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Structural and physiological mechanisms underlying abiotic stress tolerance in the Mediterranean shrub *Cistus albudus* L.

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Dissertation submitted by Marina Pérez Llorca to obtain the degree of Doctor by the University of Barcelona.

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This dissertation was conducted at the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology at the University of Barcelona under the supervision of Prof. Sergi Munné-Bosch and Dr. Maren L. Müller.

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Mecanismes estructurals i fisiològics de tolerància a l'estrès abiòtic en l'arbust mediterrani *Cistus albidus* L.

Memòria presentada per na Marina Pérez Llorca per optar al grau de Doctora per la Universitat de Barcelona.

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Marina Pérez-Llorca	Prof. Sergi Munné-Bosch	Dra. Maren L. Müller

En la ladera de un monte

más alto que el horizonte,

quiero tener buena vista.

Mi cuerpo será camino,

le daré verde a los pinos

y amarillo a la genista.

Joan Manuel Serrat

To all women in science

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ABSTRACT

Mediterranean species have to be in constant acclimation to seasonal environmental changes, including variations in water availability, air temperature and solar radiation. Due to global warming, these environmental changes are predicted to increase in frequency and intensity. Shrubs have a crucial role in plant communities such as avoiding soil erosion and, therefore, the study of shrub responses to abiotic stresses is essential to predict future changes in populations. Here, we evaluated the performance of the Mediterranean shrub, *Cistus albidus*, under several abiotic stresses in natural and controlled conditions. It was found that *C. albidus* plants have to face several combinations of abiotic stressors, with drought and cold being the main environmental cues that threaten their performance during winter. As a first line of defence, *C. albidus* diurnally changed the angle of their most apical leaves together with a decussate leaf disposition to avoid photoinhibition. Structural mechanisms were not always effective for the whole plant, and carotenoids had a primordial role during the day, dissipating excess energy as a second line of defence. They also had an important function under sustained winter stress, but α-tocopherol was the crucial antioxidant responding to stress, as a third line of defence. Jasmonates seemed to be involved in many abiotic stress responses, including cold stress and drought. High jasmonates contents, especially those of 12-*oxo*-phytodienoic acid, jasmonic acid and jasmonoyl-isoleucine, were found in natural conditions during a particularly cold day and after a winter drought event in *C. albidus* seedlings. Furthermore, it is shown that α-tocopherol and jasmonates have an important role in drought tolerance and survival of seedlings in natural field conditions. Even though seedlings have evolved numerous mechanisms to tolerate stress, there was a 30% decrease in survival after summer drought. It is concluded, given the variability in stress responses and the number of mechanisms to withstand abiotic stress, that *C. albidus* is a highly tolerant and resilient plant to abiotic stress. However, there are still tipping points that could become more frequent in the wake of global change that could cause shifts in *C. albidus* populations.

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$^1O_2$	Singlet oxygen
ABA	Abscisic acid
CKs	Cytokinins
F_v/F_m	Maximum photosystem II quantic efficiency
GAs	Gibberellins
H_2O_2	Hydrogen peroxide
IAA	Indole-3-acetic acid
iPA	Isopentenyladenosine
JA	Jasmonic acid
JA-Ile	Jasmonoyl-isoleucine
JAs	Jasmonates
$OH^{\cdot}$	Hydroxyl radical
OPDA	12-*oxo*-phytodienoic acid
PAR	Photosynthethically active radiation
PC-8	Plastochromanol-8
PSII	Photosystem II
ROS	Reactive oxygen species
SA	Salicylic acid
***t*-Z**	*trans*-zeatin
VAZ	Xanthophyll pool: violaxanthin, antheraxanthin and zeaxanthin
VPD	Vapour pressure deficit

INTRODUCTION

1. Mediterranean plant biodiversity

1.1. Shrubs role in Mediterranean ecosystems

Mediterranean habitats are biodiversity hotspots, the unique climatic conditions of which enable a high species richness. The Mediterranean-type climate regions include the Mediterranean basin, the Cape region of South Africa, southwestern Australia, Central Chile and California, areas situated on the southwest margins of big continental land masses at about 35º N and S latitude. In these regions, summers are dry and hot, and winters are mild and wet (Köppen, 1936). In terms of plant diversity, these regions are home of one-sixth of the global flora (Rundel et al., 2016). The specific reasons why Mediterranean-type ecosystems are this diverse and show such a high rate of endemic species are still not well elucidated but, besides climate, topographic and edaphic gradients are crucial and determining factors.

The resulting vegetation of the Mediterranean climate is the evergreen sclerophyllous shrubland. These shrublands appeared at similar epochs in the geological scale due to increases in aridity and seasonality, but evolved differently depending on the region (Figure 1A); hence, even though they share some similarities, they are different in all the five regions. The Cape region and Australia are the most species-rich while the Mediterranean Basin comes after, and, then, California and Central Chile (Cowling et al., 2015). These differences in species richness are believed to be a result of landscape stability, with the Cape and Australia being “older” and more stable regions (Hopper, 2009; Cowling et al., 2015). With the exception of the Cape sclerophyllous shrublands, which are believed to have first appeared at the end of the Cretaceous, the rest of the Mediterranean-type shrublands emerged during the Miocene. After their first appearance, it was geological and climate events which shaped the different habitats into what we know today. The case of the Mediterranean basin is of particular interest since it is the Mediterranean-type ecosystem with the biggest area, it has a large number of native species, an elevated topographic variability and a very high climate seasonality as well as a high presence of natural fires (Cowling et al., 1996).

In our current time, in the Mediterranean basin we find the maquis or garrigue; in the Cape region, the fynbos; in Australia, the kwongan; in Chile, the matorral; and, in California, the chaparral (Figure 1B). All of these sclerophyllous shrublands have in common their adaptation to adverse conditions but, although they are all considered high biodiversity habitats, plant diversity differs among them. In the Mediterranean basin we find the families Asteraceae (mostly herbs), Fabaceae and Cistaceae well represented whereas in the Cape region, Proteaceae, Geraniaceae and Aizoaceae are the most diversified groups (Rundel et al., 2016). In southwestern Australia, species from the groups Myrtaceae, Proteaceae and Fabaceae are very present whereas the families Asteraceae, Boraginaceae and Brassicaceae are well represented in California. The case of central Chile is particular given its strong biogeographical isolation, which has been a barrier for the extensive presence of natural fires, which, in turn, results in the absence of a high diversification of any family but in many genera represented by a single or few species such as *Montiopsis, Alstroemeria* or *Lithraea* (Rundel et al., 2016). Certainly, natural fires are thought to be one of the main causes of plant diversity in Mediterranean ecotypes (Rundel et al., 2016; He et al., 2019). Furthermore, the presence of fires has been related with a higher variability of traits at a local scale in species growing in fire-prone ecosystems in the Mediterranean basin (Moreira et al., 2012), which, in turn, has implications in shaping plant biodiversity and distributions.

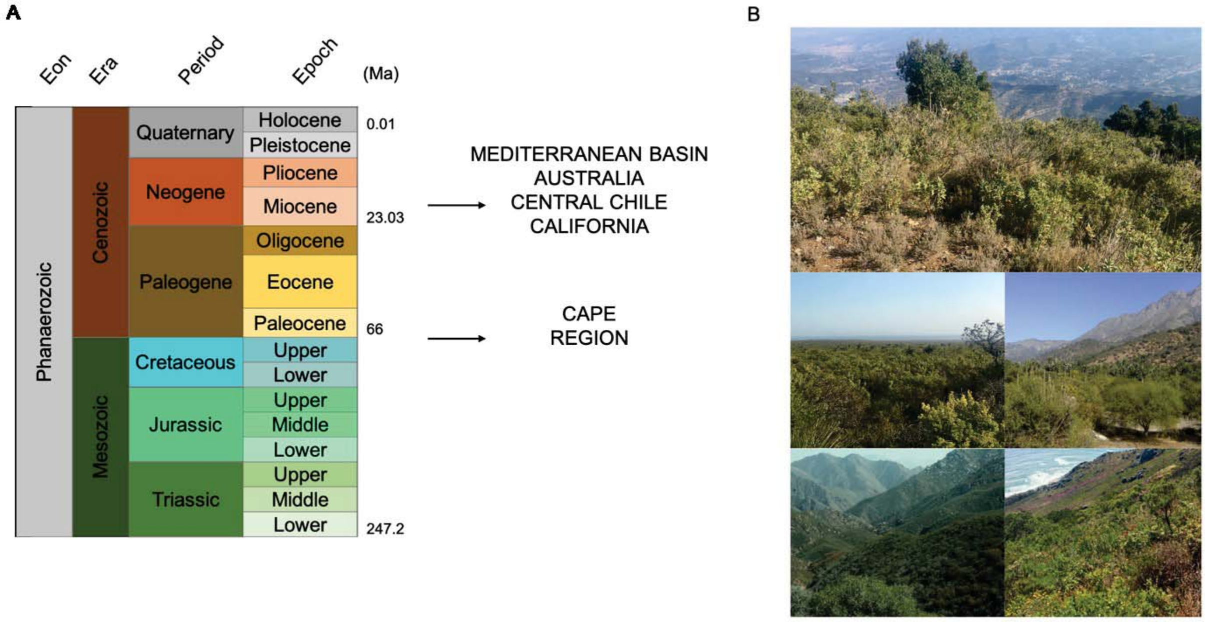

Figure 1 Mediterranean-type ecosystems. **A** The origin of the Mediterranean biomes on the geological scale. Geological scale adapted from the International Comission on Stratigraphy (stratigraphy.org). **B** Examples of every Mediterranean-type ecosystem. From top and left to right: maquis in the Montserrat mountains (SE Spain), kwongan in the northern sandplains (SW Australia), matorral in the National Park La Campana (Central Chile), matorral in the San Gabriel Mountains (S California), fynbos in the Table Mountain Natural Park (Western Cape, South Africa). Photos: M. Pérez-Llorca (SE Spain, SW Australia, South Africa), J. G. Pausas (Central Chile), P. W. Rundel (S California).

The most common life form in these shrublands, just as its name suggests, are shrubs. Shrubs are phanerophytes by the classification of Raunkiaer (1934), which have their perennial buds – *i.e.* structures that contain meristematic cells – above 25 cm of the ground. This group contains both shrubs and trees and differentiating them is not always easy (Rundel, 1991a). Generally, trees are considered to have a single stem while shrubs tend to be multi-stemmed but there are numerous intermediate forms. One of the main reasons for this controversy is that woody plants can change their growth habits – *i.e.* growth plasticity – depending on the circumstances. For instance, when there is low light availability in a forest, shrubs or small trees tend to grow taller; or, single-stemmed trees such as *Betula* *pendula* can shift their growth habits if faced with browsing or cutting damage (Götmark et al., 2016).

Shrubs dominate a biome in either the early stages of succession to later give way to bigger phanerophytes or in habitats where stress is constant, particularly drought, nutrient deficiency and frequent fires. In this manner, shrubs are extremely adapted to adverse conditions and present numerous advantages over trees such as a higher tolerance to disturbances (Götmark et al., 2016). In fact, shrubs evolution from temperate forests is believed to have been triggered by the interaction of multiple stresses (Rundel, 1991a). Shrubs are present in a 72% of the families known in the dicotyledons and in a 26% of the monocotyledons (Cronquist, 1981). Furthermore, it has been estimated that shrubs cover an 11.5% of the global land surface and, taking into consideration their coverage in forest understory, a 40% of the global land surface (Gong et al., 2013). Contrastingly, in Mediterranean shrublands, shrubs are not the dominating life form regarding the number of species, even though they might dominate in vegetation coverage. For instance, in the Mediterranean basin, Chile and California, shrubs only represent a 20-40% of all the vascular flora whereas in the Cape region and Australian shrublands, the shrub dominance can reach up to 80% of the flora, in the case of Australia (Rundel, 1991b).

Shrubs not only are well adapted to their habitats but also suppose a benefit for other organisms in the ecosystem. Shrubs provide shelter to other smaller plant life forms and animals, acting as nurse plants in more open habitats (Filazzola and Lortie, 2014) and as a refuge from high light intensities and wind for microorganisms (Berg and Steinberger, 2010). Furthermore, they can enhance the fertility of the soil contributing to the carbon cycle (Zhao et al., 2007) – given their high productivity –, and they retain water due to their big radicular systems. Thanks to their root system, they also contribute to soil stability, avoiding erosion (Mashizi and Sharafatmandrad, 2019; Zhou et al., 2019). In this manner, shrubs are considered key in the mid and late states of vegetation succession (Rolo et al., 2013; Andivia et al., 2017).

1.2. Effects of climate change in Mediterranean communities

A number of the predictions made on climate change due to human activities are already occurring. Ocean's temperatures have steadily risen 0.13ºC per decade (EPA, 2016). Chapter 10 of the Intergovernmental Panel on Climate Change (IPCC, 2013) states that the melting rate of the ice-sheets in the Arctic, Antarctica and Greenland is increasing, thinning the ice sheet by three quarters since the 1980s in the case of the Arctic. Furthermore, temperature extremes have also increased in frequency and intensity at a global scale. The near term-projections (IPCC, 2013), show that surface air temperature is going to increase at a higher rate than it has in the next few decades and that mean precipitations are going to significantly change, with wet regions getting wetter, and dry regions getting drier. The Mediterranean basin received special attention since projections showed an increase in mean temperatures of more than 1ºC in summer and of about 0.6ºC in winter, and, mean seasonal precipitations are predicted to decrease, especially in winter, by a 5–15%. Furthermore, some projections foresee a higher variability in seasonal precipitation which could derive in more inconsistent water availability during the year (Kumar et al., 2014; Konapala et al., 2020).

Desertification – *i.e.* the process of land degradation in arid, semi-arid and sub-humid areas – is also another major concern that, together with the effects of climate change, affects global species richness and food security (IPCC, 2019). In the case of northern Mediterranean countries, a 34% of the land is occupied by drylands, with Spain and Cyprus at the top of the list (Zdruli, 2011). Even though the rate of soil loss was estimated to be of 18 mm per year in Spain (López-Bermúdez, 1990), efforts have been put to prevent this soil erosion with an observed greening trend (Fensholt et al., 2012), which gives light to the mitigation of desertification in the Mediterranean basin. However, the predicted effects of climate change such as more frequent drought events or temperature extremes in a number of Mediterranean countries, could exacerbate soil erosion and hence, have detrimental consequences for Mediterranean plant biodiversity, which in drylands is estimated to represent a 20% of the global plant biodiversity (White and Nackoney, 2003). Species richness in shrublands is also threatened by desertification and climate change (García-

Fayos and Bochet, 2009). It was reported that more than 2,000 plant species from Mediterranean drylands could become extinct by the year 2100 (Malcolm et al., 2006). Shrubs being key for avoiding soil erosion, a reduction in its biodiversity could imply an increase in desertification.

Plant performance has already been reported to be affected by climate change (see Doblas-Miranda et al., 2017). For instance, growth reductions and growth variability have been related to decreases in precipitation in pine forests (Sarris et al., 2007; Vieira et al., 2010). If these changes in plant performance become more frequent and are not an isolated event, they can lead to permanent changes in vegetation structure, composition and dynamics, consequences that have already been proven to occur in certain regions (Roura-Pascual et al., 2005). Indeed, the lack of water – *i.e.* drought events – is one of the main drivers of vegetation shifts in land ecosystems (see Martínez-Vilalta and Lloret, 2016). Death is the ultimate consequence of drought and forests are particularly sensitive to it (Allen et al., 2010; Allen et al., 2015). The effects of climate change are already visible in Mediterranean forests (Peñuelas et al., 2017). The causes of these alterations in Mediterranean communities are changes in land use, an increase in pollutants, increase in fire risk and overexploitation of resources (Paula et al., 2009; Duguy et al., 2012; Peñuelas et al., 2017), and, if these changes continue at current rates or are enhanced, a massive increase in soil erosion leading to desertification is very likely to occur. As it has been previously mentioned, shrubs have a key role in avoiding soil erosion and, even though they are more abiotic stress tolerant than trees, particularly to drought (Liu et al., 2015), they are also suffering the consequences of climate change (Sapes et al., 2017; Sánchez-Salguero and Camarero, 2020), and these consequences can be aggravated with the predicted climate change scenario in the Mediterranean basin (Liu et al., 2020).

2. Mechanisms of abiotic stress tolerance and resilience

2.1. Conceptualising stress tolerance, resilience, sensitivity and avoidance

Mediterranean plant communities undergo a high environmental variability throughout their life histories, particularly those inhabiting the Mediterranean basin.

They have to withstand relatively cold winters and high temperatures in summer together with drought events. Drought, extreme temperatures, high light irradiances or nutrient deficiencies are common Mediterranean environmental stressors that cause abiotic stress and that have a negative effect on plant performance. Furthermore, in natural conditions, plants do not usually face one stressor alone but multiple ones simultaneously, making plant response to stress the result of a very complex network of structural, physiological and genetic mechanisms.

Classically, plants are denominated **tolerant**, **resilient**, or **sensitive**, depending on their strategy (or the lack of it) to face stress. Differentiating between these terms is not that easy and definitions sometimes overlap or are contradictory. On the one hand, it is generally assumed that a plant is tolerant to a stress when it is able to respond effectively without compromising its survival. On the other, resilience is the ability of an organism/system to return to initial structure and function after a disturbance (Westman, 1978). These two concepts are not exclusive, and both can be used to define a plant. Therefore, if a plant experiences a stress (considering a mature plant), suffers damage, but does not die, we can define this plant as stress tolerant. If after experiencing this damage – *i.e.* a disturbance in its normal/ optimal growth conditions –, the plant can return to the state it was before the stress, then this plant will be stress resilient.

If the damage caused by stress is too severe and persistent the plant will not be able to respond to this stress and the very probable outcome will be plant death. In other words, this plant is sensitive to stress. Being sensitive to certain environmental conditions will determine the presence or absence of a given species in a habitat – although there are other filters for species distributions such as historical and biotic (Lambers and Oliveira, 2019). There are species more sensitive to a certain stress than others. For instance, halophytes that live in dune habitats, where plants are under high-salinity conditions, are more salt stress-tolerant than, for example, species that live in a deciduous temperate forest, making the latter more sensitive to salt stress. Mediterranean species are another special example since they are very drought tolerant compared to species from equatorial regions that have higher precipitation regimes. Even though halophytes and Mediterranean plants are adapted to their particular habitats – *i.e.* with genetic characteristics acquired by a process of selection over many generations –, they eventually die if the stress is very extreme. In other words, a stress-tolerant plant could shift to stress-sensitive if a certain threshold is crossed. Certainly, halophytes are classified into different groups depending on their salt tolerance, being oligo-halophytes the group with a lower salt-tolerance (their survival is hampered if the NaCl concentration in the soil is higher than 0.1%) (Iversen, 1936). Even within a species this sensitivity varies. Cotado and Munné-Bosch (2020) recently found that sensitivity to drought differed between populations of an alpine perennial situated at different elevations. In this manner, species cannot be arbitrarily classified into tolerant or sensitive since the ability to respond to a stress depends on the intensity of the stress together with numerous other factors such as location or developmental stage. Furthermore, a plant can be more tolerant to one stress and more sensitive to another, such as Mediterranean species are more drought tolerant than chilling tolerant.

Indeed, it is difficult to separate tolerance from resilience or sensitivity since, for instance, how can a plant be stress tolerant and not resilient or *vice versa*? Considering what has been defined above, a plant would be tolerant and not resilient if the stress is constant (and not isolated) and the plant would not be able to return to optimal growth and developmental conditions. This could be the case of plant communities that live in constantly disturbed sites such as ruderal habitats. Does that mean that species that live in these habitats never have optimal growth conditions? Stress usually stops plant growth, but these plants do develop and complete their life cycles.

The key to understand the complexity of stress tolerance, resilience and sensitivity might reside in another term that has been used in the literature: stress **avoidance**. One could argue that there is no such thing since plants are sessile and cannot escape high temperatures or being bitten by an insect. However, there would be a way to avoid environmental conditions, and this would be to “not exist” during harsher periods. This is the case of annual plants, who complete their life cycle during the favourable season. Nevertheless, annual plants, even though avoiding the most stressful season, they also experience certain types of stresses (Pescador et al., 2018). There are also some plants that have deep radicular systems to reach the most humid layers of the soil, avoiding the shallowest layers that might lack water under drought (Brunner et al., 2015). This strategy could be described as drought avoidance and if we consider the species or individual, they are indeed avoiding drought and its consequences. However, if we consider these species to be adapted to their stressful habitats, it is implied that they actively allocate more carbon to roots to have a deeper radicular system, then are they really avoiding or tolerating drought? An extreme example of drought avoidance that adds to the complexity of tolerance and avoidance, are resurrection plants, species that reduce their metabolism during the dry season remaining nearly in a quiescent state without the loss of their tissues until rains reappear. However, a crucial trait of resurrection plants is that they are desiccation tolerant and the mechanisms of this tolerance are activated at the initial stages of dehydration (see Oliver et al., 2020). Taken all together, and considering that tolerance, resilience, sensitivity and avoidance are terms with distorted margins, (i) a tolerant plant is defined as one that activates a stress response under stress and survives, suffering damage or not; (ii) a resilient plant activates a stress response under a stress, survives and returns to a pre-stress state, suffering damage or not; (iii) a sensitive plant is one that activates a stress response but it is not enough to cope with stress, suffers too much damage and dies; and, (iv) an avoiding plant is the individual that does not face stress, does not activate the stress response upon stress (but that could have been already activated due to intrinsic causes), and, survives (Table 1).

Table 1 Differences between plant stress outcomes under stress.

Plant outcomes under stress	Presence of damage	Activation of stress response after stress	Survival	Return to a pre-stress state
Tolerance	Yes/No	Yes	Yes	No
Resilience	Yes/No	Yes	Yes	Yes
Sensitivity	Yes	Yes	No	-
Avoidance	No	No	Yes	-

2.2. Structural and biochemical mechanisms of abiotic stress tolerance

Abiotic stressors can cause photo-oxidative stress – *i.e.* stress caused by excess excitation energy in chloroplasts. **Oxidative stress** in lipids, proteins, nucleic acids and other molecules in the chloroplast is prompted by the action of reactive oxygen species (ROS) produced by an excess of energy during photosynthesis (Demmig-Adams and Adams, 2006). Upon abiotic stress such as drought, cold, heat or salt stress, plants close their stomata resulting in a carbon fixation limitation that imbalances the electron flow in the electron transport chain because there is an excess of photons from light compared to the demand of ATP and NADPH for carbon assimilation in the Calvin cycle (Asada, 2006). This excess energy, if not quenched by non-photochemical quenching (NPQ), will lead to the production of the highly toxic ROS, singlet oxygen ($^1O_2$) in the photosystem II (PSII). In the photosystem I (PSI), if there is an overreduction of electron transport components, hydrogen peroxide (H_2O_2) will be formed from the superoxide anion ($O_2^{\cdot -}$), and, ultimately, H_2O_2 will be able to form the hydroxy radical ($OH^{\cdot}$), the most toxic of the ROS together with $^1O_2$ (Asada, 2006). In plants, it is $^1O_2$ the ROS that causes the higher photo-oxidative damage (Triantaphylidès et al., 2008), reducing PSII efficiency because there is not enough turnover of the PSII protein subunit D1, a phenomenon also defined as **photoinhibition** (Demmig-Adams and Adams, 2018). Plants have several structural and physiological mechanisms to face photoinhibition by ROS, which will be discussed in the following sections.

