirens*. Therefore, our results suggest that other abiotic or developmental factors would have a greater influence than ambient UV-B on the seasonal and altitudinal variation in the amount of leaf phenols or flavonoids

in this species. In fact, despite we did not find differences between LMA, leaf thickness or leaf area of leaves collected in the different sampling dates, leaves from June showed a lighter green colour than leaves collected in September, suggesting that biochemical changes would occurred between these months probably related with leaf maturation. In accordance, Liakoura *et al.* (2001), after studying 14 Mediterranean plant species, concluded that seasonal fluctuations of leaf UV-absorbing compounds (UACs) seemed to be more correlated to developmental processes than to seasonal fluctuations in UV-B radiation. In the present study, higher amounts of flavonoids (and also phenolic acids) at the highest locality in June might be explained by a delay in leaf ontogeny with altitude in this season, since Sommavilla *et al.* (2012) found higher contents of phenolic acids and flavonoids in the earlier stages of leaf development in the sub-Mediterranean species *Celtis australis* L. They related higher levels of phenols in young leaves to a higher need of protection against herbivory and UV-B radiation due to less developed cuticles and cell walls. These authors also reported no significant fluctuations in the leaf content of these compounds once the leaves were mature, which would also be in agreement with our results. The fact that leaf UV-absorbing compounds have other roles besides UV radiation screening might explain the requirement of a relative seasonal stability in their levels once leaves have matured (Stephanou and Manetas 1997).

Even though the total amount of phenols or flavonoids did not show a consistent response to the seasonal or altitudinal variation in UV-B levels along the study period, foliar amounts of phenolic acids (and also neolignan) increased with altitude (Fig. 23). Accordingly, leaves from the highest and the lowest localities were segregated in the PCA analyses mainly due to their content in phenolic acids. However, the leaf content of phenolic acids (and neolignan) was not affected by the UV-exclusion treatments suggesting that the detected changes in the amount of these compounds were not caused by the altitudinal gradient in UV radiation. Many abiotic factors, other than UV irradiance, change with altitude (e.g. temperature, precipitation, soil characteristics, wind speed or snow cover) and, hence, might explain the variation in leaf content of these compounds along an altitudinal gradient. For

instance, increases with altitude in phenolic acids or neolignan might be related to the decrease in temperature (Table VII), since previous studies have related higher amounts of these compounds with low temperatures (Leyva *et al.* 1995, Kumazaki *et al*. 2009). In addition, cinnamic acids and neolignans are biosynthetic precursors of most of the other phenolic compounds, which might also explain some of the above-mentioned changes (Vermerris and Nicholson 2008).

Among phenolic acids, the leaf content of caffeic acid derivative varied with altitude, being its content greater in the highest altitude (Table X and Fig. 27). Other studies have shown increases in caffeic acid with altitude (Zidorn *et al.* 2005, Spitaler *et al.* 2006, 2008, Albert *et al.* 2009), with this effect being related to the rise in the amount of UV-B radiation (Zidorn *et al*. 2005) or to the decrease in temperature (Albert *et al*. 2009). The lack of an effect of UV-exclusion on the leaf content of this compound suggests that UV radiation is not the main factor affecting its content in *B. sempervirens* leaves. Alternatively, a greater need for its antioxidant activity (Sato *et al*. 2011 and see Shahidi and Chandrasekara 2010 for a review) due to plant exposure to low temperatures under high solar irradiation (García-Plazaola *et al*. 1999) might explain greater amounts of this compound at the highest locality. In accordance with this, the maximum overall leaf content of caffeic acid derivative was found in autumn (December) and winter (March).

The content of chlorogenic acid, an ester of caffeic acid and quinic acid (Clifford 1999), contributed to differences in leaf biochemistry between the highest and the lowest localities in September, due to the high amounts of this compound at 1750 m. However, in general, changes in the leaf content of this compound did not follow the seasonal or altitudinal gradient in UV-B radiation (Table X). Taking into account that UV exclusion did not affect the levels of chlorogenic acid in leaves, these results suggest that the detected temporal and altitudinal fluctuations in this compound were not related to changes in UV-B radiation. Variations in the content of chlorogenic acid might be related to its possible role as an endogenous signaling molecule participating in growth and developmental responses (Kotilainen *et al*. 2010).

In general, changes in gallotannins did neither follow the altitudinal or seasonal variation in UV-B radiation (Fig. 23, Table IX). The increase in digalloylglucose derivative at the end of winter (March) might be related to its antioxidant properties (Zhang *et al*. 2009) and/or to its capacity to inhibit ice nucleation of water, facilitating an adaptation to freezing temperatures, as suggested for the boreal hardwood *Cercidiphyllum japonicum* S.& Z. by Wang *et al*. (2012).

Only one flavonoid, the luteolin derivative, responded to the UV-exclusion treatments. At the end of summer (September) and at the lowest locality, the content of this compound was considerably lower in leaves grown without UV radiation than in those exposed to UV-A and UV-B radiation (Fig. 25). Previous studies have shown that luteolin is a flavonoid with a high antioxidant activity due to its orthohydroxy B-ring (Rice-Evans *et al*. 1997, Agati *et al*. 2009). Accumulation of luteolin derivatives in response to enhanced UV-B radiation has been found in willows, being related to UV-B induction of the flavone synthase that catalyzes the production of luteolins (Tegelberg and Julkunen-Tiitto 2001). However, in our study, variations in the leaf content of luteolin derivative did not follow the natural fluctuations of UV-B radiation throughout the year or along the elevational gradient (Table IX), suggesting that, there are other factors that exert a stronger control over its content. Since flavonoids play a key role in countering light-induced oxidative stress irrespective of UV radiation, it has been suggested that its antioxidative function prevails in relation to its function in screening of UV radiation (Agati *et al.* 2009, 2010, 2011).

The phenolic compounds in *B. sempervirens* cuticles differed from those found in leaves (which also contained the cuticles). This was probably because the cuticle mass was so low in relation to the whole leaf mass that cuticle phenolic compounds could not be detected in the analyses of the whole leaves. Seasonal differences in the amount of phenolic compounds in cuticles (all of them being flavonoids) did not follow seasonal changes in UV radiation. In addition, the flavonoid content of *B. sempervirens* cuticles (except in the case of rhamnetin derivative 1) decreased along the altitudinal gradient (Fig. 26, Table XII). Increases in cuticle thickness with altitude (Anfodillo *et al*. 2002,

Turunen and Latola 2005) might explain the decrease in flavonoids. However, our results do not support this idea since no differences in the cuticle mass per area were found between altitudes (data not shown). Alternatively, the reduction of flavonoids with altitude might be a consequence of an increased synthesis of wax in response to the decrease in temperature since, in some species, low temperatures induce an increase of cuticular waxes (Shepherd and Griffiths 2006) and both, cuticular waxes and flavonoids, are derived from acetyl-CoA (Kunst and Samuels 2003). Hence, an increase of wax synthesis could reduce the acetyl-CoA available for flavonoid synthesis. Moreover, higher cuticular wax contents have been associated with higher UV-B reflectance (Holmes and Keiller 2002), which could help leaves to cope with increasing levels of UV-B radiation along the altitudinal gradient. A study of the cuticlular wax content of *B. sempervirens* leaves along the altitudinal gradient would be necessary to confirm this hypothesis.

It has been suggested that some phenolic compounds of leaf cuticles, especially hydroxycinnamic acids attached to cutin polymers, might be used as biomarkers of UV-B levels (Riley and Kolattukudy 1975, Liakopoulous *et al.* 2001, see Rozema *et al*. 2009 for a revision, Willis *et al.* 2011). The present study does not support this idea in the case of *B. sempervirens* cuticles, since: 1) the only phenolic acid detected was a protochatechuic acid derivative, which was found in negligible amounts, 2) there was no effect of UV-exclusion on the cuticle content of any of the phenolic compounds detected, all of them being flavonoids, and 3) the cuticle content of these flavonoids never increased following the seasonal or altitudinal increases in UV-B radiation.

In conclusion, while the amount of phenolic acids and neolignan increased in *Buxus sempervirens* L. leaves in response to the altitudinal gradient, the amount of flavonoids in cuticles decreased. However, based on the observed seasonal changes and the results of the UV-exclusion experiment, we suggest that these variations in the leaf and cuticle content of phenols in *B. sempervirens* depended more on leaf developmental processes or on other environmental factors, such as temperature, than on natural variation in UV-B

radiation. Accordingly, any phenolic compound of the leaves or cuticles of *B. sempervirens* seems to be an appropriate biomarker of ambient UV-B levels.