2.2.1. Structural mechanisms

The first thing to consider as a structural mechanism to the stressful conditions that plants face under a Mediterranean-type climate is morphology. Mediterranean species, particularly phanerophytes, tend to be of small size and bushy. This is a result of water and nutrient impoverishment and frequent natural fires (Cowling et al., 1996), which will not allow phanerophytes to grow into very big trees since there is a constant vegetation turnover. **Sclerophylly** and **trichomes** are also common traits of Mediterranean species that are destined to avoid stress – sclerophyll leaves controlling too much transpiration (di Castri, 1981; Cowling et al., 1996) and tomentosus leaves reflecting solar radiation (Ehleringer and Björkman, 1978); hence, avoiding an excess of energy. Another important attribute of Mediterranean plants that usually accompanies sclerophylly, is their high **leaf thickness** or leaf mass per unit of area (LMA). This trait is related to nutrient and water-deficient habitats since a lower transpiration surface will reduce water losses (Wright et al., 2004). While a higher LMA might seem an advantage under water stress, a trade-off between LMA and photosynthesis rates has been proposed since a higher LMA is related with lower CO_2 diffusion (Flexas et al., 2014; Peguero-Pina et al., 2017) – *i.e.* mesophyll conductance, g_m (Flexas et al., 2008). Even though Mediterranean species usually have lower photosynthetic rates compared to other plants in other biomes, there are adaptations that counterbalance the longer mesophyll pathway for CO_2 due to leaf thickness, such as having the stomata sunken in **epidermal crypts**, which allows the formation of a microenvironment with higher relative humidity (Jordan et al., 2008; Roth-Nebelsick, 2013). Despite the drawbacks a high LMA might suppose, their advantages are clear in Mediterranean habitats: increases in LMA have been related with higher photoprotection mechanisms (Fernández-Marín et al., 2017) and with longer leaf lifespans (Wright et al., 2004).

The disposition of leaf primordia along the stem during growth, or **phyllotaxis**, is a mechanism by which plants can reduce light interception. There are three main leaf phyllotactic patterns in plants: spiral, distichous and decussate. The efficiency in capturing light by these different phyllotaxis is a rather intricate topic since some studies have reported constant fluxes of solar radiation among phyllotaxis

(Sekimura, 1995) but other works have found that the decussate disposition reduces light interception efficiency by a 40-50% (Valladares and Brites, 2004). Nevertheless, it is important to note that there are other factors that affect light interception such as **leaf inclination** relative to the sun beam or internode-to-leaf-length ratio and supporting biomass (Brites and Valladares, 2005). By reducing light interception, plants are able to avoid an excess energy that could lead to photoinhibition, especially in sites where solar radiations are quite high throughout all year such as in Mediterranean-type habitats.

Plasticity in vessel diameter is an important trait to cope with drought (Hacke et al., 2016). One of the main causes of Mediterranean forest die-off is cavitation in plant vessels due to drought (Bréda et al., 2006; McDowell et al., 2008; Mitchell et al., 2013). Bigger life forms are more prone to suffer from cavitation than smaller forms. Indeed, xylem structure has been proven to be critical for cavitation resistance both inter and intra-species (Hacke et al., 2000; Kondoh et al., 2006; Guet et al., 2015; Schreiber et al., 2015; Olson et al., 2018), with larger vessels correlating with xylem collapse and smaller vessels correlating with cavitation resistance.

2.2.2. Biochemical mechanisms

At the biochemical level, the mechanisms activated to face stress will depend on the intensity of the stress. It is generally known that plants prevent and/or counteract the detrimental effects of oxidative stress by fine-tuning a myriad of protective mechanisms such as modifying their chlorophyll levels; the production of antioxidant molecules to scavenge ROS; and variations in their phytohormone contents to regulate the response to stress. Many of these protective compounds belong to the group of plant isoprenoids (Peñuelas and Munné-Bosch, 2005). Isoprenoids are synthesised *via* two pathways: the cytosolic mevalonic acid pathway (MVA pathway, derived from the five-carbon unit isopentenyl diphosphate) and the chloroplastic 2-C-methyl-D-erythritol 4-phosphate pathway (MEP pathway). From the MVA pathway, sterols, triterpenoids, sesquiterpenes and other volatile isoprenoids are produced. The MEP pathway is where most of the photoprotective pigments, antioxidants and hormones in plants are formed (Vranová et al., 2013).

Photosynthetic pigments such as chlorophylls and carotenoids are key for photosynthesis since they are light harvesters. Chlorophylls are present in high quantities in the light-harvesting complex of both photosystems and they absorb light and transfer the excitation energy from photons to the reaction centres (Grimm et al., 2006). All higher plants hold two forms of chlorophyll, chlorophyll *a* and chlorophyll *b*, but while chlorophyll *a* is present in the reaction centres and the antennae, chlorophyll *b* is only present in the light-harvesting systems (Croce, 2012). Under a severe stress, total chlorophyll (chlorophyll *a* + chlorophyll *b*) will decrease (Esteban et al., 2015) while under a milder stress, only chlorophyll *a* will be reduced resulting in a lower chlorophyll *a* to chlorophyll *b* ratio (*e.g.* Bailey et al., 2001). In fact, the ratio chlorophyll *a*/chlorophyll *b*, which reflects the proportion of chlorophyll bound by light-harvesting complexes in relation to reaction centres, is generally used as an indicator of stress acclimation (Walters, 2005). Carotenoids, apart from participating in light harvesting, dissipate excess energy through non-photochemical quenching (NPQ) and act as antioxidants, the latter role mainly performed by β-carotene, zeaxanthin and lutein (Young et al., 1997; Peñuelas and Munné-Bosch, 2005; Ramel et al., 2012). Among the carotenoids involved in thermal dissipation we find violaxanthin (Vx), antheraxanthin (Ax) and zeaxanthin (Zx), the three conforming the xanthophyll cycle (VAZ cycle). When there is excess light, Vx is reversely converted to Ax and then to Zx (Demmig-Adams and Adams, 1996; Jahns and Holzwarth, 2012). Furthermore, another carotenoid the main function of which is to participate in the correct folding of light-harvesting proteins (Formaggio et al., 2001), lutein, is also involved in thermal dissipation in some plant species through the lutein epoxide cycle (Bungard et al., 1999; García-Plazaola et al., 2007; Li et al., 2009). Finally, neoxanthin is another carotenoid the presence of which is associated with chlorophyll *b* (Takaichi and Mimuro, 1998) and that might have a scavenging role for $O_2^{\cdot -}$ (Dall'Osto et al., 2007).

Even though the transient accumulation of ROS can have beneficial signalling effects such as the chloroplast-to-nucleus retrograde communication that confers stress acclimation (Fang et al., 2019a), abundant ROS in the chloroplast can lead to irreversible damage to the cell. The key players in mitigating the effects of ROS and maintaining an appropriate balance between their production and removal in chloroplasts are **antioxidants**. There are two types of antioxidants in the chloroplast: enzymatic and nonenzymatic. Among the latter we find ascorbate and glutathione, which form the ascorbate-glutathione cycle that scavenges H_2O_2 (Foyer and Shigeoka, 2011). In addition to the ascorbate-glutathione cycle, thylakoid 2-Cys-pirirredoxins are also believed to counterbalance H_2O_2 (Awad et al., 2015).

Within the lipophilic group of nonenzymatic antioxidants, carotenoids, as aforementioned, play an important role in scavenging ROS, with β-carotene and zeaxanthin scavenging $^1O_2$ (Young et al., 1997; Havaux et al., 2007, respectively) and lutein as a quencher of the triple excited chlorophyll ($^3Chl^*$), avoiding the formation of $^1O_2$ (Dall'Osto et al., 2006). Tocochromanols, including tocopherols and plastochromanol-8 (PC-8), are crucial in protecting thylakoid membranes (Falk and Munné-Bosch, 2010; Kruk et al., 2014). Tocopherols scavenge $^1O_2$ and lipid peroxyl radicals, with α-tocopherol being the main form accumulated in plant tissues under stress (Munné-Bosch and Alegre, 2002). Indeed, α-tocopherol was proven to be a very potent antioxidant by Fahrenholzt et al. (1974) and even though there are other antioxidants that may match its efficiency such as PC-8 (Olejnik et al., 1997), still today α-tocopherol is considered the only vitamin E due to its biological activity (Azzi, 2018). However, there are other forms of tocopherol such as γ-tocopherol – which is particularly abundant in seeds – that also perform important roles under certain abiotic stresses such as cold or salt stress (Maeda et al., 2006; Abbasi et al., 2007). Finally, it is important to note that α-tocopherol and β-carotene act synergistically to prevent peroxidation by $^1O_2$ (Trebst et al., 2002). Even though α-tocopherol seems to be the key antioxidant, it is rather its action together with the other antioxidants which provides the plant with a fine-tuned response to face and tolerate stress.

Phytohormones conform an intricate signalling network that regulates plant stress response, acting at very low concentrations. The major groups of hormones plants produce are cytokinins (CKs), gibberellins (GAs), auxins (AUX), abscisic acid (ABA), ethylene (ETH) salicylic acid (SA), jasmonates (JAs), brassinosteroids (BRs) and strigolactones (SLs). While CKs, GAs, AUX and BRs are classically considered to be growth hormones, ABA, SA, JAs and ETH are normally involved in stress responses. However, their role is not that arbitrary and many of the “growth” hormones have important roles in plant stress response and *vice versa*.

ABA is considered the abiotic stress hormone par excellence since it is induced by various stresses (Swamy and Smith, 1999). ABA increases upon stress regulating responses such as growth inhibition (Planes et al., 2015), stomatal closure (Assmann, 2003), water balance (Simonneau et al., 1998) and osmotic stress tolerance (Tuteja, 2007). **SA** is involved in an arsenal of plant processes from growth to senescence and in stress tolerance (Morris et al., 2000; Sharma, 2014; Khan et al., 2015). SA are phenolic compounds that have been reported to alleviate several abiotic stresses such as salt stress (Jayakannan et al., 2013), water stress (Chini et al., 2004) or heavy metals (Sharma et al., 2020). JAs, being fatty acid-derived hormones also have a key role in abiotic stress tolerance (Santino et al., 2013). Among the most abundant **JAs** we find jasmonic acid (JA), 12-*oxo*-phytodienoic acid (OPDA), the JA precursor, and the conjugate jasmonoyl-isoleucine (JA-Ile), all three having differential roles in stress tolerance (Wasternack and Song, 2018). ETH is a gaseous hormone that has been classically denominated a stress hormone since its synthesis and accumulation increase at the onset of an environmental stress to trigger a cascade of different stress responses (Cheng et al., 2013). **CKs** are important in plant stress response since they have been reported to have a role in the maintenance of the photosynthetic apparatus under drought (Rivero et al., 2009, 2010). GAs have multiple functions in plant growth, with GA_1 and GA_4 being the most active forms, but they also have been reported to have key roles in abiotic stress adaptation (Qin et al., 2011). Indole-3-acetic acid (**IAA**) is the main **AUX** in plants and it has a remarkable role during stress acclimation (Shani et al., 2017), particularly in roots (Di Mambro, 2017).

BRs are a group of plant steroid hormones with different roles in plant development (Peres et al., 2019) and more and more studies are arising about their positive role under abiotic stressors (*e.g.* Xia et al., 2009; Fang et al., 2019b; Setsungnern et al., 2020). SLs are mainly involved in belowground plant communication, mycorrhizal symbiosis and shoot branching (Besserer et al., 2006; Mostofa et al., 2018; Barbier et al., 2019). Another regulatory molecule with a myriad of functions in plant development and stress response, sometimes considered a plant hormone, and other times an antioxidant is **melatonin** (see Arnao and Hernández-Ruiz, 2015).

Plant stress responses are not linear but rather a complex signalling network between redox balance, antioxidants and phytohormones which are species and plant developmental stage-dependent. Furthermore, although most of these compounds are originated in the same biosynthetic pathway (*i.e.* MEP pathway), the accumulation of one or another might be related and/or triggered by different environmental cues. Elucidating the **cross-talk** between these mechanisms under specific stresses as well as combinations of stresses and what are the implications for plant performance are major plant science goals that are crucial to foresee plant responses in their natural habitat as well as in crops.

3. Model of study: *Cistus albidus* L.

3.1. *Cistus* spp.

Sclerophyllous species are characteristic of Mediterranean-type ecosystems, but these usually coexist with non-sclerophyllous Mediterranean plants, which also represent a good part of the flora of Mediterranean habitats. Mediterranean non-sclerophyllous woody plants are actually believed to have had higher diversification rates since they arose, compared to typical sclerophyllous species (Verdú and Pausas, 2013).

In the Mediterranean basin, within these non-sclerophyllous woody plants, there is the genus *Cistus* (Cistaceae), which is found in the maquis habitat and has an important role in vegetation succession (Juhren, 1966). Indeed, *Cistus* shrubs have a crucial role in their habitats since not only do they form compact heathlands after a disturbance, which can impede soil erosion (Pausas and Vallejo, 1999), but they also have been related to high species richness (Saura-Mas et al., 2015). After a disturbance, particularly fires, *Cistus* species form vegetation stands near the soil that avoid soil erosion and create a microclimate, facilitating the germination and resprouting of other species (Juhren, 1966). Furthermore, most of *Cistus* spp. are pyrophytes (Le Houerou, 1973) – *i.e.* plant species the seeds of which germinate in the presence of fire – that normally favour their autosuccession (Roy and Sonié, 1992). Furthermore, *Cistus* species synthesise huge amounts of natural secondary metabolites, which makes them a good model to study specific biosynthetic pathways (Papaefthimiou et al., 2014).

3.2. *Cistus albidus* L.

Among *Cistus* spp. we find the white-leaved rockrose, *Cistus albidus* L. *C. albidus* is a phanerogam up to 1 m tall and is present in all SW Mediterranean basin, from 0 to 1,200 m.a.s.l. It belongs to the purple/pink clade of the *Cistus* genre, the flowers of which are arranged in terminal umbelliform cymes. Flowers have 5 petals, 5 sepals, 5 carpels and numerous stamens. *C. albidus* flowers in spring and fruits are ripen by autumn (Blasco and Mateu, 1995). Germination can occur during most of the year after the fruits are ripen since seeds have been proven to germinate in a wide range of temperatures, resulting in a more water dependent germination (Thanos and Georghiou, 1988). Fruits are dehiscent capsules that either remain on the plant for most of the year or dehisce in a gradual manner (Bastida and Talavera, 2002), resulting in big seed banks. Seeds are also dispersed by ants (Bastida and Talavera, 2002), a fact that allows the sufficient distance from the mother plant and, hence, the thriving of the seedlings (Beattie, 1985).

The leaves of *C. albidus* are oblong to elliptic with an opposite and decussate disposition along the stem (Figure 2). Leaves are densely covered by hairs, particularly stellate hairs accompanied by glands that contain terpenoids (Gülz et al., 1996). This results in a greyish-white appearance of the leaves, just as its common name suggests. Highly pubescent leaves are a drought tolerance adaptation since the normally high Mediterranean light radiations are reflected on them (Gausman and Cardenas, 1973), avoiding high increases in leaf temperature and hence, water loss, just as it has been previously mentioned. Another important characteristic of *C. albidus* is that they are malacophyllous shrubs, meaning that they shed leaves during a short period of time, a distinctive feature that shares with some of its other genre counterparts. Losing leaves during the unfavourable season, also termed semi-deciduous habit, reduces the total transpiring surface in the plant and, hence, avoids an extreme water loss. For *Cistus* spp. the time of shedding is normally in early summer (Orshan et al., 1989).

C. albidus has been proven to be a highly stress tolerant and resilient species, particularly to drought (Munné-Bosch et al., 2003; Grant et al., 2005; Jubany-Marí et al., 2009; Oliván and Munné-Bosch, 2010; Galle et al. 2011), but also to salt (Torrecillas et al., 2003) and cold stress (Oliveira and Peñuelas, 2004). However, some studies have reported its sensitivity to certain environmental situations. For instance, a 12% rainfall decrease resulted in a growth and leaf production reduction (Rodríguez-Ramírez et al., 2017) and subsequent drought cycles lead to increases in mortality rates (Shihan et al., 2020). Given the already happening effects of global change – *e.g.* reductions in yearly precipitation (de Luis et al., 2009) – and the predicted increases in severity and frequency of drought events (Guiot and Crammer, 2016), these detrimental consequences that have been already observed in the performance of *C. albidus* could become even worse and further affect the survival of individuals, populations and, ultimately, whole community dynamics.

Figure 2 *Cistus albidus* L. From top and left to right: *C. albidus* growing in their natural habitat in the Montserrat Mountains (SE Spain); adult plant of *C. albidus* growing in the Experimental Fields of the Faculty of Biology (University of Barcelona); seedling of *C. albidus* growing in their natural habitat in the Montserrat Mountains in May; detail of the leaf disposition of *C. albidus*. Photos: M. Pérez-Llorca.

AIMS OF THE THESIS

This dissertation aims to gain insights into the mechanisms *Cistus albidus* L., as a model Mediterranean shrub, has to cope with abiotic stress as well as to establish when these mechanisms act and the interactions between them. Particularly, within these mechanisms, a special focus will be made on the protective function of the isoprenoid pool. Furthermore, the specific tolerance this plant has to abiotic stress is aimed to be determined through means of overall performance and survival. Particular emphasis is given to drought and winter stress (*i.e.* a combination of light and low temperatures stress), since these events are increasing in intensity and are already affecting plant communities in Mediterranean ecosystems (see section 1.2).

To accomplish the general purposes of this dissertation, specific objectives were defined:

- To evaluate the effects structural photoprotection mechanisms such as leaf angle and leaf disposition have in the performance of *C. albidus* plants under Mediterranean winter conditions, most particularly under high light stress and low temperatures. Furthermore, to establish the effects of inter-individual variability in the stress response of *C. albidus*.
- To study the changes in the isoprenoid pool of *C. albidus* diurnally and seasonally as a photoprotective regulatory mechanism to face the combination of drought and chilling stress.
- To determine the trade-offs that might exist between stress hormones (*i.e.* ABA, SA and JAs) as well as other stress defences during winter conditions in *C. albidus*. Moreover, another specific objective was to assess the inter-individual variability of these stress defences.
- To establish to what extent *C. albidus* seedlings can tolerate and/or be resilient to severe drought and through which mechanisms they can achieve this.

SUPERVISORS’ REPORT

UNIVERSITAT DE BARCELONA

Departament de Biologia Evolutiva,
Ecologia i Ciències Ambientals

Barcelona, November 19th, 2020

Maren L. Müller and Sergi Munné-Bosch, as supervisors of the Doctoral Thesis entitled **"Structural and physiological mechanisms underlying abiotic stress tolerance in the Mediterranean shrub *Cistus albidus* L."** presented by doctoral student Marina Pérez Llorca,

REPORT on the impact factor and the participation of the doctoral student in each of the articles included in this dissertation

Chapter 1. Article "**Leaf orientation as part of the leaf developmental program in the semi-deciduous shrub, *Cistus albidus* L.: diurnal, positional, and photoprotective effects during winter"**, published in the journal *Frontiers in Plant Science*, impact factor (2019) of 4,407. In this work, various mechanisms of adaptation to the Mediterranean winter in white-leaved rockrose (*Cistus albidus* L.) are described, including adaptations such as changes in foliar orientation to prevent photoinhibition processes. The study of the inter-individual variability in a natural population and how this influences not only the leaf angle and possible photoinhibition processes, but also the endogenous contents of auxin, melatonin and α-tocopherol (vitamin E) is also remarkable. The PhD student carried out all the samplings, the physiological and biochemical analyses of the samples and the statistical treatment of results, and she is, therefore, the first author of the work. The PhD student has demonstrated great working capability as well as excellent sample handling and processing, as well as an excellent predisposition to be introduced to the use of high performance liquid chromatography (HPLC) for the analysis of vitamin E and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for hormone analysis. The PhD student shows excellent abilities in analyzing and interpreting the results and actively participates in the writing of the article.

Chapter 2. Article **"Contrasting patterns of hormonal and photoprotective isoprenoids in response to stress in *Cistus albidus* during a Mediterranean winter"**, published in the journal *Planta*, impact factor (2019) of 3,390. In this work, seasonal and diurnal variations in the content of various isoprenoid compounds with hormonal function and in the photoprotection of the photosynthetic apparatus are studied, thus contributing to a better understanding of the biochemical and physiological mechanisms involved in the accumulation of defense metabolites in the *C. albidus* response to drought and low temperatures during the Mediterranean winter. It is noteworthy the original experimental approach comparing compounds of the same metabolic pathway (isoprenoids), but with very different biochemical properties and defense functions. An environmental study evaluating the factors that can influence the accumulation of these defense compounds in leaves in response to abiotic stress is carried out. The PhD student performed all the samplings and analysis of the samples and was also involved in the experimental design and discussion of the results, thus being the first author of the work. The PhD student has demonstrated great working capability and excellent skills in sample processing and performing HPLC and LC-MS/MS analyses for isoprenoid analysis with proficiency. The PhD student also shows a great ability to analyze and interpret the results and actively participates in the writing of the article.

Chapter 3. Article **"Inter-individual and sun orientation driven variability reveals antagonistic salicylate and jasmonate accumulation in white-leaved rockrose"**, published in the journal *Environmental and Experimental Botany*, impact factor (2019) of 4,027. This work describes how the composition and contents of abscisic acid, salicylic acid and jasmonate change due to environmental conditions, solar orientation and by inter-individual variability in white-leaved rockrose, which grows in its natural habitat under Mediterranean conditions. Particularly noteworthy is the original experimental approach in which a plant of great ecophysiological interest is used to study the response of plants to Mediterranean winter in its natural habitat. The PhD student carried out all the samplings, the analyses of the samples, the statistical treatment and the elaboration of the results and she was also involved in the experimental design and discussion of the results, thus being the first author of the work. The PhD student has demonstrated great working capability as well as excellent sample handling and processing, showing as well excellent skills in handling HPLC and spectrophotometric techniques for the analysis of antioxidants. The PhD student also shows a great ability to analyze and interpret the results and actively participates in the writing of the article.

Chapter 4. Article **"The threshold between life and death in *Cistus albidus* L. seedlings: mechanisms underlying drought tolerance and resilience"**, submitted to be considered for publication in the journal Tree Physiology, impact factor (2019) of 3,655. In this work the ability of *C. albidus* seedlings to respond to water stress is described in detail and the limits of their tolerance are examined. Particularly noteworthy is the original experimental approach in which large numbers of individuals are exhaustively evaluated under both natural and controlled conditions, thus carrying out a great work of great robustness and scientific value. The PhD student carried out the samplings, the analysis of the samples, the statistical treatment and the elaboration of the results and was also involved in the experimental design and discussion of the results, thus being the first author of the work. The PhD student has demonstrated an extraordinary ability to work both in the field and in the laboratory and demonstrated an excellent use of HPLC and LC-MS/MS for the analysis of antioxidants and hormones, respectively. The PhD student also demonstrates a great ability to analyze and interpret the results and, more importantly, a great ability for leadership using tools for demographic analysis and complex statistical treatments.

The PhD student thus demonstrates an excellent degree of scientific maturity and was given here the opportunity to serve as corresponding author, which is also a proof of the outstanding scientific value of the PhD candidate.

And in witness whereof, and for such purposes as may arise,

Maren L. Müller   Sergi Munné-Bosch

RESULTS

Chapter 1. Leaf orientation as part of the leaf developmental program in the semi-deciduous shrub, *Cistus albidus* L.: diurnal, positional, and photoprotective effects during winter

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Leaf Orientation as Part of the Leaf Developmental Program in the Semi-Deciduous Shrub, *Cistus albidus* L.: Diurnal, Positional, and Photoprotective Effects During Winter

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Mediterranean ecosystems harbor a very important part of Earth's biodiversity, and they are a conservation priority due to the effects of global change. Here, we examined the performance of the semi-deciduous shrub *Cistus albidus* under Mediterranean conditions during winter, including changes in leaf angle governed by diurnal, seasonal, and positional effects and their relationship with winter photoinhibition and photoprotection. We found marked diurnal variations in leaf angle during the day in autumn, which disappeared as the photoperiod shortened during winter due to a progressive decrease in the predawn leaf angle from November to January. During this period, auxin contents decreased, while those of melatonin increased, and the F_v/F_m ratio, chlorophyll, and tocopherol contents kept unaltered, thus indicating the absence of photoinhibitory damage. A marked decrease in the leaf angle toward the shoot apex occurred during winter, which was associated with slightly higher F_v/F_m ratios. An analysis of the inter-individual variability and sun orientation effects on leaf movements in a population growing in the Montserrat mountains revealed a very marked variability of 46.8% in the leaf angle, while F_v/F_m ratio showed a variation of 7.5% only. West orientation, which was associated with reduced leaf temperatures, but with no differences in the photosynthetic photon flux density, led to enhanced tocopherol contents, while leaf angle, F_v/F_m ratio, chlorophyll, auxin, and melatonin contents kept unaltered. It is concluded that (1) *C. albidus* has very effective and fine-regulated photoprotection mechanisms, including an adequate orientation of decussate leaves as part of the developmental program, (2) leaf angle is modulated on a diurnal and seasonal basis, thus contributing to prevent photoinhibition as a first line of defense, and (3) enhanced tocopherol contents help withstand combined high light with low temperature stress in *C. albidus* growing at high elevation.

Keywords: abiotic stress, *Cistus albidus* L., leaf orientation, leaf positional effects, low temperature stress, melatonin, photoinhibition, photoprotection

INTRODUCTION

The Mediterranean basin is one of the most unique biomes that harbors an important portion of Earth's plant biodiversity. Mediterranean ecosystems have been described as especially delicate upon the predicted extreme climatic events due to climate change (Peñuelas et al., 2017), and given that there is already a constant loss of biodiversity (Butchart et al., 2010), Mediterranean plant species are a conservation priority (Bellard et al., 2014). Light has been classically considered as one of the main stressors in Mediterranean-type ecosystems for plants (Joffre et al., 1999; Karavatas and Manetas, 1999), particularly excess of light, which together with drought (typically occurring during summer) or low temperatures (during winter) can cause considerable damage to the photosynthetic apparatus (Martínez-Ferri et al., 2004; Fernández-Marín et al., 2017).

Mediterranean plants have evolved different photoprotection mechanisms to avoid this excess of light, building a first line of defense. There are structural adaptations to avoid excess of light absorption such as tomentosus leaves that reflect light (Ehleringer and Björkman, 1978), chloroplast movements during the day (Haupt and Scheuerlein, 1990), changes in leaf orientation (Ehleringer and Comstock, 1987), paraheliotropism (Lambers et al., 2008), and a phyllotaxis with an opposite disposition of leaves (Brites and Valladares, 2005). In the case of leaf paraheliotropism, for instance, not all plants species exhibit it but it has developed in multiple lineages, including the well-known common bean (*Phaseolus vulgaris* L.), where this movement is triggered by an endogenous circadian oscillator and light-induced signals that lead to turgor-dependent changes in the pulvinus, which is located at the juncture of the leaf base and the petiole (Mayer et al., 1985). In several Mediterranean plants, such as the white-leaved rockrose (*Cistus albidus* L.), reduced leaf angles occur as part of the developmental program with opposite and decussate leaves (without petioles) increasing their leaf angle as they develop at the shoot apex and progressively occupy more distal positions (De Bolòs, 1993).

Once light has gone through the structural photoprotection mechanisms, it is absorbed by leaves and penetrates the chloroplasts, plants activate a second line of defense to counteract excess solar radiation. The first and one of the most flexible mechanisms to counteract the excess excitation energy and, consequently, photo-oxidative stress in chloroplasts is the xantophyll cycle (Demmig-Adams and Adams, 2006). However, if the excited triple chlorophyll (^{3}Chl*) derived from excited chlorophyll a (^{1}Chl*) is not quenched by the non-photochemical quenching (NPQ) in the photosystem II, this can lead to the production of singlet oxygen ($^1O_2$) and ultimately to the oxidation of lipids and other molecules in the chloroplast. At the same time, O_2 can also be over-reduced by the electrons originated in the photosystem I to superoxide radicals (O_2^-) and then form hydrogen peroxide (H_2O_2) and finally hydroxyl radical (OH^-), which is highly reactive (Asada, 2006). The joint effects of these ROS can cause photoinhibition, and if this is sustained, plants will experience photo-oxidative damage (Takahashi and Badger, 2011). This can be prevented by the concerted action of a myriad of antioxidant compounds. Among them, lipophilic compounds, such as tocopherols (vitamin E), have been shown to play a major role in photoprotection (Havaux et al., 2005) acting as a sentinel for stress sensing and signaling (Munné-Bosch, 2019). On the other hand, melatonin, a tryptophan-derived compound (sharing part of the biosynthesis pathway with the phytohormone indole-3-acetic acid, Pérez-Llorca et al., 2019) has been postulated as a putative antioxidant and photoprotective compound in plants (Ding et al., 2017). However, it is still not fully elucidated whether or not melatonin always acts directly as an antioxidant or indirectly through its modulatory effects on gene expression (Arnao and Hernández-Ruiz, 2019).

The aim of this study was to get new insights into the performance of the semi-deciduous shrub, *C. albidus* during high light stress and suboptimal (low) temperatures during the Mediterranean winter, with a particular emphasis on the causes and consequences of changes in leaf orientation as part of the developmental program. We hypothesized that leaf movements may be governed on a diurnal and seasonal basis and that they may significantly influence the extent of photoinhibition and photoprotective demand of leaves during winter. To test this hypothesis, we measured (1) leaf angles during predawn and midday from November to January, (2) leaf positional effects on leaf angles during winter (January), and (3) the inter-individual variability and sun orientation influence on leaf angles and the F_v/F_m ratio in a natural population growing at 1,100 m.a.s.l., which was exposed to a combination of high light and low temperatures. Furthermore, we studied the endogenous variations in melatonin in relation to a biosynthesis-related phytohormone (auxin) and a well-known chloroplastic antioxidant (α-tocopherol) to get new insights into the possible protective effects of this compound against environmental stress in plants.

MATERIALS AND METHODS

Experimental Design and Sampling

Three independent, complementary experiments were performed using white-leaved rockrose (*Cistus albidus* L.). The first experiment was focused on the study of diurnal variations in leaf angle from autumn to winter, including measurements at predawn (1 h before sunrise) and midday (at maximum diurnal photosynthetic photon flux density; PPFD). The samplings were performed on November 6 and December 4, 2017 (autumn), and January 16, 2018 (winter) in a population growing in the experimental garden of the Faculty of Biology at the University of Barcelona (41.384 N, 2.119E, 59 m.a.s.l.). All measurements from this study were made using leaves situated on the second whorl of the plant (second leaf position) from the top of shoots. The second experiment was focused on the study of leaf positional effects on leaf angle during winter. Leaves situated on the first, second, third, fourth, and fifth whorl (designated here, arbitrarily, as positions 1–5 and corresponding to two different leaf dispositions on the stem) from the top of shoots of the same plants as those used for the first experiment were selected for measurements during January 16, 2018 at midday (at maximum diurnal PPFD). The third experiment focused on sun orientation and inter-individual driven variability in the leaf angle of a natural population in the Natural Park of the Montserrat mountains, Spain (41.586 N–1.830 E, 1100 m.a.s.l.) during March 22, 2018. A stressful cold and sunny day was selected for measurements, with maximum and minimum air temperatures during the day of 14.7°C and −0.2°C, respectively (mean relative humidity was 30% and maximum diurnal PPFD 1570 μmol m^{-2} s^{-1}, while no precipitation during the day was recorded). Sixty individuals of the same population, but with different sun orientations, were sampled; 30 were East oriented and 30 were West oriented (with an average leaf temperature of 11°C and 19°C, respectively, with no difference in the average relative humidity and maximum diurnal PPFD). All measurements for this study were made using leaves situated on the second whorl (position 2) from the top of shoots in all individuals. Details of soil characteristics of both sites (experimental garden from the University of Barcelona and the Montserrat mountains) can be found in Müller et al. (2013).

For the three experiments, all samplings at each time point were performed using two leaves of the same whorl per shrub from same-aged individuals that were randomly selected at the beginning of the study. Aside from leaf angle, which was described with the zenith and the azimuth angles of the surface normal to a small flat plate (Itakura and Hosoi, 2018), leaf biomass, leaf mass per area ratio, leaf hydration, chlorophyll contents, F_v/F_m ratio, indole-3-acetic acid (IAA), melatonin, and α-tocopherol contents were measured as described below. For all biochemical analyses, samples were immediately snap frozen in liquid nitrogen and stored at −80°C until subsequent processing in the laboratory.

Environmental Conditions, Photoinhibition, and Other Physiological Markers

PPFD, leaf temperature, and the F_v/F_m ratio (after 1 h of darkness), the latter used as an indicator of photoinhibition (Bolhar-Nordenkampf et al., 1991), were measured with a Mini-PAM II (Photosynthesis Yield Analyzer, Walz, Germany) *in situ*. Leaves were immediately weighed to estimate leaf biomass and leaf area was measured using a flatbed scanner (model Officejet Pro 8610, HP, California, USA). Then, dry mass was estimated by weighing the sample after oven drying it at 65°C to constant weight. Leaf hydration (H) was calculated as (fresh mass-dry mass)/dry mass, and leaf mass per area (LMA) was calculated as dry mass/area.

Biochemical Analyses

The chlorophyll a + b content of leaves was determined spectrophotometrically in methanol extracts using the equations described by Lichtenthaler (1983). Melatonin and auxin contents were determined by ultrahigh-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS), and α-tocopherol was determined by high-performance liquid chromatography (HPLC). In short, 50 mg per sample was extracted with 250 μl of cold methanol using ultrasonication and vortexing (Branson 2510 ultrasonic cleaner, Bransonic, Danbury, CT, USA) for 30 min. Deuterium-labeled indole-3-acetic and melatonin were then added, and after centrifugation at 12,000 rpm for 10 min at 4°C, the pellet was re-extracted using the same procedure. Supernatants were pooled and filtered through a 0.22-μm PTFE filter (Waters, Milford, MA, USA) before UHPLC-MS/MS and HPLC analyses. Indole-3-acetic and melatonin contents were analyzed by using UHPLC-ESI-MS/MS as described in Müller and Munné-Bosch (2011). Quantification was made considering recovery rates for each sample by using deuterium-labeled internal standards. α-Tocopherol contents were determined by using a normal-phase HPLC system coupled to a fluorescent detector as described in Cela et al. (2011). α-Tocopherol was identified by co-elution with an authentic standard (Sigma-Aldrich, Steinheim, Germany) and quantified using a calibration curve.

Statistical Analysis

To assess the combined effects of month ("November," "December," "January") and daytime ("Predawn," "Midday") on leaf angle, leaf biomass, leaf mass per area, hydration, chlorophyll a + b, F_v/F_m ratio, IAA, melatonin, and α-tocopherol, we used a linear mixed model (LMM). Different combinations of "Month," "Daytime," and their interaction were tested as fixed terms using Akaike information criterion (AIC), i.e., the model that best fitted the data should have the lowest AIC value. "Plant" was included in all models as a random term to account for repeated measures (Zuur et al., 2009). Models were fitted with maximum likelihood (ML) for model comparison. Final models were fitted using restricted maximum likelihood (REML). Additionally, when "Daytime" had a significant effect, an LMM with "Daytime" as an explanatory variable was used to test for significant differences within each month. To assess significant differences within leaf position on the response variables above mentioned, we used a LMM where "Leaf position" was fitted as the fixed term and "Plant" as a random term. To assess the effect of sun orientation in the Montserrat natural-growing population, a linear regression was used with "Orientation" as explanatory variable. In all models, the p of the main fixed effects were estimated using conditional F-tests (generally, ANOVA type II or ANOVA type III when an interaction term was included), and when main effects were significant, multiple comparisons between "Month" and "Daytime," or "Leaf position" levels, were tested with the Tukey HSD post hoc test. All models were validated by visually checking the distribution of residuals for normality and homoscedasticity (Zuur et al., 2009). Before analyses, leaf angle, chlorophyll a + b, IAA, and melatonin were $\log_{10}$-transformed; leaf mass per area and α-tocopherol were square root-transformed; and F_v/F_m ratio was logit-transformed. Finally, to explore possible relationships between all response variables, we used the Spearman's rank correlation test. All analyses were performed using the computing environment R (R Core Team 2019).

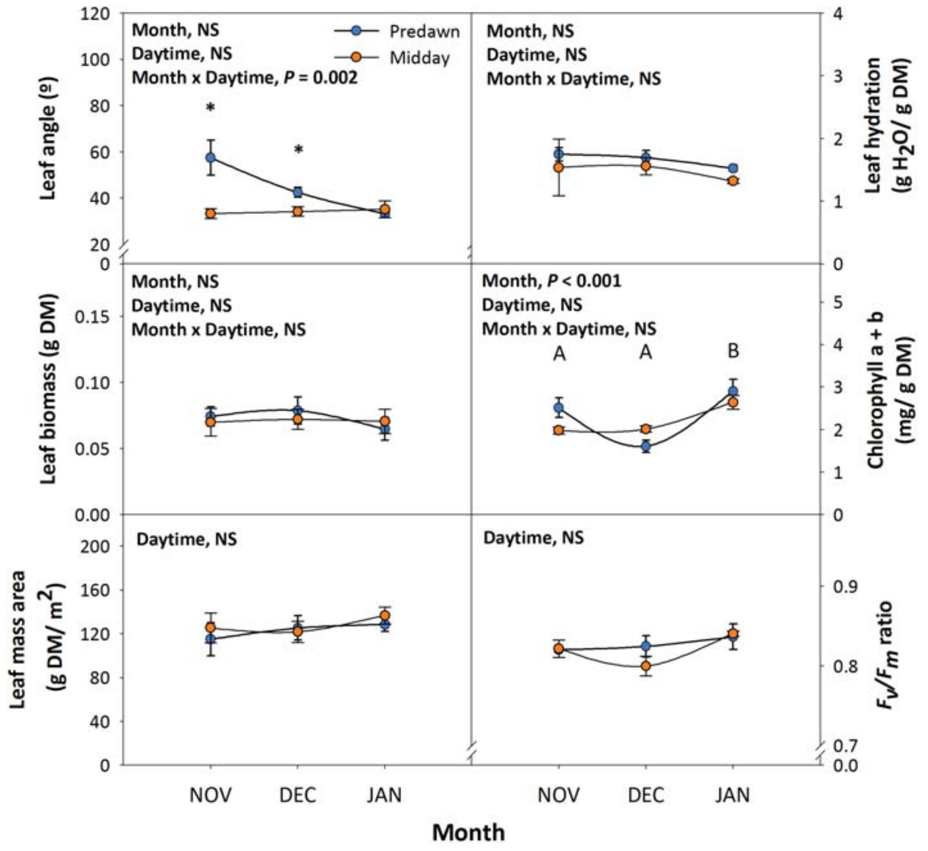


FIGURE 1 | Diurnal and seasonal changes in leaf orientation in the Mediterranean shrub, *C. albidus*. A comparison of leaf angle, biomass, mass per area ratio, hydration, chlorophyll contents, and the F_v/F_m ratio between predawn and midday from autumn (November) to winter (January) in plants growing at the experimental garden of the University of Barcelona is shown. Data correspond to leaf position 2 from the apex (see photograph in **Figure 3** for details) and represent the mean ± SE of $n = 6$ individuals. After model selection, significant differences in "Month" and "Daytime" were tested by conditional Fisher tests ($p \leq 0.05$). Different capital letters indicate significant differences in Tukey's HSD multiple comparison test ($p \leq 0.05$) with all data. Asterisks indicate significant differences in Tukey's HSD multiple comparison test in "Daytime" within "Month". NS, not significant; DM, dry matter.

TABLE 1 | Environmental conditions during the sampling days both in the Experimental garden at the Faculty of Biology of the University of Barcelona and in the Natural Park of the Montserrat mountains.

	Min *T*ᵉ (°C)	Max *T*ᵉ (°C)	Mean *T*ᵉ (°C)	Mean RH (%)	Mean PPFD (μmol/m² s⁻¹)	Max PPFD (μmol/m² s⁻¹)
Experimental garden						
6th November	10.0	17.8	13.2 ± 0.34	33.56 ± 2.05	671 ± 79.45	989
4th December	3.9	15.2	9.2 ± 0.45	41 ± 1.37	459 ± 84.80	892
16th January	7.9	18.0	12.8 ± 0.46	58.2 ± 0.67	330 ± 85.25	936
Montserrat mountains						
22nd March	−0.2	14.7	4.18 ± 0.93	51.09 ± 3.53	510 ± 68.35	2531

Min Tᵉ, minimum temperature; Max Tᵉ, maximum temperature; Mean Tᵉ, mean temperature; Mean RH, mean relative humidity, Mean PPFD, mean daily photosynthetic photon flux density; Max PPFD, maximum photosynthetic photon flux density. Climate data were obtained from a public database (Servei meteorològic de Catalunya).

RESULTS

Leaf Angle Is Modulated on a Diurnal and Seasonal Basis in *C. albidus*

Leaf angle measurements at predawn and midday during 6th November, 4th December, and 16th January revealed significant diurnal variations in leaf orientation. Leaves had a reduced angle during the day in autumn (November and December) but not in winter (January), when maximum orientation parallel to sun's rays was attained already at predawn (**Figure 1**). It is interesting to note that despite diurnal variations in leaf orientation in autumn, maximum orientation parallel to the sunbeam was always attained at midday, with leaf angles around 35°, irrespective of the day of measurements (**Figure 1**). Diurnal PPFD during

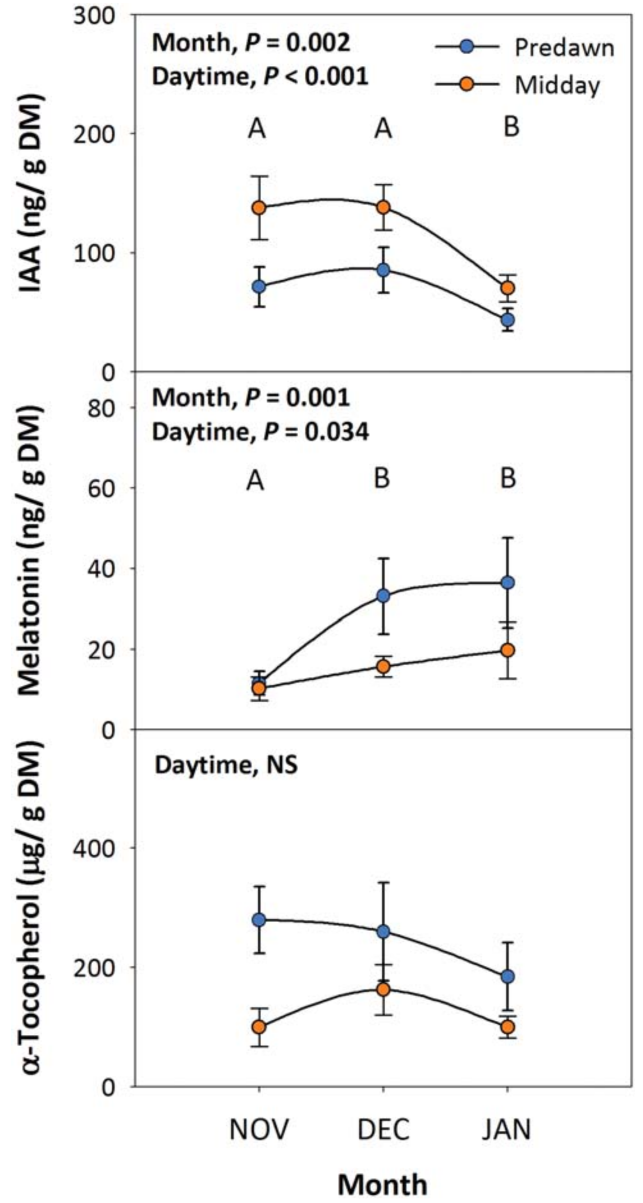


FIGURE 2 | Diurnal and seasonal changes in endogenous melatonin contents in the Mediterranean shrub, *C. albidus*. A comparison between melatonin contents relative to those of the biosynthesis-related phytohormone auxin and the well-known chloroplastic antioxidant α-tocopherol between predawn and midday from autumn (November) to winter (January) in plants growing at the experimental garden of the University of Barcelona is shown. Data correspond to leaf position 2 from the apex (see photograph in **Figure 3** for details) and represent the mean ± SE of *n* = 6 individuals. After model selection, significant differences in "Month" and "Daytime" were tested by conditional Fisher tests ($p \leq 0.05$). Different capital letters indicate significant differences in Tukey's HSD multiple comparison test ($p \leq 0.05$) with all data. Asterisks indicate significant differences in Tukey's HSD multiple comparison test in "Daytime" within "Month". NS, not significant; DM, dry matter.

this period changed from non-detectable values at predawn to values ranging between 892 and 989 μmol m^{-2} s^{-1} at midday, while the photoperiod was shortened from 10.3 h on 6th November to 9.5 h on 16th January. Leaf biomass, leaf mass per area ratio, and leaf hydration kept unaltered throughout the study (**Figure 1**). Despite chlorophyll contents decreased slightly on 4th December at predawn, which might be associated with temperatures reaching minimum values of 3.9°C (**Table 1**), the F_v/F_m ratio, an indicator of photoinhibition, kept unaltered throughout the study (**Figure 1**).

To investigate the possible protective effects of melatonin against environmental stress in plants, we studied the endogenous variations in melatonin in relation to its biosynthesis-related phytohormone auxin (indole-3-acetic acid, IAA) and a well-known chloroplastic antioxidant (α-tocopherol). Endogenous auxin contents decreased as the season progressed from autumn to winter, and values at midday were higher than those at predawn, whereas melatonin contents increased slightly during the same period with higher values at predawn than at midday (**Figure 2**). While melatonin contents were on the same order of magnitude than those of auxin, they were, however, four orders of magnitude smaller than those of α-tocopherol, the levels of which kept unaltered throughout the study (**Figure 2**).