Chapter V. General discussion

The main results of this thesis are discussed in this chapter in relation to the initial objectives:

5.1) UV-B radiation effects on Mediterranean species and its interaction with water availability

1.1) Photoprotective mechanisms

In the Mediterranean species studied, one of the most consistent responses to the increase in UV-B radiation levels was a rise in leaf thickness (Chapter II) or in leaf mass area (LMA, Chapter I). This is in agreement with previous studies suggesting that an increase in leaf thickness is an adaptive response to enhanced levels of UV radiation since thicker leaves attenuate the penetration of this harmful radiation into the mesophyll protecting the photosynthetic apparatus (Wand 1995, Fagerberg and Bornman 2005).

Although it is widely accepted that a rise in leaf UV-B-absorbing compounds, especially phenols, is a plant response mechanism to cope with UV-B radiation increases (Jenkins 2009), due to the antioxidant and UV-screening properties of these compounds (Close and McArthur 2002), we did not find this response in the species studied. In accordance, it has been suggested that Mediterranean species have a high constitutive content of phenolic compounds (resulting from their adaptation to conditions of marked oxidative pressure typical of the Mediterranean climate) which confer them protection against UV radiation (see Paoletti 2005 for a revision). In *P. lentiscus* seedlings, it was even found a decrease in the total amount of phenols when plants were grown under UV-A and UV-A+UV-B radiation compared to those grown without UV radiation. In this case, such results also suggest that UV-A radiation might play a role in phenol synthesis or degradation (Chapter I). Accordingly, when the content of specific phenolic compounds of *Laurus nobilis* leaves was determined, we found that supplementation of UV-A radiation decreased the vacuolar amount of quercetin and kaempferol derivatives in this species (Chapter II). This pattern agrees with Wilson *et al.* (2001) who

suggested a general down-regulation of extractable flavonoids mediated by UV-A radiation. Fahlman and Krol (2009) associated decreases in quercetin glycoside under conditions of enhanced UV-A radiation to possible UV-A-mediated breakdown of quercetin. They also found a UV-B-mediated breakdown of quercetin but only if high doses of UV-B radiation were applied (516 kJ m^{-2}). Strikingly, we found that, in the case of kaempferol, the addition of UV-B radiation counteracted the effect of UV-A radiation, suggesting a wavelength-specific response. In some species it has been reported that some UV-absorbing compounds were more affected by UV-A than by UV-B radiation (Kotilainen *et al.* 2008 and 2009, Victório *et al.* 2011). A wavelength-specific effect on the amount of phenolic compounds agrees with wavelength-specific effects found on plant growth and morphology in some previous studies (Cooley *et al*. 2000, Shinkle *et al.* 2004).

Supporting a wavelength-specific plant response, the activation of photoprotective mechanisms to cope with an excess of light energy also varied depending on whether plants were exposed to enhanced UV-A or to enhanced UV-A+UV-B radiation (Chapter II). Indeed, an increase in UV-A radiation, but not in UV-A+UV-B radiation, reduced the leaf content of light-absorbing pigments, which is a common plant response to excess light displayed to avoid the imbalance between light absorption and the energy required by photosynthesis (Munné-Bosch and Alegre 2000). Accordingly, UV-A-supplemented plants, but not UV-A+UV-B-supplemented ones, showed a higher de-epoxidation of the xanthophyll cycle in relation to control plants. This response has previously been related to an increase in the dissipation of excess energy as heat (Demmig-Adams 2003, García-Plazaola *et al.* 2007). In agreement, higher values of non-photochemical quenching (NPQ) were found in UVA-supplemented plants at midday although only under conditions of low water availability. An increase in zeaxanthin content in response to enhanced UV-A radiation has also been observed in the cells of the alga *Dunaliella bardawill* (Mogedas *et al.* 2009). Overall, our results suggest a higher oxidative stress in plants grown under enhanced UV-A radiation.