Positional Effects on Leaf Orientation in *C. albidus*

Leaf angle measurements at midday during 16th January revealed a marked positional effect on leaf orientation, with opposite, decussate leaves progressively showing an increased leaf angle from top to bottom of the shoot (**Figure 3**). Leaf biomass and leaf mass per area ratio kept unaltered with leaf position, while leaf hydration slightly increased by a 20% from leaf position 1 to 5 (**Figure 3**). Despite chlorophyll contents kept unaltered with leaf position, the F_v/F_m ratio decreased slightly with leaf opening. It is noteworthy, however, that this ratio kept always above 0.80 in all studied leaf positions (**Figure 3**).

Positional effects on the endogenous variations in melatonin in relation to auxin and α-tocopherol revealed that, although endogenous auxin contents were different between even and odd whorls, neither auxin nor melatonin contents were altered by increased leaf angle from top to bottom (**Figure 4**). In contrast, the contents of α-tocopherol increased concomitantly with less steep leaf angles from top to bottom (**Figure 4**).

Influence of Sun Orientation and Inter-Individual Variability on Leaf Angle in *C. albidus*

Leaf angle measurements at midday during 22nd March in a natural population of *C. albidus* growing in the Montserrat mountains at 1,100 m.a.s.l. revealed a strong variability – quantified as percentage deviation (standard deviation/population mean) × 100 – on leaf orientation. Leaf at position 2 from top of the shoot from 60 individuals showed angles ranging from 10 to 80° (**Figure 5**). The variation in the leaf angle of both West-oriented and East-oriented individuals was much higher than that of leaf biomass, leaf mass per area ratio, leaf hydration, chlorophyll contents, and the F_v/F_m ratio (**Figure 5**). Indeed, variability in leaf angle was 46.8%, while that of the F_v/F_m ratio was 7.5% only. West orientation, which was associated with markedly reduced leaf temperatures and slightly lower leaf water contents compared to the East – but

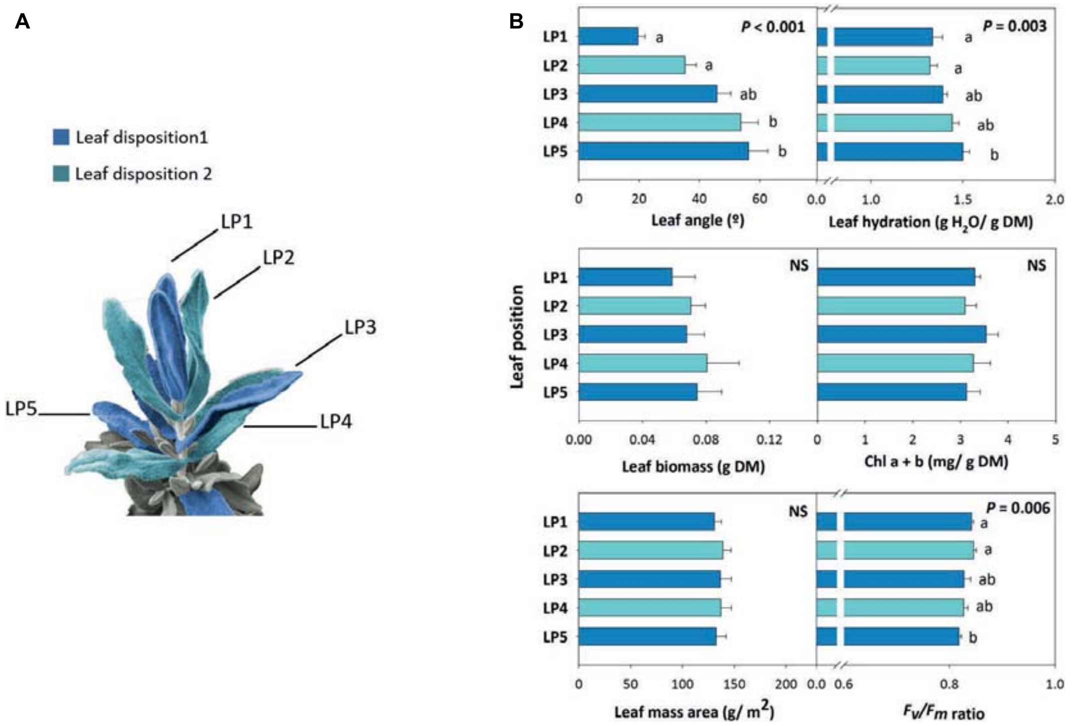


FIGURE 3 | Positional effects in leaf orientation in the Mediterranean shrub, *C. albidus*. **(A)** Schematic representation of the disposition on the shoot (Leaf disposition 1 and 2 shown in dark blue and light blue, respectively) of the five leaf positions (LP1…LP5). **(B)** A comparison between leaf positions on leaf angle, biomass, mass per area ratio, hydration, chlorophyll contents, and the F_v/F_m ratio in plants growing at the experimental garden of the University of Barcelona in January at midday is shown. Data represent the mean ± SE of $n = 6$ individuals. Significant differences in "Leaf position" were tested by conditional Fisher tests ($p \leq 0.05$), and different letters indicate significant differences in Tukey's HSD multiple comparison test ($p \leq 0.05$). NS, not significant; DM, dry matter.

with no differences in the PPFD (**Figure 5**, see also materials and methods for details) –, led to enhanced tocopherol contents, while leaf angle, F_v/F_m ratios, chlorophyll, IAA, and melatonin contents kept unaltered (**Figures 5, 6**).

DISCUSSION

Several semi-deciduous shrubs that represent an important hotspot of the Mediterranean flora, such as the white-leaved rockrose (*Cistus albidus* L.), show changes in leaf angle as part of their developmental program with opposite and decussate leaves without petioles increasing their leaf angle as they develop from top to bottom of the shoot apex. An enhanced leaf angle in more distal positions from the uppermost side of the shoot progressively leads to a leaf orientation more perpendicular to the sun's rays, which results in an increased exposure to high light. Therefore, the youngest leaves, which develop later in time, are the ones with smaller leaf angles and, therefore, enhanced photoprotection due to reduced light incidence. There are several studies in *Cistus* spp. that report that higher leaf inclinations (i.e., steeper leaf angles) prevent photoinhibition and confer a higher stress tolerance during summer drought (*C. incanus*, Grattani and Bombelli, 2000; *C. monspeliensis*, Werner et al., 1999, 2001; *C. ladanifer*, Brites and Valladares, 2005; *C. salviifolius* and *C. monspeliensis*, Puglielli et al., 2017). However, the implications of these leaf movements to prevent winter photoinhibition have not been considered in detail thus far, with the exception of the study performed by Oliveira and Peñuelas (2002), which showed reduced cold-induced photoinhibition in leaves with smaller leaf angles and therefore less sun-exposed leaves in *C. albidus*. Here, we show that changes in leaf angle are governed by leaf position, as part of the leaf developmental program of shoots, and by environmental conditions both diurnally and seasonally, all providing a first line of defense to prevent photoinhibition in this plant species. At the same time, we also show that, in nature, the variability in leaf angles is so high that other mechanisms operating at the chloroplast level, such as an enhanced vitamin E biosynthesis, are required for counteracting the potential harmful effects of combined high light with low temperatures in populations growing at high elevation.

Leaf orientation was not only influenced by the intrinsic shoot developmental program in *C. albidus,* but it also changed on a diurnal and seasonal basis, both factors interacting between them. As it occurs with paraheliotropism in some legumes (Mayer et al., 1985; Raeini-Sarjaz, 2011), the leaf angle seemed to be governed to some extent, at least, by circadian rhythms in *C. albidus* since leaves had a steeper angle at midday compared to predawn, particularly in autumn. However, the absence of a petiole and

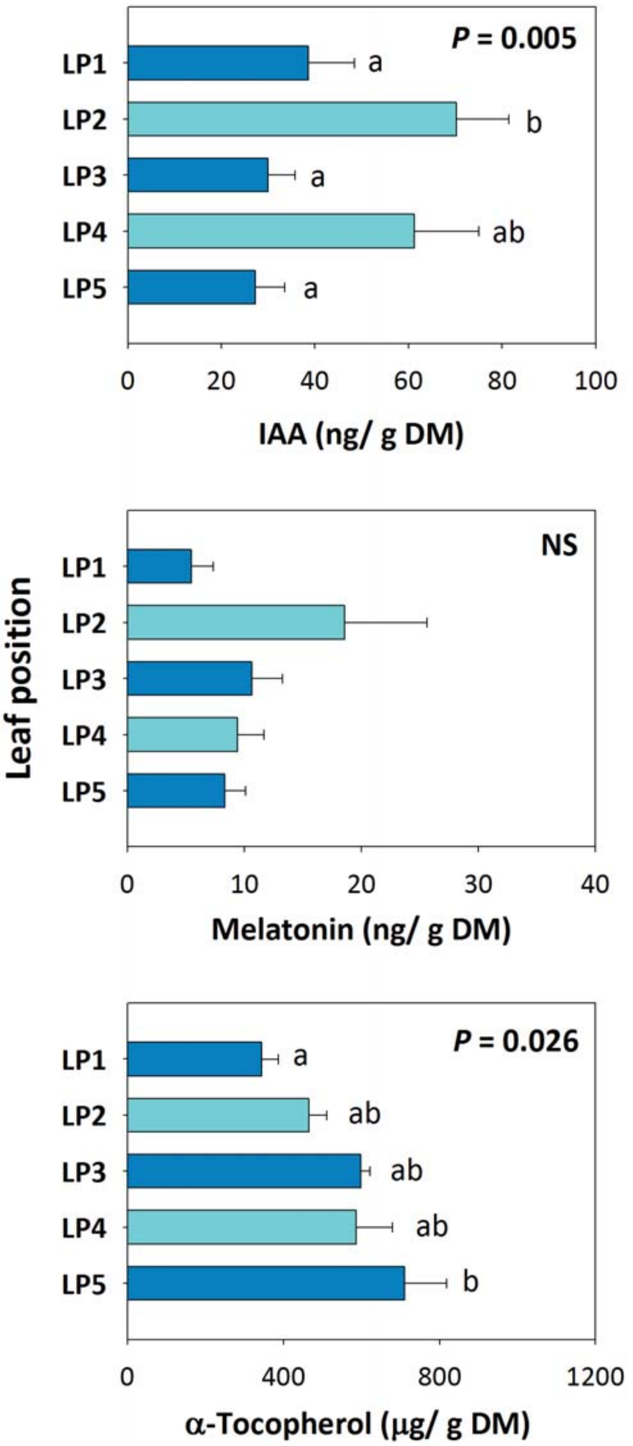


FIGURE 4 | Positional effects in endogenous melatonin contents in the Mediterranean shrub, *C. albidus*. A comparison between melatonin contents relative to those of the biosynthesis-related phytohormone auxin and the well-known chloroplastic antioxidant α-tocopherol in plants growing at the experimental garden of the University of Barcelona during January at midday is shown. Data represent the mean ± SE of $n = 6$ individuals. Significant differences in "Leaf position" were tested by conditional Fisher tests ($p \leq 0.05$), and different letters indicate significant differences in Tukey's HSD multiple comparison test ($p \leq 0.05$). NS, not significant; DM, dry matter.

most probably of a pulvinus in *C. albidus* leaves suggests that this movement is not as "active" as in some legumes such as common bean (Lambers et al., 2008), but the result of a more "passive" process associated with the plant developmental program. However, the fact that leaves move on a diurnal basis indicates that this leaf movement responds to light and can, therefore, be considered a type of paraheliotropism, as recently suggested by Puglielli et al. (2017) and *sensu* Häder and Lebert (2001).

Enhanced tocopherol contents were observed in the West-oriented individuals compared to the East-oriented ones, which might help withstand combined high light with low temperature stress in *C. albidus* growing at high elevation. Mean leaf temperature during samplings in the West orientation was 11°C, in contrast to the 19°C in East orientation, while the PPFD did not differ between both orientations. Reduced leaf temperature in the West led to a slightly reduced leaf hydration, which may be associated with dehydration caused by chilling (Hussain et al., 2018). In turn, low temperatures have been shown to increase α-tocopherol contents in other plant species as a mean to counteract ROS production and allow stabilization of membranes (Munné-Bosch, 2005; El Kayal et al., 2006). Indeed, tocopherols may not only protect the photosynthetic apparatus during plant exposure to extreme temperatures but also influence retrograde signaling, thus acting in stress sensing and signaling (Fang et al., 2019; Munné-Bosch, 2019). Interestingly, the F_v/F_m ratio did not differ between the West and East orientation, thus suggesting that enhanced tocopherol contents in the West orientation served as a photoprotective strategy, as it has been previously shown in model plant species (Havaux et al., 2005). By contrast, melatonin contents did not increase in response to low temperature stress in the West-oriented individuals, despite exogenous applications of this compound have been shown to protect the photosynthetic apparatus from excess light stress, either caused by drought (Fleta-Soriano et al., 2017) or low temperatures (Ding et al., 2017). Inter-individual variability in melatonin contents was very high, as it occurred with leaf angles, which might mask a possible protective effect of these two mechanisms in populations growing under natural conditions. Indeed, the endogenous contents of melatonin found (in the order of ng/g dry matter, even lower than those found for indole-3-acetic acid) in *C. albidus* leaves suggest a modulatory role of this compound in plants, rather than a direct function as an antioxidant, which is in agreement with previous studies (Fleta-Soriano et al., 2017, see also Arnao and Hernández-Ruiz, 2019).

Correlation analyses revealed interesting relationships between various parameters in the natural population, both when the analysis included all data and the East and West individuals separately (**Figure 7**). In the natural population from the Montserrat mountains, leaf angle did not correlate with any of the other studied parameters but the F_v/F_m ratio correlated very significantly, strongly, and positively with leaf hydration ($\rho = 0.68$, $p < 0.001$), which might indicate a prevalent occurrence of increased photoinhibition in the more water-stressed leaves. Small reductions in leaf hydration, which may be caused by chilling stress in the West-oriented individuals, could explain this result. This correlation was confirmed when all data were pooled together, although the relationship was

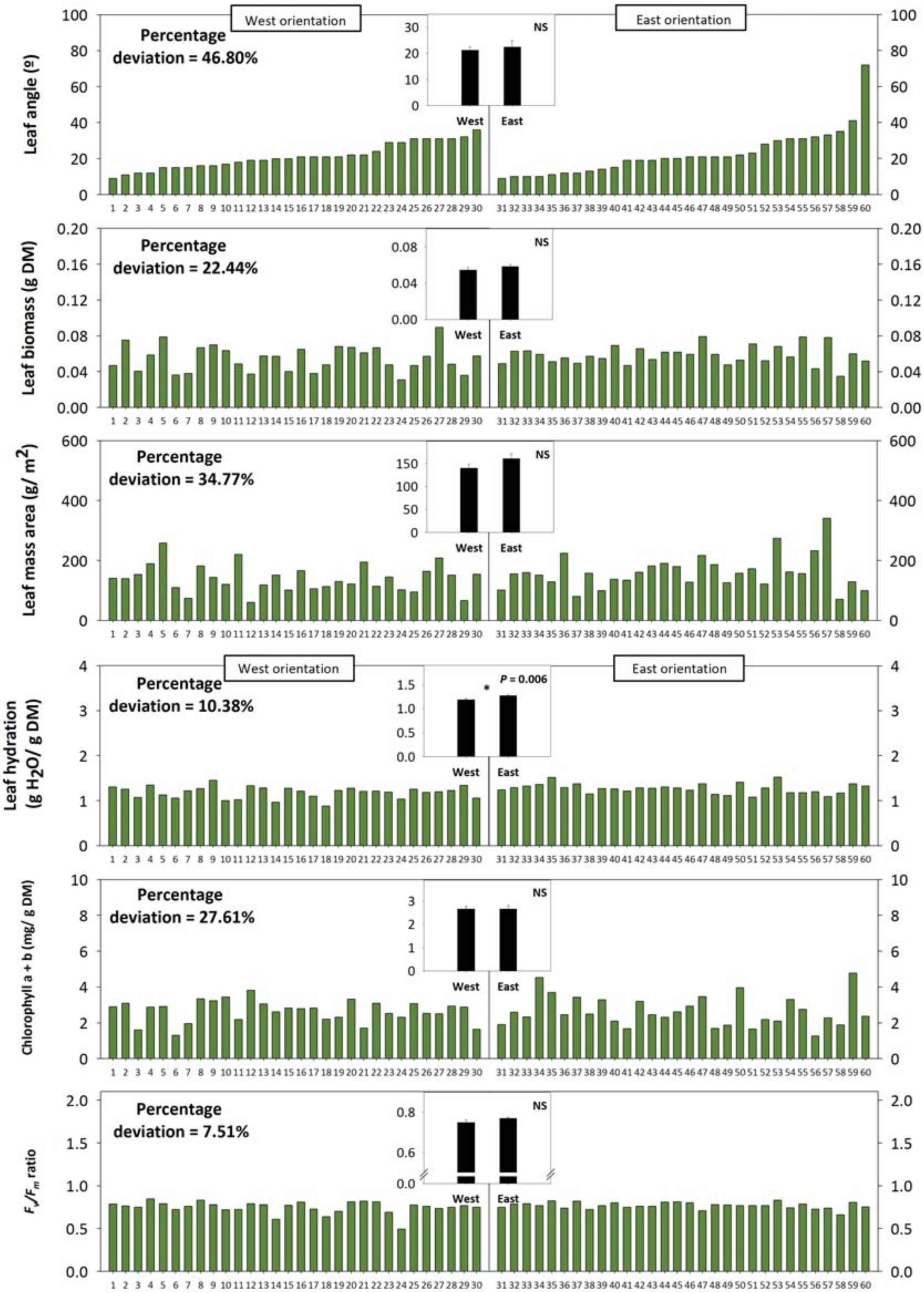


FIGURE 5 | Effects of sun orientation and inter-individual variability on leaf orientation in the Mediterranean shrub, *C. albidus*. A comparison between leaf angle, biomass, mass per area ratio, hydration, chlorophyll contents, and the F_v/F_m ratio in a *C. albidus* population growing at 1,100 m.a.s.l. in the Natural Park of the Montserrat Mountains with different sun orientation (East orientation, $n = 30$ individuals; West orientation, $n = 30$ individuals) during winter (22nd March 2018) is shown. Data correspond to leaf position 2 from the apex (see photograph in **Figure 3** for details) for all studied individuals. The number of the individual (ordered from lower to higher leaf angle) is indicated in the x-axis. Percent deviation for every variable is given as (SD/Population mean) × 100, where SD corresponds to standard deviation. The mean ± SE for each sun orientation is shown in the graphs located in the inlets. Significant differences between groups were tested by a one-way analysis of variance (ANOVA, $p \leq 0.05$) and indicated with an asterisk. NS, not significant.

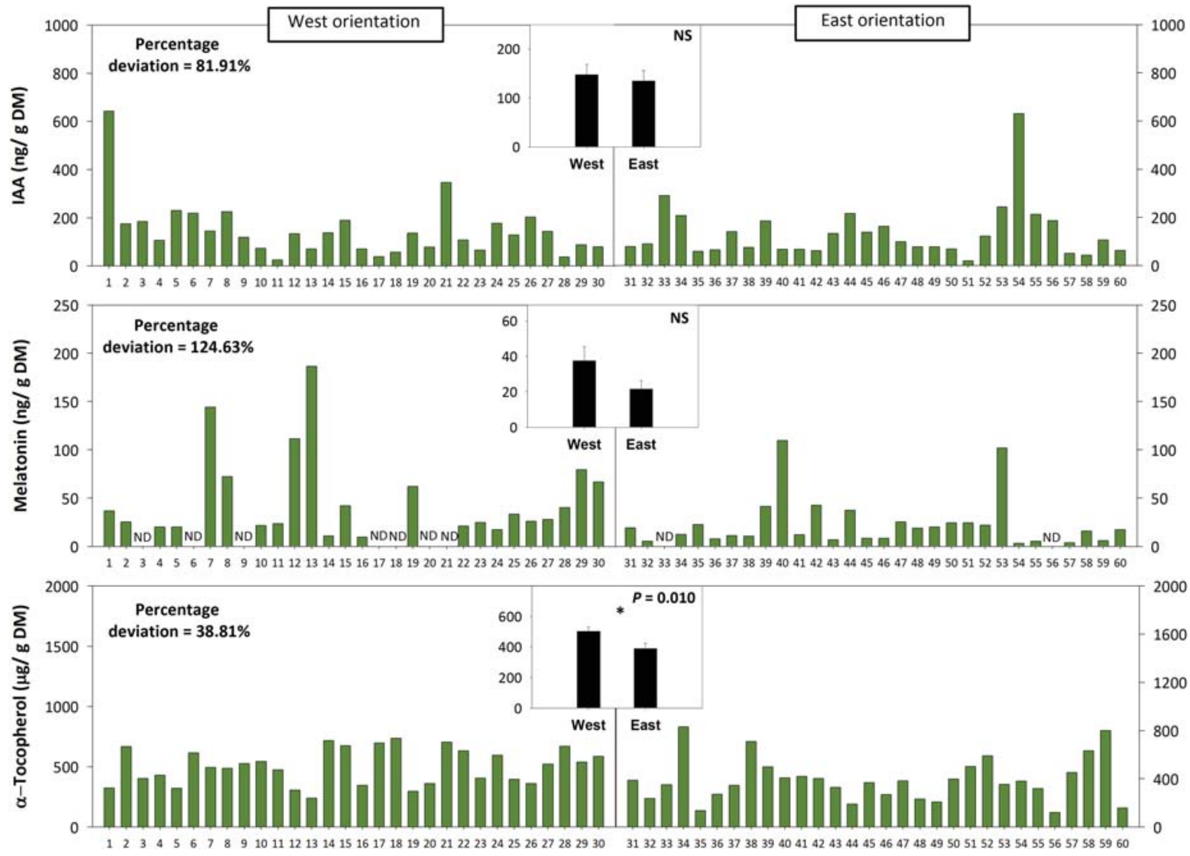
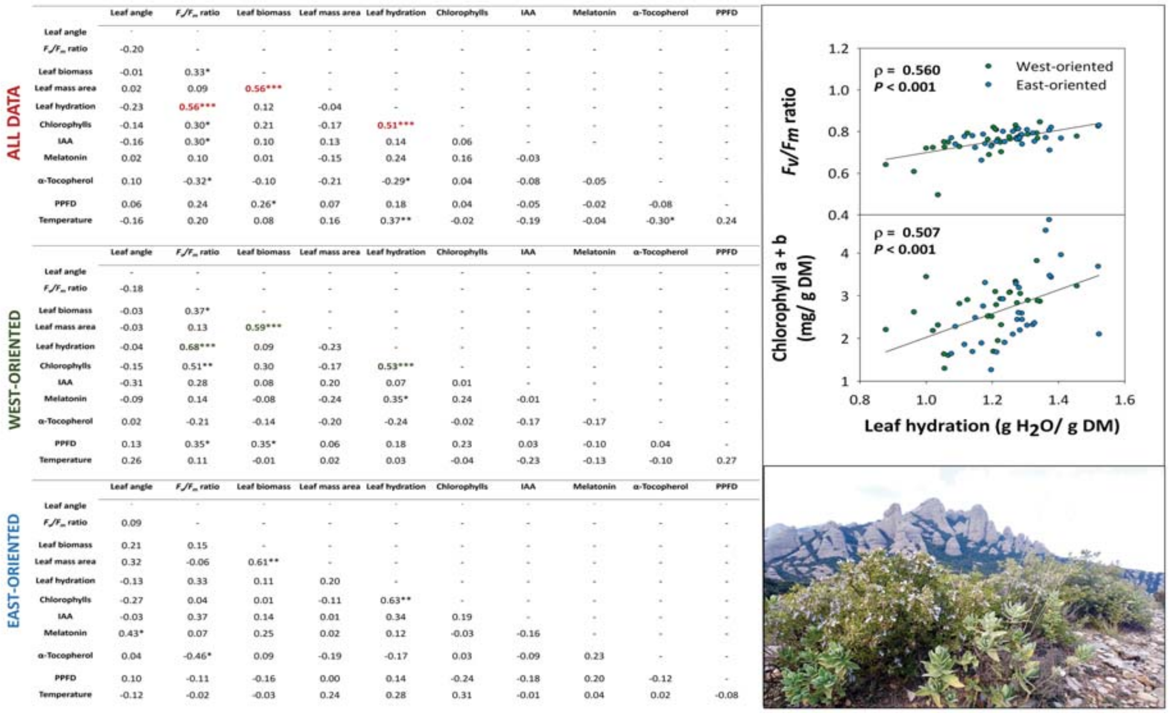


FIGURE 6 | Effects of sun orientation and inter-individual variability on endogenous melatonin contents in the Mediterranean shrub, *C. albidus*. A comparison of melatonin contents relative to those of the biosynthesis-related phytohormone auxin and the well-known chloroplastic antioxidant α-tocopherol in a *C. albidus* population growing at 1,100 m.a.s.l. in the Natural Park of the Montserrat Mountains with different sun orientation (East orientation, *n* = 30 individuals, West orientation, *n* = 30 individuals) during winter (22nd March 2018) is shown. Data correspond to leaf position 2 from the apex (see photograph in **Figure 3** for details) for all studied individuals. The number of the individual (ordered from lower to higher leaf angle) is indicated in the x-axis. Percent deviation for every variable is given as (SD/population mean) × 100, where SD corresponds to standard deviation. The mean ± SE for each sun orientation is shown in the graphs located in the inlets. Significant differences between groups were tested by a one-way analysis of variance (ANOVA, $p \leq 0.05$) and indicated with an asterisk. NS, not significant; DM, dry matter.

more moderate ($\rho = 0.56$, $p < 0.001$). Furthermore, a moderate, but very significant, positive correlation was found between leaf hydration and total chlorophylls ($\rho = 0.51$, $p < 0.001$) pooling all data together that were consistent in both West and East-oriented individuals ($\rho = 0.53$, $p < 0.001$; $\rho = 0.63$, $p < 0.001$, respectively). Simultaneously, the relationship between F_v/F_m ratio and α-tocopherol was also significant, although not too strong, in both orientations but this time being negative ($\rho = -0.32$, $p < 0.05$; $\rho = -0.46$, $p < 0.05$, respectively). In this manner, given that leaf hydration was slightly lower and tocopherol contents were higher in the West-oriented individuals than in the East-oriented ones, α-tocopherol might be acting as an important photoprotection compound under abiotic stress conditions. Therefore, these results suggest that *C. albidus* plants activate two photoprotection mechanisms. First, reductions in leaf angle, which might serve as a first line of defense from winter photoinhibition in leaves, particularly occurring in the uppermost position of shoots; and second, increased accumulation of α-tocopherol, which might serve as a stress sensing and signaling mechanism to counteract combined high light and low temperature stress during winter. The increased accumulation of α-tocopherol appears to be particularly relevant when photoprotection by leaf orientation is minimal and leaves consequently require more sophisticated biochemical, photoprotection mechanisms. In this study, higher levels of α-tocopherol are observed under more stressful conditions (e.g., West-oriented individuals exposed to a combination of abiotic stresses) and when leaves occupy positions more distal to the top of the shoot, due to the developmental program, and they orient more perpendicular to sun's rays.

It is concluded that *C. albidus* has very effective and fine-regulated photoprotection mechanisms, including (1) an adequate orientation of decussate leaves as part of the developmental program, in which the leaf angle is additionally modulated on a diurnal and seasonal basis, therefore contributing to prevent photoinhibition as a first line of defense and (2) enhanced tocopherol contents that may help withstand



ALL DATA

	Leaf angle	F_v/F_m ratio	Leaf biomass	Leaf mass area	Leaf hydration	Chlorophylls	IAA	Melatonin	α-Tocopherol	PPFD
Leaf angle	-	-	-	-	-	-	-	-	-	-
F_v/F_m ratio	-0.20	-	-	-	-	-	-	-	-	-
Leaf biomass	-0.01	0.33*	-	-	-	-	-	-	-	-
Leaf mass area	0.02	0.09	0.56***	-	-	-	-	-	-	-
Leaf hydration	-0.23	0.56***	0.12	-0.04	-	-	-	-	-	-
Chlorophylls	-0.14	0.30*	0.21	-0.17	0.51***	-	-	-	-	-
IAA	-0.16	0.30*	0.10	0.13	0.14	0.06	-	-	-	-
Melatonin	0.02	0.10	0.01	-0.15	0.24	0.16	-0.03		-	-
α-Tocopherol	0.10	-0.32*	-0.10	-0.21	-0.29*	0.04	-0.08	-0.05	-	-
PPFD	0.06	0.24	0.26*	0.07	0.18	0.04	-0.05	-0.02	-0.08	-
Temperature	-0.16	0.20	0.08	0.16	0.37**	-0.02	-0.19	-0.04	-0.30*	0.24

WEST-ORIENTED

	Leaf angle	F_v/F_m ratio	Leaf biomass	Leaf mass area	Leaf hydration	Chlorophylls	IAA	Melatonin	α-Tocopherol	PPFD
Leaf angle	-	-	-	-	-	-	-	-	-	-
F_v/F_m ratio	-0.18	-	-	-	-	-	-	-	-	-
Leaf biomass	-0.03	0.37*	-	-	-	-	-	-	-	-
Leaf mass area	-0.03	0.13	0.59***	-	-	-	-	-	-	-
Leaf hydration	-0.04	0.68***	0.09	-0.23	-	-	-	-	-	-
Chlorophylls	-0.15	0.51**	0.30	-0.17	0.53***	-	-	-	-	-
IAA	-0.31	0.28	0.08	0.20	0.07	0.01	-	-	-	-
Melatonin	-0.09	0.14	-0.08	-0.24	0.35*	0.24	-0.01	-	-	-
α-Tocopherol	0.02	-0.21	-0.14	-0.20	-0.24	-0.02	-0.17	-0.17	-	-
PPFD	0.13	0.35*	0.35*	0.06	0.18	0.23	0.03	-0.10	0.04	-
Temperature	0.26	0.11	-0.01	0.02	0.03	-0.04	-0.23	-0.13	-0.10	0.27

EAST-ORIENTED

	Leaf angle	F_v/F_m ratio	Leaf biomass	Leaf mass area	Leaf hydration	Chlorophylls	IAA	Melatonin	α-Tocopherol	PPFD
Leaf angle	-	-	-	-	-	-	-	-	-	-
F_v/F_m ratio	0.09	-	-	-	-	-	-	-	-	-
Leaf biomass	0.21	0.15	-	-	-	-	-	-	-	-
Leaf mass area	0.32	-0.06	0.61**	-	-	-	-	-	-	-
Leaf hydration	-0.13	0.33	0.11	0.20	-	-	-	-	-	-
Chlorophylls	-0.27	0.04	0.01	-0.11	0.63**	-	-	-	-	-
IAA	-0.03	0.37	0.14	0.01	0.34	0.19	-	-	-	-
Melatonin	0.43*	0.07	0.25	0.02	0.12	-0.03	-0.16	-	-	-
α-Tocopherol	0.04	-0.46*	0.09	-0.19	-0.17	0.03	-0.09	0.23	-	-
PPFD	0.10	-0.11	-0.16	0.00	0.14	-0.24	-0.18	0.20	-0.12	-
Temperature	-0.12	-0.02	-0.03	0.24	0.28	0.31	-0.01	0.04	0.02	-0.08

FIGURE 7 | Relationship between all measured parameters in *C. albidus* from a natural population in the Natural Park of the Montserrat Mountains. Tables show the results of the Spearman's rank correlation analyses (including the correlation coefficient, ρ, and *p* indicated with asterisks) for all data pooled together, for West-oriented individuals only and for East-oriented individuals only. All correlations were considered statistically significant at $p \leq 0.05$, with one, two, and three asterisks indicating *p* below 0.05, 0.01, and 0.001, respectively. Correlations with a correlation coefficient, $\rho \geq 0.50$, are shown in red, green, and blue for all data pooled together, West-oriented individuals, and East-oriented individuals, respectively. The plots in the right side of the figure show a graphical representation of the most biologically significant correlations. IAA, indole-3-acetic acid; PPFD, photosynthetic photon flux density.

combined high light and low temperature stress in *C. albidus* growing under Mediterranean field conditions.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

MP-L and SM-B conceived and designed the experiment. MP-L, AC, and MM performed the experiments. MP-L analyzed the data. SM-B wrote the manuscript with the help of MP-L. All authors revised and approved the final manuscript.

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Chapter 2. Contrasting patterns of hormonal and photoprotective isoprenoids in response to stress in *Cistus albidus* during a Mediterranean winter

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ORIGINAL ARTICLE

Contrasting patterns of hormonal and photoprotective isoprenoids in response to stress in *Cistus albidus* during a Mediterranean winter

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Abstract

Main conclusion **Seasonal accumulation of hormonal and photoprotective isoprenoids, particularly α-tocopherol, carotenoids and abscisic acid, indicate their important role in protecting *Cistus albidus* plants from environmental stress during a Mediterranean winter. The high diurnal amounts of α-tocopherol and xanthophylls 3 h before maximum light intensity suggest a photoprotective response against the prevailing diurnal changes.**

Abstract The timing to modulate acclimatory/defense responses under changing environmental conditions is one of the most critical points for plant fitness and stress tolerance. Here, we report seasonal and diurnal changes in the contents of isoprenoids originated from the methylerythritol phosphate pathway, including chlorophylls, carotenoids, tocochromanols, and phytohormones (abscisic acid, cytokinins, and gibberellins) in *C. albidus* during a Mediterranean winter. Plants were subjected not only to typically low winter temperatures but also to drought, as shown by a mean plant water status of 54% during the experimental period. The maximum PSII efficiency, however, remained consistently high ($F_v/F_m > 0.8$), proving that *C. albidus* had efficient mechanisms to tolerate combined stress conditions during winter. While seasonal α-tocopherol contents remained high (200–300 μg/g DW) during the experimental period, carotenoid contents increased during winter attaining maximum levels in February (minimum air temperature ≤ 5 °C for 13 days). Following the initial transient increases of bioactive *trans*-zeatin (about fivefold) during winter, the increased abscisic acid contents proved its important role during abiotic stress tolerance. Diurnal amounts of α-tocopherol and xanthophylls, particularly lutein, zeaxanthin and neoxanthin including the de-epoxidation state, reached maximum levels as early as 2 h after dawn, when solar intensity was 68% lower than the maximum solar radiation at noon. It is concluded that (1) given their proven antioxidant properties, both α-tocopherol and carotenoids seem to play a crucial role protecting the photosynthetic apparatus under severe stress conditions; (2) high seasonal amounts of abscisic acid indicate its important role in abiotic stress tolerance within plant hormones, although under specific environmental conditions, accumulation of bioactive cytokinins appears to be involved to enhance stress tolerance; (3) the concerted diurnal adjustment of α-tocopherol and xanthophylls as early as 3 h before maximum light intensity suggests that plants anticipated the predictable diurnal changes in the environment to protect the photosynthetic apparatus.

Keywords ABA · Carotenoids · Cold stress · Drought · Isoprenoids · MEP-pathway · Phytohormones · Tocopherols

Abbreviations

2iP	Isopentenyladenine
DPS	De-epoxidation state
IPA	Isopentenyladenosine
MEP	Methylerythritol phosphate
PC-8	Plastochromanol-8
RWC	Relative water content
t-Z	*trans*-Zeatin
t-ZR	*trans*-Zeatin riboside
VAZ	Xanthophyll cycle pool

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00425-019-03234-y) contains supplementary material, which is available to authorized users.

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Introduction

Climate change is a major global concern with a wide range of impacts on the environment. Prolonged exposure to different environmental stresses at the same time results in altered metabolism and damage to biomolecules. Plants, however, evolve defense mechanisms to withstand these stresses. Isoprenoids represent the most diverse natural product family and have crucial functions in the adaptation to environmental challenges. Their production and consumption are not only controlled by developmental factors, but also by daily and seasonal environmental conditions (Hemmerlin et al. 2012; Vranová et al. 2013). The universal precursors for all isoprenoids are isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The recently disclosed plastid-localized methylerythritol phosphate (MEP) pathway uses the central carbon intermediates pyruvate and glyceraldehyde 3-phosphate to produce IPP and DMAPP (Lichtenthaler et al. 1997). Specific isoprenoid examples synthesized by the MEP pathway include, among others, photosynthetic pigments (chlorophylls, carotenoids), plant hormones [abscisic acid (ABA), gibberellins (GAs), and cytokinins], and photoprotective and antioxidant compounds (carotenoids, tocopherols, and plastochromanol-8) (Fig. 1) (Estévez et al. 2001; Vranová et al. 2013; Kruk et al. 2014).

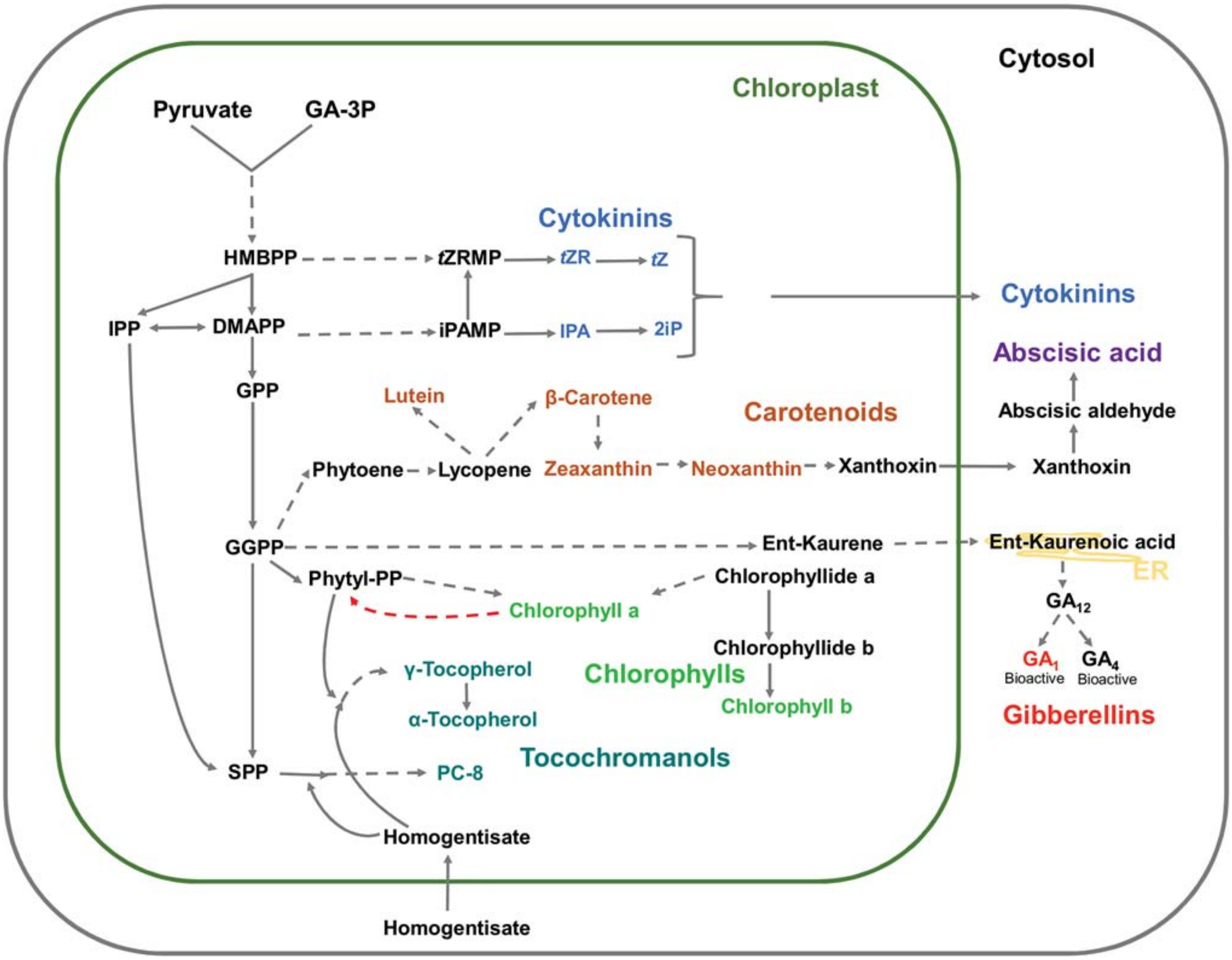

Fig. 1 Schematic representation of isoprenoid biosynthesis including chlorophylls, carotenoids, tocopherols, abscisic acid, cytokinins, and gibberellins through chloroplastic MEP pathway (adapted from Joyard et al. 2009; Mène-Saffrané et al. 2010; Meier et al. 2011; Vranová et al. 2012; Bahaji et al. 2015). *GA-3P* D-g-glyceraldehyde 3-phosphate, *HMBPP* 1-hydroxy-2-methyl-2-butenyl 4-diphosphate, *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate, *GPP* geranyl diphosphate, *GGPP* geranylgeranyl diphosphate, *SPP* solanesyl diphosphate, *t-ZRMP trans*-zeatin riboside (*t*-ZR) 5′-monophosphate, *t-Z trans*-zeatin, *iPAMP* isopentenyladenosine (IPA) 5′-monophosphate, *2iP* isopentenyladenine, *GA* gibberellin, *ER* endoplasmic reticulum, *PC-8* plastochromanol-8. Solid lines indicate a single enzymatic step, dashed lines indicate several enzymatic steps, and red dashed line indicates chlorophyll degradation pathway

Light has the most profound influence on the MEP pathway given that several isoprenoids from this pathway are part of the pigment-protein complex in the chloroplasts. In addition, several studies suggested that the biosynthesis of isoprenoids such as carotenoids, tocochromanols, ABA, cytokinins, and GAs follow a diurnal rhythm (Hedden and Kamiya 1997; Dudareva et al. 2005; Hsieh and Goodman 2005; Nováková et al. 2005; Barta and Loreto 2006; Covington et al. 2008). Evidence has been found that MEP pathway genes were co-expressed with core circadian clock genes from the "morning loop" (Vranová et al. 2012) and that the circadian clock may significantly contribute to plants' ability to tolerate environmental stress (Grundy et al. 2015).

As sessile organisms in a dynamic environment, plants are inevitably subjected to a diverse array of abiotic stresses such as drought, salinity, cold or light stress. Although these stresses may impose cellular and oxidative stress, including massive generation of reactive oxygen species (ROS), they differ in other effects, and therefore the adequate responses are not identical (Foyer and Shigeoka 2011; Finkelstein 2013). Isoprenoids from the MEP pathway form a flexible system designed to cope with disruptions in cellular ROS homeostasis during abiotic stress. Tocopherols together with tocotrienols and plastochromanol-8 (PC-8), belong to the group of tocochromanols that play pivotal roles as antioxidant agents deactivating photosynthesis-derived ROS and scavenging lipid peroxyl radicals in the thylakoid membranes, depending on the magnitude of the environmental stress and species susceptibility to stress (Demming-Adams and Adams 1996; Munne-Bosch et al. 1999; Munne-Bosch and Alegre 2000a, b; Falk and Munné-Bosch 2010; Kruk et al. 2014). All tocochromanols contain a chromanol ring that is mainly responsible for their strong antioxidant properties. Tocochromanols are formed from condensation of homogentisate with phytyl diphosphate in the case of α-tocopherol (the most abundant isoform) and solanesyl diphosphate in the case of PC-8 (Fig. 1). The prenyl chain of PC-8, however, is much longer and unsaturated, which may result in higher quenching activity toward ROS compared to tocopherols (Gruszka et al. 2008; Kruk et al. 2014). Carotenoids, including xanthophylls and carotenes, are involved in the protection of the photosynthetic apparatus with roles in the detoxification of the cell from free radicals and ROS in plants exposed to a wide range of abiotic stress and not exclusively to high solar radiation. For instance, β-carotene plays a key role during low-temperature tolerance; and due to the absence of hydroxyl groups on its carbon chain, it is homogenously distributed in thylakoid membranes, maintaining a fluid state during stress (Demming-Adams and Adams 1996; Havaux 1996). Moreover, a reversible light-driven de-epoxidation reaction, also known as the xanthophyll cycle, is involved in the protection of the photosynthetic apparatus. In this cycle, violaxanthin is converted into antheraxanthin and zeaxanthin when there is an excess of light energy of that absorbed by chlorophyll used during photosynthetic processes, and this conversion is reversed when there is no longer an excess of light. The accumulation of antheraxanthin and zeaxanthin in the photosynthetic apparatus is associated with harmless energy dissipation in the form of heat (Demming-Adams 1990; Horton et al. 1996). ABA, which can be considered as the end product of the MEP–carotenoid pathway (Fig. 1), acts as an endogenous messenger in plant growth and development under environmental stresses (Melcher et al. 2010; Brunetti et al. 2014). Water stress, for instance, triggers ABA synthesis leading to the production of H_2O_2 and nitric oxide (NO), two essential components in ABA-mediated stomatal regulation (Lee and Luan 2012). In contrast, cytokinins act as scavengers of NO and have been reported to be directly involved in modulating the ABA response (Liu et al. 2013). Cytokinins are known as potent inducers of cell division and key regulators of the cell cycle (Schaller et al. 2014). Several studies also indicate a role of cytokinins to modulate the regulatory mechanisms of photosynthetic processes under abiotic stress (Rivero et al. 2009, 2010). Gibberellins are a large group of tetracyclic diterpenoid carboxylic acids with the predominant bioactive forms being GA_1 and GA_4. They are known to be growth-promoting plant hormones and play major roles to control plant growth and development—recent data have shown that gibberellins also play roles in plant adaptation to abiotic stress (Qin et al. 2011). It has been reported that reduced gibberellin signaling improves drought tolerance as the available water is used more slowly due to the reduced leaf area, and therefore reduced transpiration (Lawlor 2013). In addition, a major role of gibberellin signaling in response to abiotic stress is to integrate information from a number of other hormone signaling pathways, such as ABA and ethylene (Achard et al. 2006).

The Mediterranean Basin is one of the most vulnerable regions in the world to the impact of climate change (IPCC 2013). Recent Mediterranean climate studies indicate decreased precipitation in all seasons, however, increased temperatures mostly in summer but not in winter (Ozturk et al. 2015). In view of these changing climatic conditions, in which drought stress could occur more frequently even in Mediterranean winter, it is of great importance studying combined stresses to understand plant species stress response in their natural habitat. The Mediterranean semi-deciduous shrub *Cistus albidus* L. was chosen in the present study due to its high content of isoprenoids in leaves and its high tolerance to abiotic stresses (Fernández-Marín et al. 2017). We studied both diurnal and seasonal changes in the contents of isoprenoids derived from the MEP pathway, including chlorophylls, carotenoids, tocochromanols (α-tocopherol and plastochromanol-8), ABA, cytokinins, and GAs to get novel insights into the role of isoprenoids in balancing combined drought and cold stress during Mediterranean winter.