The lack of significant effects of enhanced UV-A+UV-B radiation on the photoprotective mechanisms studied, together with the lack of negative effects on plant photosynthetic efficiency and growth (see below), indicates that UV-B radiation might have activated other protective mechanisms, different of those activated by UV-A radiation and not studied in this thesis. Such mechanisms might include the biosynthesis of antioxidant compounds or repair enzymes. Supporting this hypothesis, Yang *et al*. (2007) found higher activities of superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase, as well as lower thermal energy dissipation as heat, in UV-A+UV-B- than in UV-A-supplemented plants of winter wheat (*Triticum aestivum* L.).

The above-mentioned protective mechanisms required to cope with UV-A or UV-B radiation seem to have been effective, since neither the leaf potential photochemical efficiency (Fv/Fm) nor the leaf apparent electron transport rates (ETR), which are indicators of photosystem II (PSII) activity, were negatively affected by UV radiation treatments in any of the experiments performed (Chapters I and II). These results agree with other studies on Mediterranean species showing no effects of UV radiation on photochemical efficiency of PSII (e.g. Grammatikopoulous *et al*. 1998, Nogués and Baker 2000). In accordance with this, plant biomass, an integrated parameter commonly used as a reliable indicator of plant sensitivity to stressful conditions (Smith *et al.* 2000), was not affected negatively by the UV radiation treatment. In fact, we have found a beneficial effect of UV radiation, especially UV-A, on plant biomass production under water shortage (see below).

Finally, results show that the xerophytic or mesophytic character of the species studied was not related to different UV radiation effects. The species selected as xerophytes and mesophytes had a similar range of leaf sclerophylly. Therefore the degree of leaf sclerophylly might play a more important role in the adaptation of Mediterranean plant species to UV radiation than habitat characteristics (see also Verdaguer *et al*. 2012).

In summary, information gathered from the research described in this thesis suggest that Mediterranean plant species are able to cope with the expected increases in UV radiation by means of the activation of specific

photoprotective mechanisms, such as the increase in leaf thickness and/or the regulation of the leaf content of certain chloroplastic pigments. Contrary to what was expected, the results suggest that these plants are not likely to change the total amount of phenols produced in response to increases in UV-B radiation, although the leaf content of some individual compounds may be altered.

1.2) Plant water relations and growth

Previous studies have suggested that plant responses to UV radiation can be modulated by other factors to which plants are concomitantly exposed, such as photosynthetically active radiation (PAR), nutrient availability, predation by herbivores or water availability (see Caldwell *et al.* 2007 for a review, UNEP 2008). Thus, it is not surprising that different effects in response to UV radiation depending on the water supplied to the plants were found. Indeed, results showed a beneficial effect of UV radiation on production of plant biomass when plants were grown under low-water availability, but not when they were well-watered. Under low-water availability, plants produced more roots when grown under UV-A or UV-A+UV-B radiation than when grown in a UV-free environment (Chapter I). Similarly, laurel seedlings exposed to above-ambient UV-A and UV-A+UV-B radiation and limited water availability also showed greater above- and below-ground biomass productions compared to those grown under ambient levels of UV radiation (Chapter II). Therefore, our results point to a beneficial effect of UV-radiation, mainly UV-A, under water shortage, since we did not find differences in biomass production between UV-A- and UV-A+UV-B-treated plants in any of the two experiments (Chapters I and II).

Beneficial effects of UV-A radiation on plant growth have been reported for well-watered plants of *Cyamopsis tetragonoloba* L. (Lingakumar and Kulandaivelu 1998) and *Betula pubescens* E. (Weih *et al.* 1998). However, to our knowledge, this thesis is the first report suggesting a beneficial effect of UV-A radiation under conditions of low water availability (Chapters I and II). This beneficial effect might be related to a UV-improvement of plant water relations (as indicated by the higher relative water content of these plants) which, in turn, might be related to an increase in leaf thickness (Gitz and Liu-Gitz 2003,

Ennajeh *et al.* 2010). In agreement with this, UV-A and UV-A+UV-B supplementations tended to increase leaf water use efficiency (WUE) of plants when they grew under low irrigation.