Materials and methods

Experimental design and samplings

The experiments were carried out on a population of *C. albidus* plants (~ 14-year-old) growing in semi-controlled conditions at the Experimental Fields of the Faculty of Biology at the University of Barcelona (41.384N, 2.119E, 59 m.a.s.l.). To assess seasonal effects on *C. albidus*, we performed five samplings at predawn from November through March. The samplings took place on November 6, December 4, January 16, February 27, and March 7 of 2017–2018. Additionally, in January, which is typically the coldest month of the year [State Meteorological Agency (AEMET) of the Spanish Government (1981–2010)], we evaluated diurnal variations. The sampling day was a clear sunny day and consisted of five sampling points: predawn, morning, midday, afternoon, and evening. The predawn and evening samplings occurred 1 h before sunrise and 1 h after sunset, respectively, and the total hours of light were ~ 10 h. On each sampling, maximum photosystem II (PSII) efficiency and relative water content (RWC) were measured on a fully apical developed leaf of a spring ramet of six homogenous individuals. At the same time, the opposite leaf was snap frozen in liquid nitrogen and stored at − 80 °C until analyses to assess the contents of photosynthetic pigments, tocochromanols, and plant hormones.

Climatic conditions

Environmental conditions were monitored throughout the study by means of a weather station situated 300 m from the experimental plot. The weather station consisted of a photon flux density (PPFD) pyranometer sensor CM11 (Kipp and Zonen, Delft, The Netherlands) and a HMP35AC thermo-hygrometer (Väisala, Finland). Precipitation was measured with a standard rain gauge. Temperature measurements were taken every hour from three ECHO sensors (Decagon Devices, Inc., Pullman, WA, USA).

During the study, the *C. albidus* population was subjected to Mediterranean climate conditions. During the measurement days, plants were exposed to a maximum diurnal PPFD ranging between 903 µmol m^{-2} s^{-1} (January) and 1358 µmol m^{-2} s^{-1} (March).

The month before the first sampling, air temperatures (T_{air}) reached a maximum mean T_{air} of 26 °C and a minimum mean of 12 °C. Then maximum and minimum mean T_{air} decreased in the month before the second sampling (19 °C and 10 °C, respectively). In addition, temperatures dropped to minimum mean T_{air} of 2.9 °C and maximum mean of 10.7 °C 2 days before the second sampling. In the month before the third sampling, the temperature decrease continued to maximum and minimum mean T_{air} of 15 °C and 7 °C, respectively, including the last week when the minimum mean T_{air} dropped to 5 °C. The month before the fourth sampling was the coldest with a minimum mean T_{air} of 5 °C and maximum mean of 13 °C. Temperatures dropped to minimum mean T_{air} of 2 °C and maximum mean of 6 °C 3 days before the fourth sampling. Air temperatures recovered the week before the last sampling with maximum mean T_{air} of 17 °C and the minimum mean of 9 °C. Accumulated precipitation was scarce during the experimental period (283 mm from November to March). Maximum relative humidity (RH) occurred in February (91%) and the minimum in November (19%) (Fig. S1).

Plant water status, maximum PSII efficiency, and leaf mass area

Plant water status was assessed measuring relative water content (RWC) of leaves. RWC (%) was determined as: 100 (FW − DW)/(TW − DW). FW is fresh weight just after collecting the leaves, TW is turgid weight after re-hydrating the leaves, fully immersed in water, for 24 h at 4 °C, in darkness; and DW is dry weight after drying the samples at 65 °C until constant mass (Turner 1981).

Maximum PSII efficiency (F_v/F_m) was measured on leaves adapted to darkness overnight for the seasonal measurements with a portable fluorimeter mini-PAM (Walz Mini-PAM II, Effeltrich, Germany) whereas for the diurnal measurements, it was assessed on detached leaves adapted to darkness, at least for 1 h (Werner et al. 1999). Leaf mass per unit area was calculated as DW by leaf surface (m^2) measured with a flatbed scanner.

Photosynthetic pigment contents

Samples were extracted as described in Cotado et al. (2018) with some modifications. In short, 250 µl of methanol were added to 50 mg of frozen sample, then sonicated in an ultrasonic bath (Bransonic 2800, Emerson Industrial, Danbury, CT, USA) for 30 min at 4 °C and centrifuged for 10 min at 14,196*g* at 4 °C (centrifuge MR18-22, Jouan, Saint-Herblain, France). The supernatant was collected, the pellet was re-extracted as above and all the supernatants were pooled to proceed with the measurements. The content of photosynthetic pigments (chlorophyll a, chlorophyll b, and total carotenoids) was determined spectrophotometrically (Cecil Aquaris CE7400, Cecil Instruments, Cambridge, UK) as described by Lichtenthaler and Wellburn (1983).

Determination of tocochromanols and carotenoids

The extractions for the tocopherols and PC-8 analyses were performed as above. The pooled supernatants were filtered through a 0.22 PTFE filters and transferred to vials for analyses. High performance liquid chromatography (HPLC) analyses for α-tocopherol and PC-8 were carried out exactly as described in Siles et al. (2017). Quantification for α-tocopherol was based on the fluorescence signal response compared with authentic standards of α-tocopherol (Sigma-Aldrich, Steinheim, Germany) and PC-8 (gifted by Jerzy Kruk's lab). On the other hand, HPLC analyses for carotenoids were performed exactly as in Morales et al. (2016) and then quantified as in Munne-Bosch and Alegre (2000a, b). De-epoxidation state (DPS) was calculated as (Z + 0.5A)/V + Z + A where Z is zeaxanthin, A antheraxanthin, and V is violaxanthin.

Phytohormone profiling

The extractions for the phytohormone profiling were performed as above. The levels of cytokinins, including *t*-Z, *t*-ZR, IPA and 2-iP, ABA, GA_1 and GA_4 were determined by UPLC–MS/MS as described by Müller and Munné-Bosch (2011). Internal standards were used for estimating recovery rates for quantification. GA_1 was identified as the active form of gibberellins due to the unchanged contents of GA_4 (data not shown).

Statistical analysis

To assess the effect of Month ('November', 'December', 'January', 'February', 'March') on RWC, F_v/F_m, chlorophyll (a + b), chlorophyll (a/b), total carotenoids, α-tocopherol, PC-8, ABA, GA_1, total cytokinins, *t*-Z, *t*-ZR, IPA, and 2iP, we used a linear mixed-effects (LMM) model using the *lme* function within the *nlme* package. 'Month' was fitted as a fixed term and 'Plant' as a random term, allowing for repeated measures. All models were fitted using restricted maximum likelihood (REML) and validated by visually checking the distribution of residuals for normality and homoscedasticity (Zuur et al. 2009). *P* values of the fixed effects were estimated using conditional *F* tests and multiple comparisons were tested with the Tukey HSD test. To assess the effect of Time '7', '10', '13', '16', and '18.30' on the response variables mentioned above as well as on lutein, β-carotene, zeaxanthin, neoxanthin, xanthophyll cycle pool (VAZ), and DPS, we used a LMM model using the *lme* function from the *nlme* package for January. 'Time' was fitted as a fixed term and 'Plant' as a random term, allowing for repeated measures. All models were fitted with REML and validated by visually checking the distribution of residuals for normality and homoscedasticity. *P* values were estimated as above. For seasonal variations, 'chlorophyll (a + b)' was $\log_{10}$ transformed; 'IPA' and '2iP' were square root transformed. For diurnal variations, 'chlorophyll (a + b)', 'α-tocopherol', 'lutein', 'DPS', and '2iP' were square root transformed while 'PC-8', 'β-carotene', 'ABA', and '*t*-Z' were $\log_{10}$ transformed. Correlations between response variables were tested using Spearman's Rank-Order Correlation test at a significance level of $\alpha = 0.05$.

Results

Effect of drought and cold stress on plant water status, maximum PSII efficiency, and leaf mass area during winter

Mean air temperature ranged between 12.1 and 10.1 °C from December to March, while January and February were the coldest months throughout the study (see Climate conditions, Suppl. Fig. S1). The low seasonal plant water status with minimum and maximum RWC values of 47.9% (January) and 63.4% (December), respectively, was associated with accumulated rainfall of 283 mm throughout the study (Table 1, Suppl. Fig. S1). Therefore, plants were subjected to drought and cold temperatures during Mediterranean winter. In January, the plant water status did not show significant differences, except on mid day when RWC values decreased significantly to 40.8% coinciding with maximum diurnal solar radiation (1103 μmol m^{-2} s^{-1}) and maximum diurnal temperature (21 °C) (Table 2). In contrast, no chronic photoinhibition was observed throughout the study and the

Table 1 Seasonal variations in maximum PSII efficiency (F_v/F_m), relative water content (RWC), and leaf mass area (LMA) from November to March in *Cistus albidus*

	Seasonal variations				
	November	December	January	February	March
F_v/F_m	0.82 ± 0.003	0.82 ± 0.013	0.84 ± 0.016	0.82 ± 0.003	0.82 ± 0.008
RWC (%)	56.03 ± 3.58 ab	63.35 ± 4.36 a	47.86 ± 1.69 b	53.20 ± 3.17 ab	51.93 ± 1.36 ab
LMA (g/m^2)	114.97 ± 0.56	125.27 ± 0.41	128.51 ± 0.23	108.61 ± 0.19	96.62 ± 0.13

Data correspond to the mean ± SE of six individuals. Significant differences between months were tested by Fisher tests ($P \leq 0.05$) and different letters indicate significant differences between groups by Tukey's HSD multiple comparison test ($P \leq 0.05$)

Table 2 Diurnal variations in photosynthetic photon flux density (PPFD), temperature (T^e), maximum PSII efficiency (F_v/F_m), and relative water content (RWC) in January in *Cistus albidus*

	Diurnal variations			
	PPFD (μmol m^{-2} s^{-1})	T^e (°C)	F_v/F_m	RWC (%)
5.30 h	0	11.70	0.82 ± 0.003	56.03 ± 3.58 a
9 h	351	14.80	0.83 ± 0.005	57.86 ± 2.72 a
12 h	1103	21.00	0.84 ± 0.011	40.77 ± 6.00 b
15 h	560	18.30	0.85 ± 0.010	63.32 ± 4.81 a
19 h	0	14.14	0.86 ± 0.006	59.04 ± 4.50 a

PPFD and T^e are single data points at each sampling point. Data for F_v/F_m and RWC correspond to the mean ± SE of six individuals. Significant differences between months were tested by Fisher tests ($P \leq 0.05$) and different letters indicate significant differences between groups by Tukey's HSD multiple comparison test ($P \leq 0.05$)

diurnal and seasonal maximum PSII efficiency (F_v/F_m) were kept constant with values equal or above 0.82 (Tables 1, 2). Leaf mass area (LMA) did not show significant variations during the experimental period (Table 1).

Contrasting responses of isoprenoids during winter

Chlorophylls a and b decreased about 36% in December (Fig. 2a) coinciding with a short-term temperature drop of about 10 °C of the mean air temperature during 2 days before the sampling (reaching minimum air temperature of 2.9 °C; Suppl. Fig. S1). In January, however, chlorophylls returned to similar contents as in November and remained constant until the end of the experimental period, although mean air temperatures remained ≤ 10 °C for 18 days in January and February and the plant water status was below 53.2%. Seasonal chlorophyll a/b ratio increased during winter with increasing cold stress. Changes in carotenoid contents were associated with increasing stress throughout the study. Their contents remained low during November and December, when plants suffered predominantly drought stress (RWC values between 56.0 and 63.4%; Table 1). However, carotenoids increased about 77% in January having a peak in February (0.43 mg/g DW), when air temperatures were at their lowest values (minimum air temperatures ≤ 5 °C for 13 days) and the plant water status reached 53.2%. In March, temperatures recovered and carotenoid contents declined again to 0.18 mg/g DW (Fig. 2a, Suppl. Fig. S1), although RWC values remained low (51.9%).

Diurnal chlorophyll a and b contents ranged between 2.9 mg/g DW and 3.7 mg/g DW (Fig. 2a)—without significant changes—while chlorophyll a/b ratio remained relatively constant during the day in January (Fig. 2a). Since diurnal carotenoid contents indicated a slight non-significant increase during the morning, the levels of specific carotenoids were determined by HPLC, including the levels of

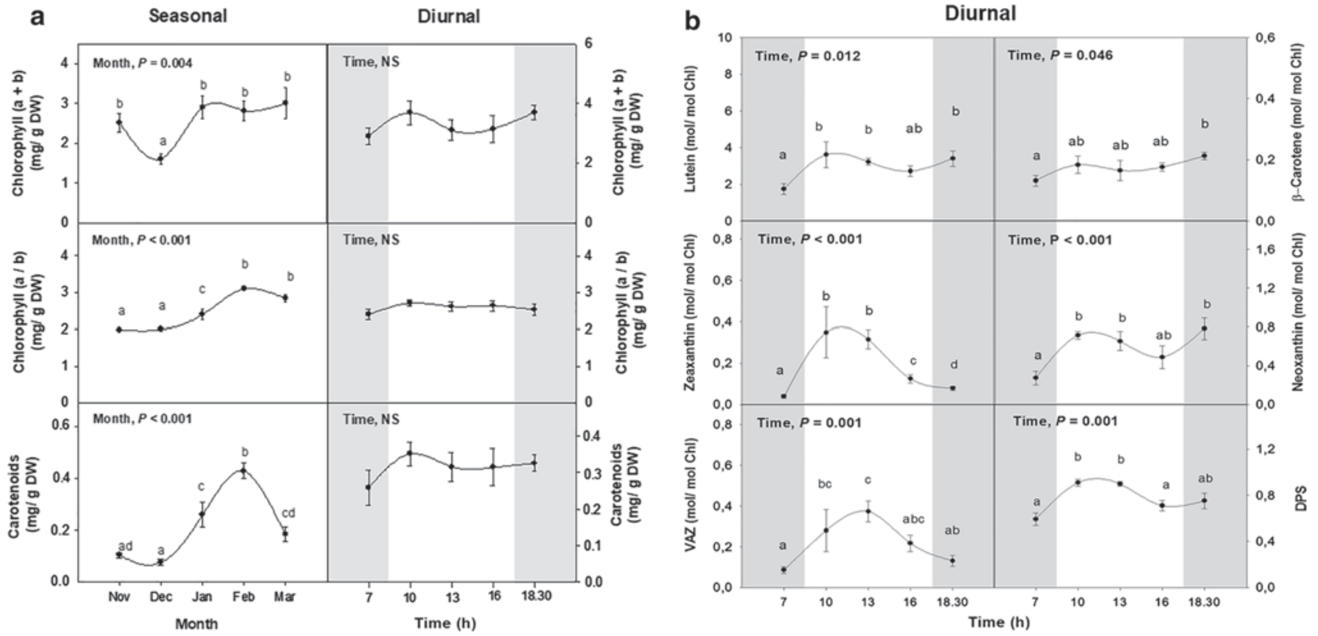

Fig. 2 **a** Seasonal and **b** diurnal variations in photosynthetic pigments in *Cistus albidus* during Mediterranean autumn and winter conditions. Seasonal variations correspond to measurements performed 1 h before dawn and diurnal variations were performed during a sunny day in January. Data represent the mean ± SE of six individuals and differences within 'Month' were tested by conditional Fisher tests ($P \leq 0.05$). Different letters indicate differences in Tukey's HSD multiple comparison test ($P \leq 0.05$). *VAZ* xanthophyll cycle pool, *DPS* depoxidation state, *NS* not significant

xanthophylls (lutein, the xanthophyll cycle pool (VAZ), zeaxanthin, and neoxanthin), de-epoxidation state of the xanthophyll cycle (DPS), and β-carotene (Fig. 2). Xanthophyll concentrations reached significantly higher contents 2 h after dawn at a light intensity of 351 µmol m^{-2} s^{-1}, which remained high during mid-day (PFFD 1103 µmol m^{-2} s^{-1}). After mid-day, however, xanthophyll contents decreased to similar contents to those of predawn, except for lutein and neoxanthin the concentrations of which remained relatively constant until sunset. Consequently, in the morning, DPS increased to values around 0.9, which remained relatively stable until noon. In the afternoon, DPS values decreased significantly by 22% when light intensity was 560 µmol m^{-2} s^{-1}. In contrast, β-carotene reached significant higher contents (0.21 mol/mol Chl) 1 h after sunset (Fig. 2b).

During winter months, α-tocopherol contents remained high, reaching mean values of 258 µg/g DW, but without significant changes. In January, diurnal α-tocopherol contents also reached a peak 2 h after dawn, such as xanthophylls, but then decreased to initial contents in the afternoon (PFFD 560 µmol m^{-2} s^{-1}; Table 1). This peak (419 µg/g DW) was 1.6-fold higher than α-tocopherol contents which reached under seasonal environmental conditions (Fig. 3). The seasonal results of PC-8 showed a contrary pattern to total carotenoids: being high during November and December (mean 70 µg/g DW). In January, however, these contents decreased about 75% and then remained low until the end of the experiment. Contrary to α-tocopherol, PC-8 contents did not accumulate during the day, with very low contents (12–22 µg/g DW) found in January (Fig. 3).

Drought in combination with decreasing temperatures was reflected by a twofold increase in seasonal ABA contents over November and December until January (Fig. 4). In February, ABA contents transiently decreased, when the highest RH (91%) was observed. In March, however, with reduced RH (51.3%) and remaining low RWC values (51.9%), ABA contents rose again to similar concentrations to those in January. Diurnal ABA contents increased reaching 1.6-fold higher contents before sunset (light intensity of 560 µmol m^{-2} s^{-1}) then under seasonal environmental conditions, although this increase was not significant throughout the day. In addition, IPA and *t*-Z showed a non-significant increase during the day in January. Contrasting seasonal variations within cytokinins indicate their different roles during Mediterranean winter. Whereas IPA contents showed similar patterns to ABA over the months, *t*-Z was peaking in December by a fivefold change, when chlorophylls a and b contents decreased. However, in January, their contents returned to constant, low contents (~2 ng/g DW; Figs. 2, 4). In contrast, seasonal and diurnal *t*-ZR, 2iP, and GA_1 did not show significant variations during the experimental period, except for a decrease in GA_1 after sunrise in January (Fig. 4, Suppl. Fig. S2).

Diurnal and seasonal isoprenoid interactions

Since contrasting isoprenoid accumulations were observed during the experimental period, it was evaluated, here, whether or not isoprenoid interactions were associated to seasonal or diurnal patterns. Seasonal carotenoid levels correlated positively with both total chlorophyll (a + b) and chlorophyll a/b ratio, while seasonal ABA contents correlated positively with IPA. In contrast, seasonal PC-8 correlated negatively with carotenoids and chlorophyll a/b ratio

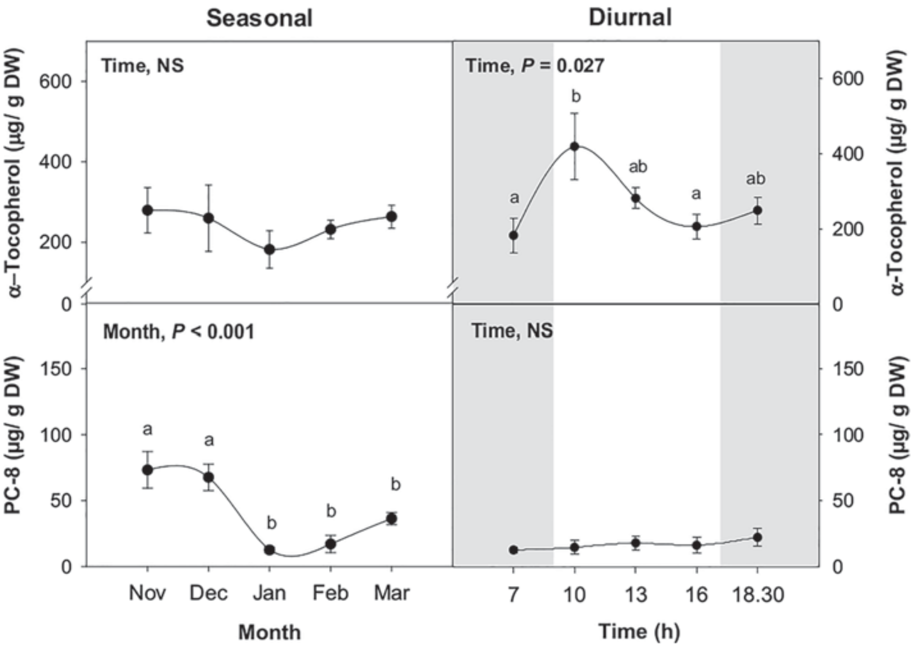

Fig. 3 Seasonal and diurnal variations in tocochromanols in *Cistus albidus* during Mediterranean autumn and winter conditions. Seasonal variations correspond to measurements performed 1 h before dawn and diurnal variations were performed during a sunny day in January. Data represent the mean ± SE of six individuals and differences within 'Month' were tested by conditional Fisher tests ($P \leq 0.05$). Different letters indicate differences in Tukey's HSD multiple comparison test ($P \leq 0.05$). *PC-8* plastochromanol-8, *NS* not significant

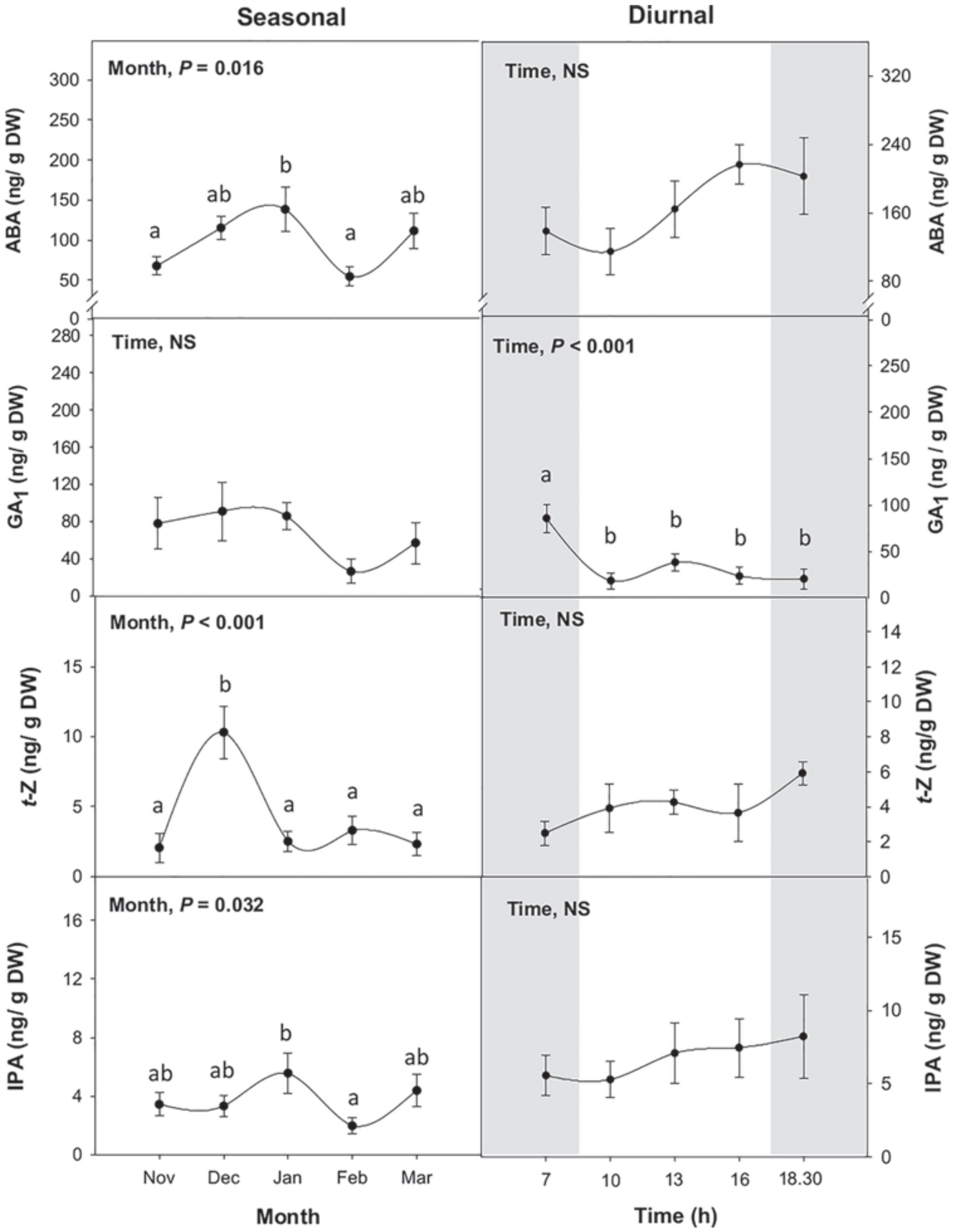

Fig. 4 Seasonal and diurnal variations in ABA, GA_1, and cytokinin contents in *Cistus albidus* during Mediterranean autumn and winter conditions. Seasonal variations correspond to measurements performed 1 h before dawn and diurnal variations were performed during a sunny day in January. Data represent the mean ± SE of six individuals and differences within 'Month' were tested by conditional Fisher tests ($P \leq 0.05$). Different letters indicate differences in Tukey's HSD multiple comparison test ($P \leq 0.05$). *ABA* abscisic acid, *GA_1* gibberellin 1, *CKs* cytokinins, *t-Z trans*-zeatin, *IPA* isopentenyladenosine, *NS* not significant

(Fig. 5, Suppl. Fig. S3). Although diurnal correlation patterns for carotenoids and chlorophylls as well as ABA and IPA were similar to the seasonal ones, diurnal ABA levels correlated negatively with α-tocopherol. Diurnal IPA contents correlated also positively with total chlorophyll, chlorophyll a/b ratio, and carotenoids (Fig. 5, Suppl. Fig. S3).

Discussion

The Mediterranean shrub *C. albidus* is adapted to grow under marked seasonal variation throughout the year. In summer, plants are typically subjected to drought and in winter, to cold temperatures (Jubany-Marí et al. 2009; Brossa et al. 2015). The mean RWC of 54% obtained in this study during winter in *C. albidus*, however, indicates that plants were subjected not only to low temperatures typical of winter, but also to drought due to the scarce rainfall during these months. Nevertheless, consistently high F_v/F_m values (above 0.80) throughout the study suggest that *C. albidus* possesses an efficient mechanism including hormonal and photoprotective isoprenoids to cope with different environmental stresses at the same time.

Water deficit triggers the ABA production in plants, and in turn causes stomatal closure and the expression of drought-related genes (Bray 2002; Seki et al. 2007). In addition, several studies revealed the involvement of ABA in the regulation of cold stress response (Daie and Campbell

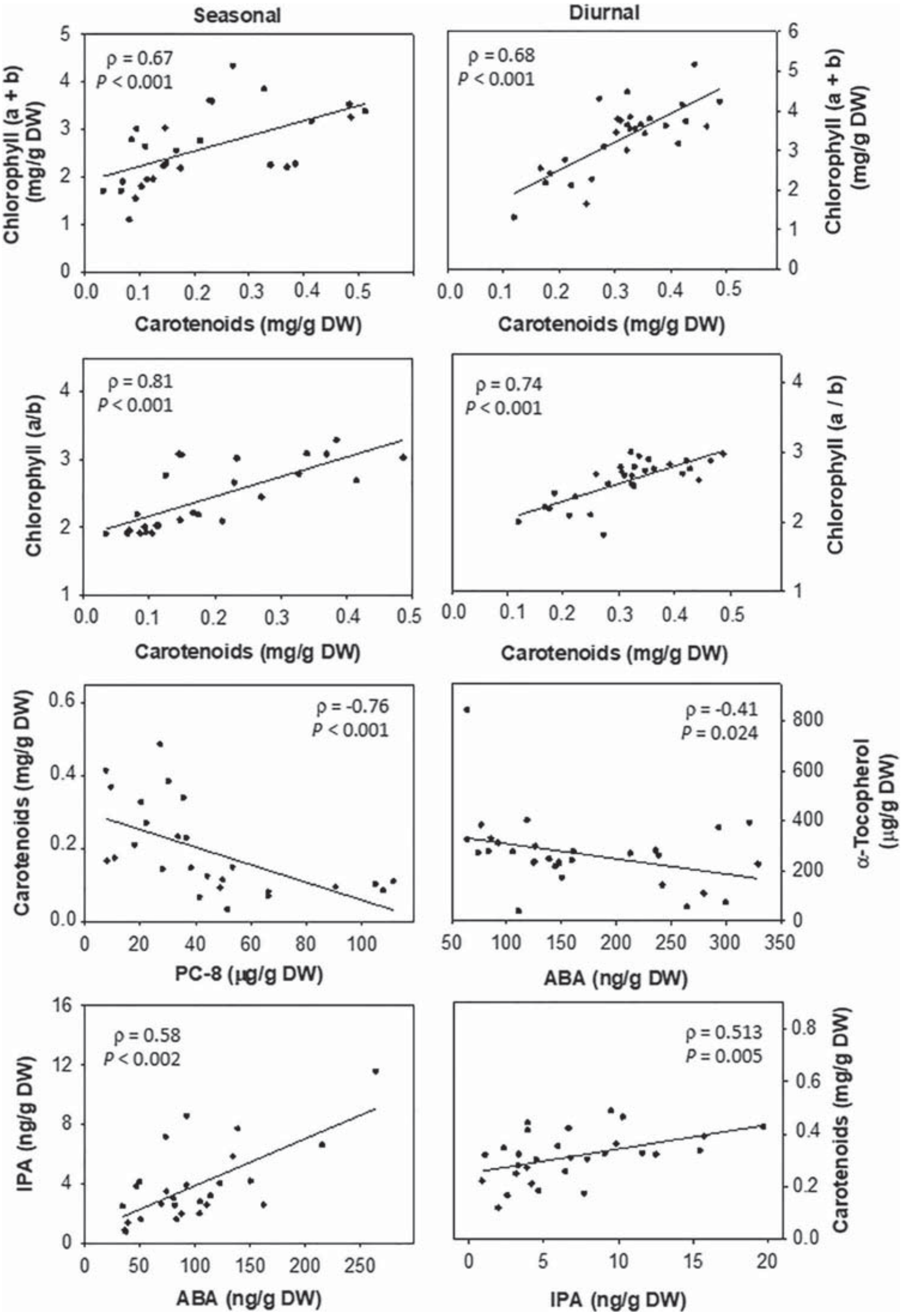

Fig. 5 Spearman's rank correlation analyses of seasonal and diurnal isoprenoids in *Cistus albidus*. Significant *P* values (<0.05) and corresponding regression coefficients are shown. *ABA* abscisic acid, *IPA* isopentenyladenosine

1981; Lalk and Dörffling 1985) and exogenous applications of ABA improved cold resistance in cucumber and bermudagrass (Flores et al. 1988; Huang et al. 2017). Our results showed a marked increase in ABA contents during the experimental period, reaching a peak in January (138.5 ng/g DW), suggesting a pivotal role of ABA in the regulation of combined drought and cold stress tolerance (Fig. 4). In February, however, we observed a transient decrease in ABA, which could be caused by the high RH values of ~ 91%. Plant hormones action depends often on specific hormone combinations rather than on the independent activities of each (Weiss and Ori 2007). Thus, the similar seasonal and diurnal hormonal patterns of ABA and IPA, which are reflected in their positive correlations (Figs. 5, 6), may have contributed coordinately to the improvement of drought and cold stress tolerance in *C. albidus*. Nevertheless, ABA signaling promoted either by single or combined water and cold stresses needs further research. Several

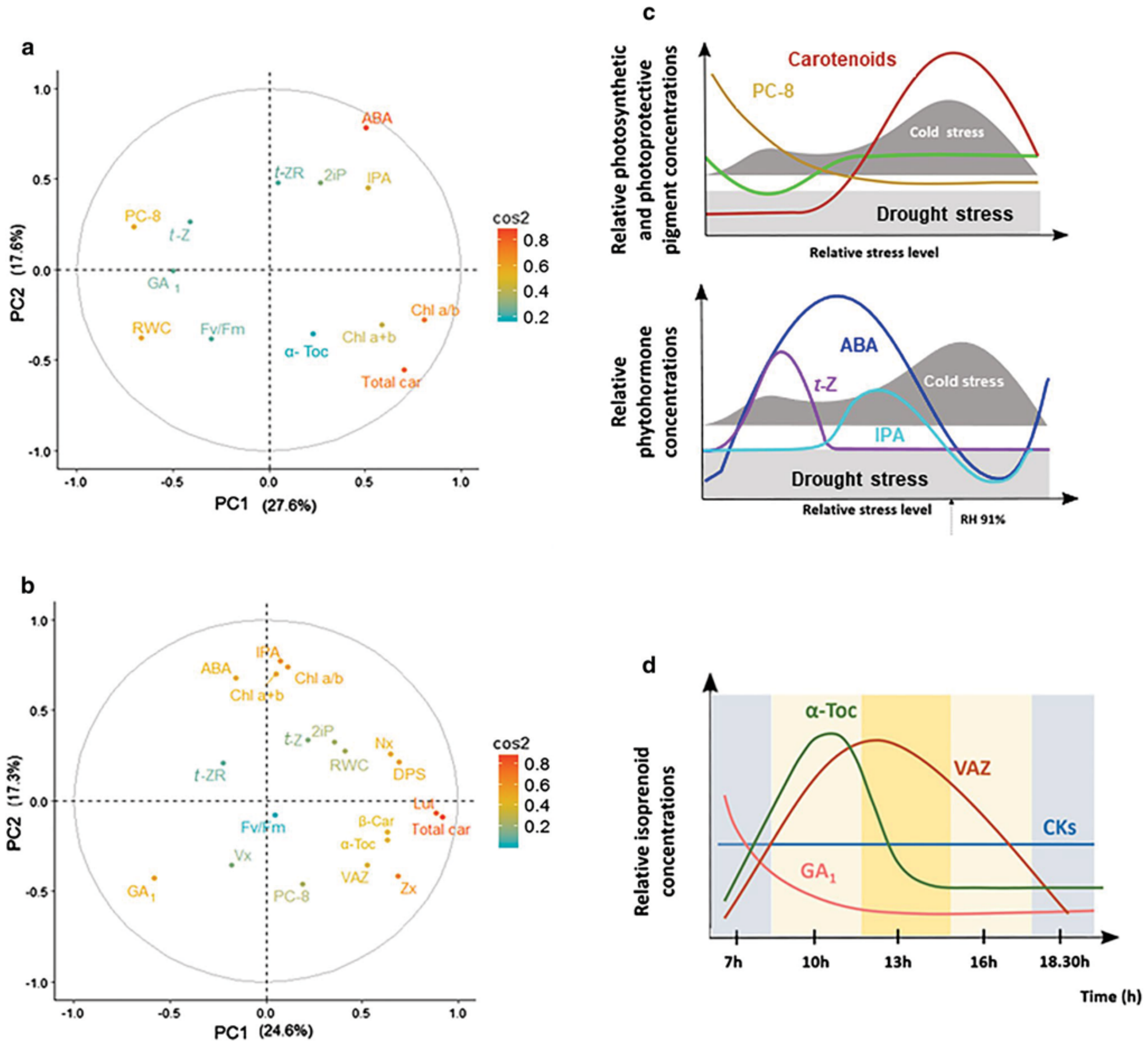

Fig. 6 **a** Bioplot showing multivariate analysis of the seasonal and **b** diurnal data from principal component analysis (PCA). **c** Graphical representation of possible effects of combined stresses on seasonal and **d** diurnal isoprenoid contents. Principal component analysis (PCA) plots represent the relationships between all variables and the quality of the variables (i.e., square cosine, square coordinates, cos2) on the factor map. A higher cos2 (red in the cos2 color scale) indicates a good representation of the variable on the principal component whereas a low cos2 (blue in the cos2 color scale) indicates that the variable is not perfectly represented by the principal components. Studies measuring isoprenoid concentrations during changing environmental conditions including combined drought (in relation to the constant RWC values of 54%) and cold stress (in relation to the mean air temperature) suggest that, depending on stress, severity-specific isoprenoid production/accumulation is rapidly elevated. *RWC* relative water content, *Chl* chlorophyll, *Total Car* total carotenoids, *α-Toc* α-tocopherol, *PC-8* plastochromanol-8, *ABA* abscisic acid, *GA$_1$* gibberellin 1, *t-Z trans*-zeatin, *t-ZR trans*-zeatin riboside, *IPA* isopentenyladenosine, *2iP* 2-isopentenyladenine, *Lut* lutein, *β-Car* β-carotene, *Nx* neoxanthin, *Vx* violaxanthin, *Zx* zeaxanthin, *VAZ* xanthophyll cycle pool, *DPS* depoxidation state, *PC* principal component, *RH* relative humidity

studies have shown that water stress responses cannot be elicited by ABA alone, suggesting that other factors are also required to modulate acclimatory/defense responses under water stress (Dat et al. 2000; Vranová et al. 2002). One important inevitable modification induced by drought as well as cold stress is the enhanced reactive oxygen species (ROS) production, where photoprotective isoprenoids from the MEP pathway play a crucial role in the modulation of these intracellular ROS concentrations and redox status (Munne-Bosch and Alegre 2000a, b; Munné-Bosch 2005; Peñuelas and Munné-Bosch 2005). Oliván and Munné-Bosch (2010) reported the photoprotective function of α-tocopherol at concentrations of approximately 65 µg/g DW under drought combined with high solar radiation (PPFD, ~ 1800 µmol m^{-2} s^{-1}) in *C. albidus* plants. In this work, much higher α-tocopherol contents (~ 200–300 µg/g DW) were observed, along with high F_v/F_m values (> 0.8); thus, indicating an important role of this antioxidant even under drought (mean RWC values of 53%) in combination with cold stress (mean maximum and minimum air temperature of 15.8 °C and 7.8 °C, respectively) and moderate solar radiation (mean maximum PFFD of 880 µmol m^{-2} s^{-1}; Fig. 3; Table 1; Suppl Fig. S1). Hormaetxe et al. (2008) found a similar interaction between these stressors in *Buxus sempervirens* L. during winter acclimation, where photoprotective responses under drought and low temperatures were magnified by light. While α-tocopherol contents did not show significant variations throughout the study, total carotenoid contents increased progressively during winter whereas PC-8 decreased in January, keeping at the lowest levels afterward. Regression analyses revealed a negative correlation between PC-8 and carotenoids and PC-8 and chlorophyll a/b (Fig. 5). From these results, it appears that protection to the photosynthetic apparatus during prolonged winter stress was achieved by the action of carotenoids and α-tocopherol (Fig. 6a, c), rather than PC-8, while the initial high levels of PC-8 may be associated much more with drought as happened predominately during November and December (mean air temperature ≤ 10 °C for only 4 days; Fig. 3, Suppl. Fig. S1).

Given that cytokinins from the MEP pathway are located in the chloroplast, several studies indicate a role of cytokinins to modulate the regulatory mechanisms of photosynthetic processes under abiotic stress (Rivero et al. 2009, 2010). Rivero et al. (2007) reported that enhanced cytokinin levels improved drought tolerance in flowering plants. Our results suggest that *t*-Z may have temporary protecting effects as indicated by the increase in *t*-Z levels in December (fivefold compared to November), when plants seemed to suffer a transient stress as indicated by decreased chlorophyll a and b contents (~ 40% for chlorophyll a and ~ 30% for chlorophyll b compared to November that recovered in January). This relation was also reflected in the negative correlation between *t*-Z and chlorophyll a and b (Fig. 6a, c). Arabidopsis mutants with decreased endogenous cytokinin production showed decreased ROS scavenging efficiency under high-light stress, suggesting that cytokinins play a role in protecting the photosynthetic apparatus (Cortleven et al. 2014).

To better understand how plants balance their isoprenoid pool to changing light levels, the diurnal variations in MEP-derived photosynthetic pigments, tocochromanols, and plant hormones were evaluated in January. α-Tocopherol (1.6-fold higher compared to seasonal levels) and xanthophyll contents (including VAZ pool) reached high levels 2 h after dawn at a light intensity of 351 µmol m^{-2} s^{-1}, when solar radiation was 68% lower compared to the maximum noon solar radiation. The increased VAZ pool was accompanied by an increased de-epoxidation state (DPS), which might be an adaptive measure allowing energy dissipation at maximum solar radiation. Our results suggest that plants anticipated the predictable changes in the solar radiation by adjusting α-tocopherol, xanthophylls, and DPS values already 3 h before maximum light intensity. This is in agreement with the study reported by Covington and Harmer (2007) where key enzymes of the carotenoid biosynthesis are clock controlled to prepare plants in advance for excessive solar energy absorption. The high diurnal F_v/F_m values (> 0.8) confirm that the increased amounts of α-tocopherol and xanthophylls completely protected the photosynthetic apparatus at mid day in January under drought (RWC of 40.8%), also reflected in their positive correlations (Fig. 6b, d). In the afternoon, under lower light intensity (560 µmol m^{-2} s^{-1}), the zeaxanthin contents decreased markedly, and were converted into violaxanthin, which consequently resulted in reduced DPS values (Fig. 2). This ZEP-catalyzed (zeaxanthin epoxidase) epoxidation is also required for the biosynthesis of ABA (Seo and Koshiba 2002). It, therefore, appears that the decrease in zeaxanthin 1.5 h before sunset, in turn, was converted into ABA through a series of isomerization and dehydrogenation reactions. Diurnal ABA contents reached 216 ng/g DW in the afternoon that were 1.6-fold higher compared to the maximum seasonal ABA contents. This suggests its important role to cope with diurnal environmental changes during winter. For instance, dark-grown bean accumulated ABA under water stress conditions, while non-stressed plants did not show reduced xanthophyll levels and consequently did not accumulate ABA (Li and Walton 1990). The high α-tocopherol contents in the morning (1.6-fold higher than seasonal) and high abscisic acid contents in the afternoon (1.6-fold higher than seasonal) appear to indicate different photoprotective and hormonal strategies through using environmental signals or overcoming environmental stress. In previous studies, positive correlations have been observed between ABA and tocopherol levels in aging Arabidopsis plants and in *C. creticus* plants subjected to drought (Munné-Bosch et al. 2007, 2009). Evidences have been reported that ABA signaling may be involved in the formation of homogentisate, needed for the biosynthesis of tocochromanols (Chaudhary and Khurana 2009; Falk and Munné-Bosch 2010). Our results, however, suggest that ABA was not involved in the diurnal transient increase in α-tocopherol (2 h after sunrise). In addition, seasonal increased ABA contents did not lead to enhanced α-tocopherol levels (Fig. 4). These evidences suggest that the levels of α-tocopherol may additionally be modulated by other factors, which could include low temperatures or light intensity.

In conclusion, seasonal changes of isoprenoids derived from the MEP pathway likely play a pivotal role overcoming the harmful effects of drought and cold stress under moderately high light intensity in the semi-deciduous Mediterranean shrub *C. albidus*. The high amounts of isoprenoids, particularly α-tocopherol, carotenoids, and ABA, indicate their important role in coping with environmental changes during a Mediterranean winter. The α-tocopherol contents remained high from November to March suggesting that they play a predominant role as antioxidants to protect the photosynthetic apparatus. Enhanced accumulation of carotenoids seemed to prevent damage to the photosynthetic apparatus under severe stress conditions. Following the initial transient increases of bioactive cytokinins during winter, the increased ABA contents indicate its important role during abiotic stress tolerance. The marked increase of the photoprotective isoprenoids, α-tocopherol, and xanthophylls, in the morning (3 h before maximum solar radiation) suggests that plants anticipated predictable changes in the environment by a concerted adjustment of α-tocopherol and xanthophylls. These contrasting seasonal and diurnal isoprenoid patterns in *C. albidus* appear to be finely modulated to respond to the prevailing environmental conditions to tolerate different environmental stresses at the same time.

Author contribution statement MPL, SMB, and MM designed the experiment. MPL and AC performed the biochemical analyses with the help of MM. MPL analyzed the data. MPL and MM wrote the manuscript with the help of SMB.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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Chapter 3. Inter-individual and sun orientation driven variability reveals antagonistic salicylate and jasmonate accumulation in white-leaved rockrose

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Inter-individual and sun orientation driven variability reveals antagonistic salicylate and jasmonate accumulation in white-leaved rockrose

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ABSTRACT

Salicylates and jasmonates may act synergically or antagonically depending on the plant growth conditions, biotic and abiotic stress factors to which plants are exposed to, and the tissue and plant species. Although possible tradeoffs between salicylate and jasmonate accumulation have been documented in model plants, information is still scarce in Mediterranean plant species, particulary in studies considering plants growing in their natural habitat. We evaluated to what extent inter-individual variability, sun orientation and diurnal variations in environmental conditions modulate not only the accumulation of salicylic acid and jasmonates (both free and conjugated forms), but also the contents of abscisic acid, hydrolyzable tannins and free phenolics, in white-leaved rockrose (*Cistus albidus* L.), a semi-deciduous shrub typically found in the Mediterranean basin. We found that jasmonates, most particularly the biologically active jasmonoyl-isoleucine and the jasmonic acid precursor, 12-*oxo*-phytodienoic acid showed a much more marked inter-individual variability than salicylic acid, abscicic acid, hydrolyzable tannins and free phenolics. East orientation reduced both abscisic acid and salicylic acid contents, while increased the accumulation of both jasmonates and hydrolyzable tannins, without affecting total free phenolic contents. Furthermore, salicylic acid and jasmonates contents, in particular those of 12-*oxo*-phytodienoic acid, changed throughout the day, while those of abscisic acid, hydrolyzable tannins and free phenolics remained constant. We conclude that sun orientation and inter-individual variability strongly influence stress-related phytohormones and chemical defense accumulation, with East-oriented individuals presenting higher amounts of jasmonates and hydrolyzable tannins, but less abscisic acid and salicylic acid than West-oriented ones. Results suggest a tradeoff between salicylic acid and jasmonates accumulation under stress conditions in *C. albidus* growing in their natural habitat.