5.2) Changes in leaf content of specific phenolic compounds in response to seasonal and elevational gradients of UV radiation

The seasonal and altitudinal variation in the content of phenolic compounds of *Buxus sempervirens* leaves seemed to be more related to other factors, such as ontogenetical or temperature changes, than to the natural variations in ambient levels of UV radiation (Chapter III). The increase in the total amount of neolignan and phenolic acids in leaves of *B. sempervirens* along the altitudinal gradient was more likely to be related to a decrease in temperature rather than to an increase in UV radiation, since foliar amounts of these compounds were not affected by UV radiation treatments. Furthermore, published studies have related the induction of biosynthesis of these compounds with low temperatures (Leyva *et al.* 1995, Kumazaki *et al*. 2009). Only one phenolic compound, the flavonoid luteolin derivative, varied in September and at the lowest locality in response to UV-exclusion treatments, with higher concentrations in leaves exposed to UV-A+UV-B than in those grown in a UV-free environment. However, no variations in the leaf content of this compound was found in response to the seasonal or altitudinal gradient in UV radiation, suggesting that there are other factors that exert a stronger control of its content under ambient levels of UV radiation.

Considering the content of phenolic compounds of leaf cuticles from *B. sempervirens*, the only consistent pattern was a reduction in the amount of flavonoids along the altitudinal gradient. Although this unexpected result could be explained by an increase in the cuticle thickness along the altitudinal gradient (Anfodillo *et al*. 2002), results do not support this, since the mass per area of *B. sempervirens* cuticles did not vary among altitudes (data not shown).

Alternatively, since it has been shown that low temperatures can increase cuticular waxes in some species (Shepherd and Griffiths 2006) and that both, cuticular waxes and flavonoids, are derived from acetyl-CoA (Kunst and Samuels 2003), the decrease in flavonoids of *B. sempervirens* cuticles with increasing altitude might be a consequence of an increased synthesis of waxes at higher altitudes in response to lower temperatures, which would reduce the acetyl-CoA available for flavonoid synthesis. Moreover, because a higher cuticular wax content has been associated with greater reflectance of UV-B radiation (Holmes and Keiller 2002), an increase in the cuticular content of wax along the altitudinal gradient could help leaves to reduce penetration of UV-B radiation. However, an intensive study of the cuticular wax content of leaves from different altitudes would be needed in order to confirm this hypothesis.

Overall, we did not find any phenolic compound that could in *B. sempervirens* leaves or cuticles that could be an appropriate biomarker of ambient levels of UV-B radiation. Previous studies using leaf cuticles indicated that certain phenolic acids belonging to the group of hydroxicinnamic acids, such as *p*-coumaric (Rozema *et al.* 2009) could be used as UV-B biomarkers. However, any of these compounds were present in the leaf cuticles of *B. sempervirens*, where only flavonoids were detected at significant amounts.

Chapter VI. Conclusions

1) Plant exposure to UV-A and UV-A+UV-B radiation under water shortage had beneficial effects on the production of plant biomass of the species studied. Since no differences in plant biomass production were found between UV-A- and UV-A+UV-B-treated plants, the beneficial effect on plant growth under low water availability seems to be mainly driven by UV-A radiation. Consequently, UV-A supplementation to seedlings might improve their resistance to drought.

2) Despite the increase in biomass of plants grown with additional UV-A radiation under low water availability, these plants activated photoprotective mechanisms, including a reduction of light-harvesting pigments in leaves or an increase of energy dissipation as heat.

3) Above-ambient levels of UV-B radiation counteracted the effects of enhanced UV-A on the studied photoprotective mechanisms without changing its beneficial effect on plant biomass production.

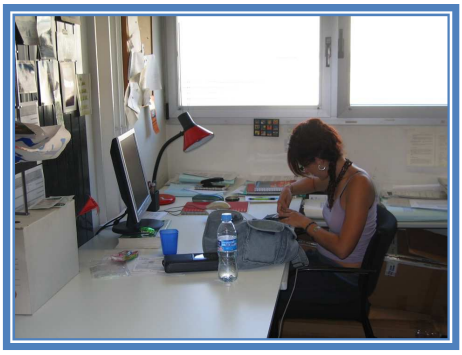
4) An increase in the index of leaf sclerophylly (LMA) is one of the most consistent response to the rise in UV radiation levels.

5) Plant responses to UV radiation are mostly species-specific, but a differential response was not related to the xerophytic or mesophytic character of the species.

6) None of the phenolic compounds of *Buxus sempervirens* leaves or cuticles seemed to be an appropriate biomarker of ambient levels of UV-B radiation, since the leaf or cuticle content of phenols depended more on leaf developmental processes or on other abiotic factors than on natural seasonal or altitudinal variations in UV-B radiation.

7) In the case of the Mediterranean woody species studied, UV radiation seemed to be, rather than a stress factor, a regulator factor, since it activated different response mechanisms which allowed plants to cope with a stress characteristic of the Mediterranean environment, low water availability.

Chapter VII. References



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Chapter VIII. Annexes

Annex I. Map of the nine selected meteorological stations (red) and the 4 sampling sites (yellow).

Annex II. Detected phenolic compounds by HPLC and mass spectrometry analyses for leaves and cuticles of *B. sempervirens*.

Phenolic compound	Type	Retention time (m)	Detection UV	Quantification UV-spectrum (nm)	MS-ions Single quadruple
Leaves					
Digalloylglucose der.	Gallotannin	4.95	x	220	-
Caffeic acid der.	Phenolic acid	5.66	x	320	-
Unknown		6.55	x	270	193↑,493,767,390,395
Cinnamic acid der. (1)	Phenolic acid	7.93	x	320	434
Cinnamic acid der. (2)	Phenolic acid	8.58	x	320	177,369,407
Chlorogenic acid	Phenolic acid	9.60	x	320	355(M+1),377(M+23),
Neolignan 1	Neolignan	16.13	x	270	545(M+23),235,219 (Cedrusin-Me-4-o-glucoside)
Neolignan 2	Neolignan	19.02	x	220	Methyl-17-stock=methyl-sccoisolariciresinol 9-o-p-glucopyranoside= lignan 561 (M+23,545(M+1),265,219,180
Isorhamnetin-dirhamnoside+Kaempferol3-glucoside	Flavonoid	20.21	x	320	Iso-rhamnetin dirhamnoside 793(M+23),771(M+1,625(isorhamnetin-rhamnoside+1),479(rhamnetin+1),317(rhamnetin+1) Kaempferol 3-glucoside 471(M+23),449(M+1), 287 (Kaempferol+1)
Quercetin 3-arabinoside	Flavonoid	21.13	x	320	435(M+1),457(M+23),Quercetin 303(M+1)
Pentagalloylglucose	Gallotannin	23.38	x	220	-
Quercetin-aglycon	Flavonoid	24.29	x	320	303 (quercetin+1)
Isorhamnetin-rhamnoside	Flavonoid	24.79	x	320	647(M+23),625(M+1),479(isorhamnetin+1),317(rhamnetin+1)
Cinnamic acid der. (3)	Phenolic acid	26.90	x	320	-
Cinnamic acid der. (4)	Phenolic acid	27.36	x	320	-
Luteolin der.	Flavonoid	34.73	x	320	-
Cinnamic acid der. (5)	Phenolic acid	39.94	x	220	-
Cinnamic acid der. (6)	Phenolic acid	40.75	x	220	-
Cuticles					
Eriodictyol	Flavonoid	19.37	x	220	273
Protocatechuic acid der.	Phenolic acid	23.81	x	220	-
Quercetin der. (1)	Flavonoid	30.65	x	220	303 (quercetin+1)
Rhamnetin der. (1)	Flavonoid	33.00	x	220	317(M+1)
Rhamnetin der. (2)	Flavonoid	33.91	x	220	361(M+1),383(M+23)
Apigenin der.	Flavonoid	35.07	x	220	331(M+1),353(M+23)
Apigenin+luteolin der.	Flavonoid	38.32	x	220	Apigenin der. 345 (M+1),367(M+23). Luteolin der. 375(M+1),397(M+23)
Quercetin der. (2)	Flavonoid	45.37	x	220	345(M+1),367(M+23)

SEGUIRÉ CAMINANDO

Seguiré caminando por el quebrajoso
camino de estos sueños míos.
Pararé de vez en cuando para sacarme
de las albarcas chinas y guijarros.
Limpiaré con mi manga desilusionada
el rojo sudor de mi frente ilusionada.
Apartaré las zarzas del camino, sacaré
alguna espina clavada en mi carne y
comeré negras zarzamoras para aliviar mi alma.
Tras una loma habrá otra loma y,
tras un valle, una montaña, otros pequenos valles
y más montañas.
Alimentaré mi cansado cuerpo
de los infinitos y dulces sueños de mi alma
e irá quedando en el viento
un maravilloso, triste e ilusionado camino
de indefinibles suspiros.

Manolillo Chinato