1. Introduction

Mediterranean-type ecosystems are important biodiversity hotspots - the 4,3% of plant species on Earth are Mediterranean endemics. Climate change is predicted to increase the frequency of extreme climatic events such as heat waves and drought periods (Peñuelas et al., 2017; Valliere et al., 2017) and Mediterranean-type ecosystems are especially sensitive to these global changes (Matesanz and Valladares, 2013). Therefore, Mediterranean-type ecosystems are nowadays a conservation priority (Myers et al., 2000). Shrubs are essential to moderate the negative impacts of drought on these ecosystems (Rodriguez-Ramirez et al., 2017) and play an important role in preventing biodiversity loss due to an acceleration of desertification (Cabal et al., 2017).

Changes in environmental conditions can cause variations in stress-related phytohormones and chemical defenses and, in turn, these variations have an impact on shrubland composition in Mediterranean-type ecosystems (Mancilla-Leytón et al., 2014). Hence, climate change is likely to alter the chemical defense composition of some plant species and, given that Mediterranean ecosystems are already being affected by global changes (Maxime and Hendrik, 2011; Maya-Manzano et al., 2016; López-Moreno et al., 2017), it is imperative a better understanding of the environmental and genetic factors that modulate chemical defenses accumulation. Abiotic stressors, such as temperature, have been shown to have an effect on chemical defenses accumulation (Graglia et al., 2001; Stark et al., 2015; Abdala-Roberts et al., 2016a, 2016b). For instance, accumulation of secondary metabolites like hydrolyzable tannins is an important adaptation of plants to grazing

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(Peters and Constabel, 2002). Nevertheless, the effects of inter-individual variability on their production in woody perennials growing under Mediterranean field conditions is still limited. Tannins play an important role as constitutive defenses against herbivory (Peters and Constabel, 2002; Mancilla-Leytón et al., 2014; Abdala-Roberts et al., 2016a; Büchel et al., 2016) due to their binding capacity to proteins and consequently difficulting digestion to herbivores, particularly vertebrates (Iason, 2005; Mueller-Harvey, 2006; Barbehenn and Constabel, 2011; Top et al., 2017). Free phenolic compounds also play a role in the interaction between plants and biotic stressors. While being affected by environmental factors such as temperature, evapotranspiration (Bautista et al., 2016), or altitude (Rasmann et al., 2014), they are also decisive to fight herbivory in Mediterranean-type ecosystems (Massei et al., 2000; Mancilla-Leytón et al., 2014).

Compared to tannins and phenolics, jasmonates (JAs) are a much more rapid defense response, so much so that these lipid-derived signals increase within minutes in response to wounding and necrotrophic pathogens (Peters and Constabel, 2002; Ibrahim et al., 2018). JAs are signaling phytohormones related with many functions in plants, being typically involved in plant responses to biotic stress, but also to abiotic stress (Santino et al., 2013; Cotado et al., 2018). JAs effects are complex and depend, among other factors, on the accumulation of specific compounds. In this manner, 12-*oxo*-phytodienoic acid (OPDA), which is a jasmonic acid (JA) precursor, plays, in some cases, a differential role from JA or its amino acid conjugated form, jasmonoyl isoleucine (JA-Ile), which is generally considered the main biologically active form (Wasternack and Song, 2018). Other amino acid conjugates are jasmonoyl-methionine (JA-Met), jasmonoyl-valine (JA-Val), jasmonolyl-leucine (JA-Leu) and jasmonoyl-phenylalanine (JA-Phe) (Santino et al., 2013; Widemann et al., 2015; Yan et al., 2016; Cotado et al., 2018; Wasternack and Song, 2018). While JAs are typically involved in plant responses to necrotrophic pathogens, salicylates are involved in the resistance to biotrophic pathogens, so that the accumulation of these compounds is tightly regulated to fine-tune defense responses to build a robust plant immune system against a great number of different microbes (Davies, 2010; Giménez-Ibañez and Solano, 2013). However, both JAs and salycilates have also been involved in the plant response to abiotic stresses, a role they play in cooperation with abscisic acid (ABA). ABA is a phytohormone typically involved in osmotic adjustment and the induction of stomatal closure, but it may also play a role in the defense to some biotic stresses (Davies, 2010; Lim et al., 2015).

With the aim of better understanding possible tradeoffs between the activation of various types of chemical defenses, here we assessed the effects of inter-individual and environmentally driven variability on stress-related phytohormones and chemical defenses accumulation (including not only salicylic acid – SA – and JAs, but also ABA, hydrolyzable tannins and free phenolics) in *Cistus albidus* growing in Mediterranean field conditions during winter. Particular emphasis was given to the study of different JAs, including both free and conjugated forms. We hypothesized that there might be tradeoffs between the accumulation of various types of stress-related phytohormones and chemical defenses in white-leaved rockrose. To test this hypothesis, we evaluated the inter-individual and sun orientation driven variability in these compounds in a natural mountain population, as well as their diurnal variations in a population growing in an experimental garden.

2. Material and methods

2.1. Experimental design and sampling

Two independent but complementary studies were performed using white-leaved rockrose, *Cistus albidus* L, a semi-deciduous shrub typical of Mediterranean-type ecosystems. In a first experiment, a natural population of *C. albidus* was studied *in situ* in the Natural Park of the Montserrat Mountains, Spain (41.586 N- 1.830 E, 1100 m.a.s.l.). The effects of sun orientation and inter-individual variability on stress-related phytohormones (ABA, SA and JAs), hydrolyzable tannins and free phenolics was assessed. In a second experiment, we evaluated the diurnal variations in the same stress-related phytohormones and chemical defenses in a population growing in the experimental garden of the Faculty of Biology at the University of Barcelona (41.384 N, 2.119E, 59 m.a.s.l.) to facilitate repeated sample collection during the day. Both locations were exposed to a Mediterranean climate with mean winter temperatures under 18 °C but above 0 °C and with an annual rainfall usually concentrated on spring (Köppen, 1936). Winters in this region are generally associated with cloudless skies so that plants are generally exposed to > 1000 μmol/m^2 s of photosynthetically-active photon flux density at midday. Details of soil characteristics of both sites can be found in Müller et al. (2013).

The first study was performed on 22nd March 2018 at midday during a cold day to evaluate the effects of sun orientation and inter-individual variability during winter in a natural population. Maximum and minimum air temperatures during the day were 7.7 °C and -0.5 °C, respectively. Mean relative humidity was 30%, while no precipitation during the day was recorded. Sixty individuals of the same population but with different sun orientations were sampled; thirty were East oriented and thirty were West oriented. In some parts of the West-oriented area, a little snow remained in the soil forming patches (snow rainfall occurred two days prior to samplings). In addition to the monitoring of environmental parameters, maximum PSII efficiency, temperature and hydration, of one apical, fully developed and sun-exposed leaf of each plant were measured. The opposite leaf, which was exposed to the exact same conditions, was immediately frozen in liquid nitrogen and kept at -80 °C until biochemical analyses.

The second study was carried out during 27th February 2018 and 7th March 2018 to evaluate diurnal variations in chemical defenses accumulation. Samplings for diurnal effects evaluation were performed at predawn (1 h before sunrise), morning (exactly between the predawn and midday time points), midday (at maximum diurnal PPFD), afternoon (between the midday and evening time points), and evening (1 h after sunset). Six homogeneous individuals were selected for the study. The exact same measurements and biochemical analyses as in the first study were performed.

2.2. Environmental conditions, maximum PSII efficiency and water content

Leaf temperature and the maximum PSII efficiency (after 1 h of darkness for experiment 1 and at predawn for experiment 2) were measured with a Mini-PAM II (Photosynthesis Yield Analyser, Walz, Germany) *in situ*. Leaf hydration (H) was calculated as (fresh wt-dry wt)/dry wt, where fresh wt corresponds to the fresh mass and dry wt to the dry mass after oven-drying the sample at 80 °C to constant mass.

2.3. Stress-related phytohormones

Stress-related hormone profiling, including ABA, SA, OPDA, JA, JA-Ile, JA-Met, JA-Phe, JA-Val and JA-Leu was performed by ultrahigh-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) as previously described (Müller and Munné-Bosch, 2011; Cotado et al., 2018). Briefly, 50 mg per sample were extracted with 250 μl methanol, adding deuterium-labelled hormones, using ultrasonication (Branson 2510 ultrasonic cleaner, Bransonic, Danbury, CT, USA) for 30 min. After centrifugation at 12 000 rpm for 10 min at 4 °C, the pellet was re-extracted using the same procedure. Supernatants were pooled and filtered through a 0.22 μm PTFE filter (Waters, Milford, MA, USA) before analyses. Hormone contents were analyzed by using UHPLC-ESI-MS/MS as described in Müller and Munné-Bosch (2011). Quantification was made considering recovery rates for each sample by using the deuterium-labelled internal standards.

2.4. Hydrolyzable tannins and free phenolics

Hydrolyzable tannins and free phenolics content were determined based on the Folin-Ciocalteu method as described in Tambe and Bhambar (2014). Pure methanolic extractions were performed with 50 mg of powdered leaves for both hydrolyzable tannins and free phenolics analyses. For hydrolyzable tannins analyses, 100 µl of extract were mixed with 1.5 ml of distilled water, 200 µl of 35% Na_2CO_3 and 100 µl of Folin-Ciocalteu phenol reagent (Sigma-Aldrich). After 30 min at room temperature, absorbance was read spectrophotometrically at 725 nm (CE Aquarius UV/Visible Spectrophotometer, Cecyl Instruments Ltd, Cambridge, UK). Standard solutions of gallic acid (200, 400, 600, 800 and 1000 µg/ml) were used to quantify the hydrolyzable tannins contents of samples. For free phenolics analyses, 100 µl of extract were mixed whith 1 ml of Folin-Ciocalteu phenol reagent (Sigma-Aldrich). After 4 min at room temperature and dark conditions, 800 µl of 5% Na_2CO_3 were added. The absorbance was read spectrophotometrically at 765 nm after 1 h at room temperature and dark conditions. Standard solutions of gallic acid (200, 400, 600, 800 and 1000 µg/ml) were used to quantify the free phenolics content of samples.

2.5. Statistical analysis

To assess the effect of Sun orientation (‘East’, ‘West’) on leaf temperature, PPFD, maximum PSII efficiency (F_v/F_m ratio), leaf hydration, OPDA, free JA, JA-Ile, JA-Phe, JA-Val, hydrolyzable tannins and free phenolics, we used a generalized least squares (GLS) model and *P*-values were estimated using conditional *F*-tests. All models were validated by visually checking the distribution of residuals for normality and homoscedasticity (Zuur et al., 2009). To assess the effect of Time (‘Predawn’, ‘Morning’, ‘Midday’, ‘Afternoon’, ‘Evening’) in February and March on leaf temperature, PPFD, leaf hydration, OPDA, free JA, JA-Ile, JA-Phe, hydrolyzable tannins and free phenolics, we used a Linear Mixed Model (LMM) using the *lme* function within the *nlme* package. Time was fitted as a fixed term and Plant was fitted as a random term, to allow for repeated measures. The *P*-values of the fixed effects were estimated using conditional *F*-tests and multiple comparisons were tested with Tukey HSD test. All models were fitted using Restricted Maximum Likelihood (REML) and validated by visually checking the distribution of residuals for normality and homoscedasticity. Additionally, correlations between ABA, SA, OPDA, JA, and all the JA-amino acid conjugated forms and hydrolyzable tannins and free phenolics were tested with Spearman’s rank correlation tests. All results were considered significant at a probability level of $P \leq 0.05$. All statistical analyses were conducted using the R statistical software (R Development Core Team, 2018).

3. Results

3.1. Inter-individual variability and sun orientation drive antagonistic changes in jasmonates and salicylic acid

An analysis of micro-environmental conditions within the randomly selected 60 individuals of *C. albidus* grown in the Natural Park of the Montserrat Mountains at 1100 m.a.s.l. revealed a moderate inter-individual variability in the micro-environmental conditions the plants were exposed to. In particular, the standard deviation (SD) relative to the mean value of the population (in percentage) was around 28% for leaf temperature and 30% for the photosynthetically-active photon flux density (PPFD, Fig. 1). However, while leaf temperature was on average a 58% higher in East-oriented individuals than in the West-oriented ones (with values of *ca.* 19 °C and 12 °C, respectively), there were not significant differences on average in the PPFD between West- and East-oriented individuals (Fig. 1). Inter-individual variability for leaf hydration and the F_v/F_m ratio was smaller ($SD_{population}$ of 8% and 10%, respectively), and either of these parameters differed on average between West- and East-individuals (Fig. 1).

Among the stress-related phytohormones studied (ABA, SA and JAs), JAs were the ones showing the highest inter-individual variability, a large part of this variability being caused by sun orientation (Fig. 2). Among the JAs analyzed, the most biologically active form (JA-Ile) was the one showing the highest variability with values ranging between non-detectable values and 60 µg/g dry wt (with a $SD_{population}$ of 516%, Fig. 2). The hormonal profiling also revealed a high biological variation in the contents of OPDA, JA, JA-Val and JA-Phe (in decreasing order), the two latter JA-amino acid conjugates being present at the lowest amounts (always below 60 ng/g dry wt). OPDA, a precursor of free JA, was particularly variable and found at high concentrations (up to 8 µg/g dry wt) in the East-oriented population (Fig. 2). Indeed, we found a significant effect of sun orientation on OPDA, JA, JA-Phe and JA-Val contents, with East-oriented individuals presenting on average significantly higher values than the West ones (Fig. 2). In the case of OPDA, the mean of East-oriented individuals was 1444 ng/g dry wt whereas the mean of the West-oriented ones was 78 ng/g dry wt. JA contents were an 80% higher in East-oriented individuals. JA-Phe and JA-Val contents were a 52% and 77% higher in the East-oriented individuals than in the West individuals, respectively (Fig. 2). JA-Met and JA-Leu were found below the detection limt (0.80 ng/g dry wt and 2.4 ng/g dry wt, respectively). In contrast to JAs contents, those of SA and ABA decreased in East-oriented individuals compared to West-oriented ones (Fig. 2).

In the case of constitutive defenses, including hydrolyzable tannins and free phenolics, the inter-individual variability in their contents (both expressed as mg equiv. gallic acid/g dry wt) was much smaller compared to that of phytohormones, and most particularly in relation to JAs, but still very high in the case of hydrolyzable tannins ($SD_{population}$ = 303%). The contents of hydrolyzable tannins in the *C. albidus* population growing at 1100 m.a.s.l. in the Natural Park of the Montserrat Mountains ranged between 7 and 25 mg equiv. gallic acid/g dry wt, showing on average a significant sun orientation effect (Fig. 3). As it occurred with JAs, hydrolyzable tannins content was also on average higher in East- compared to West-oriented individuals, although differences were relatively smaller (17%). Free phenolic contents, which ranged between 10 and 140 mg equiv. gallic acid/g dry wt among the 60 individuals studied, did not differ on average between East- and West-oriented individuals (Fig. 3).

3.2. Diurnal variations in stress-related phytohormones and chemical defenses

An analysis of environmental conditions, including leaf temperature and incident PPFD, on 27th February and 7th March 2018 in *C. albidus* grown in the experimental garden at 59 m.a.s.l. was consistent with a snowy, cloudy day and a cold (but relatively milder), sunny day, respectively. Both incident PPFD and leaf temperature were consistently much higher during 7th March than 27th February (Fig. 4). There were not significant differences on average in the maximum PSII efficiency between both days (with mean values above 0.8 in both cases, data not shown). Among all stress-related phytohormones and chemical defenses studied, SA and OPDA were the only compounds consistently showing diurnal variations during both days (Figs. 5 and 6). OPDA was the compound found at the highest concentrations among JAs, and OPDA contents increased during the morning reaching maximum contents during the afternoon during both 27th February and 7th March (Fig. 5), when diurnal leaf temperature was the highest (Fig. 4). The most biologically active form (JA-Ile) was the one showing the highest variability among the two days studied, and although variations in JA-Ile were significant during 27th February, values were low and ranged between 2 and 8 µg/g DW (Fig. 5). SA contents also increased during the day, the maximum diurnal peak coinciding with that of OPDA during 7th March, but occurring earlier during 27th February. Contents of ABA and constitutive defenses, including both hydrolyzable tannins

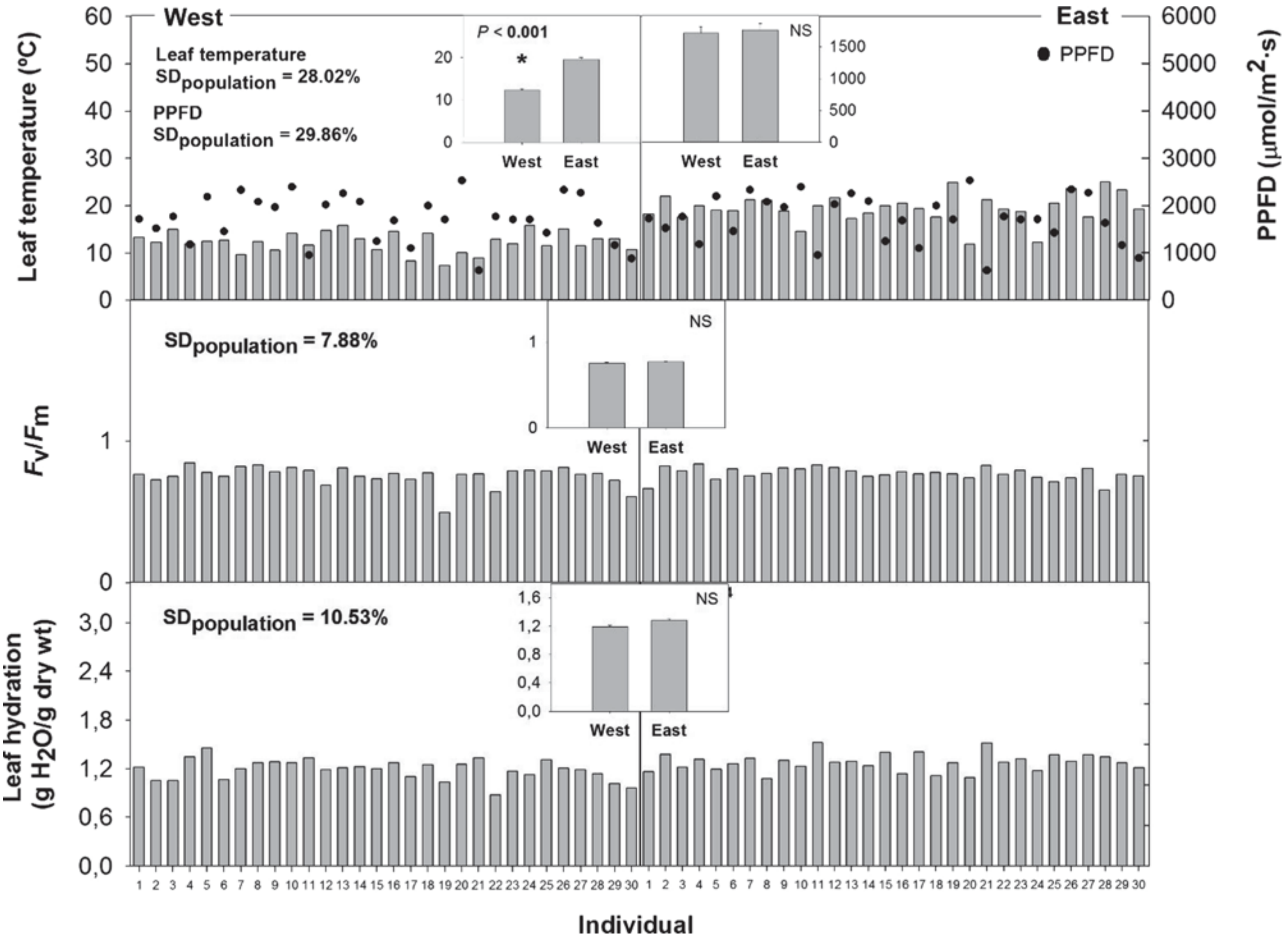


Fig. 1. Inter-individual variability in leaf temperature and photosynthetically-active photon flux density (PPFD), maximum efficiency of PSII photochemistry (F_v/F_m ratio), and leaf hydration (H) in a *C. albidus* population growing at 1100 m.a.s.l. in the Natural Park of the Montserrat Mountains with different sun orientation (East orientation, n = 30 individuals, and West orientation, n = 30 individuals) during winter (22nd March 2018). Percent deviation for every variable is expressed as $SD_{population}$. The mean ± SE for each sun orientation are shown in the graphs located in the inlets. Significant differences between groups were tested by one-way analysis of variance (ANOVA, $P \leq 0.05$) and indicated with an asterisk. NS, not significant; dry wt, dry matter.

and free phenolics, kept constant throughout the day, both during 27th February and 7th March (Figs. 5 and 6).

4. Discussion

Plants have evolved different defense strategies to adapt to Mediterranean-type ecosystems, including the modulation of both stress-related phytohormones and chemical defenses. Environmental factors may have an important impact on these strategies, mainly changing investment priorities between constitutive and inducible defenses (Graglia et al., 2001; Garbero et al., 2011; Mancilla-Leytón et al., 2013, 2014; Rasmann et al., 2014; Bautista et al., 2016). In the present study, we show that white-leaved rockrose presents a tradeoff between the accumulation of stress-related phytohormones such as SA and ABA, and the accumulation of JAs, most particularly OPDA. This occurred particularly in the study of inter-individual and sun orientation driven variability in the Montserrat Mountains, but not on a diurnal basis during the winter in the experimental garden. Furthermore, OPDA and JA-Ile seem to respond very differently to environmental and genetic factors compared to free JA or other amino acid conjugated forms, thus suggesting possible specific roles for different JAs forms in white-leaved rockrose.

OPDA, JA and its conjugated forms are all stress-related phytohormones and their biosynthesis especially changes under both extreme temperatures (Sharma and Laxmi, 2016) and biotic pressures, particularly herbivore attack and wounding (see Bari and Jones, 2009, and references therein). OPDA is the precursor of JA and it is usually found in higher concentrations than other JAs. Indeed, the contents of OPDA were higher than those of JA and the other amino acid conjugated forms in both study sites, evidencing its high accumulation in leaves of white-leaved rockrose. A strong sun orientation effect was observed on JAs in the population found in the Montserrat Mountains. This is in agreement with previous studies showing a differential effect between sun and shade individuals in *Asclepias syriaca* herbivore-induced JAs, where shade-plants had significantly lower contents (Agrawal et al., 2012). Greater contents of OPDA, JA, JA-Ile, JA-Phe and JA-Val were observed in East-oriented individuals than in West-oriented ones. JA-Ile contents were higher than those of JA-Phe and JA-Val in most of the East-oriented individuals. Although JA-Ile has been described to be the most bioactive JA-amino acid conjugated form in plant stress responses (Fonseca et al., 2009), not only JA and JA-Ile but also JA-Phe have been shown to participate in the response upon wounding (Glauser et al., 2009; Widemann et al., 2015). As all JA forms were higher in the East-oriented individuals, it is very likely that East-oriented plants were exposed to a higher wounding pressure caused by biotic stressors that West-oriented ones. On the other hand, SA and ABA contents were higher in West-oriented individuals. Interstingly, inter-individual and sun orientation driven variability revealed a tradeoff between SA and JAs accumulation, most particularly between SA and OPDA, which strongly negatively correlated (Fig. 7). It is worthy to note that SA correlated more strongly with OPDA than with other JAs, including JA-Ile (Fig. 7), thus suggesting a tradeoff between SA accumulation and the

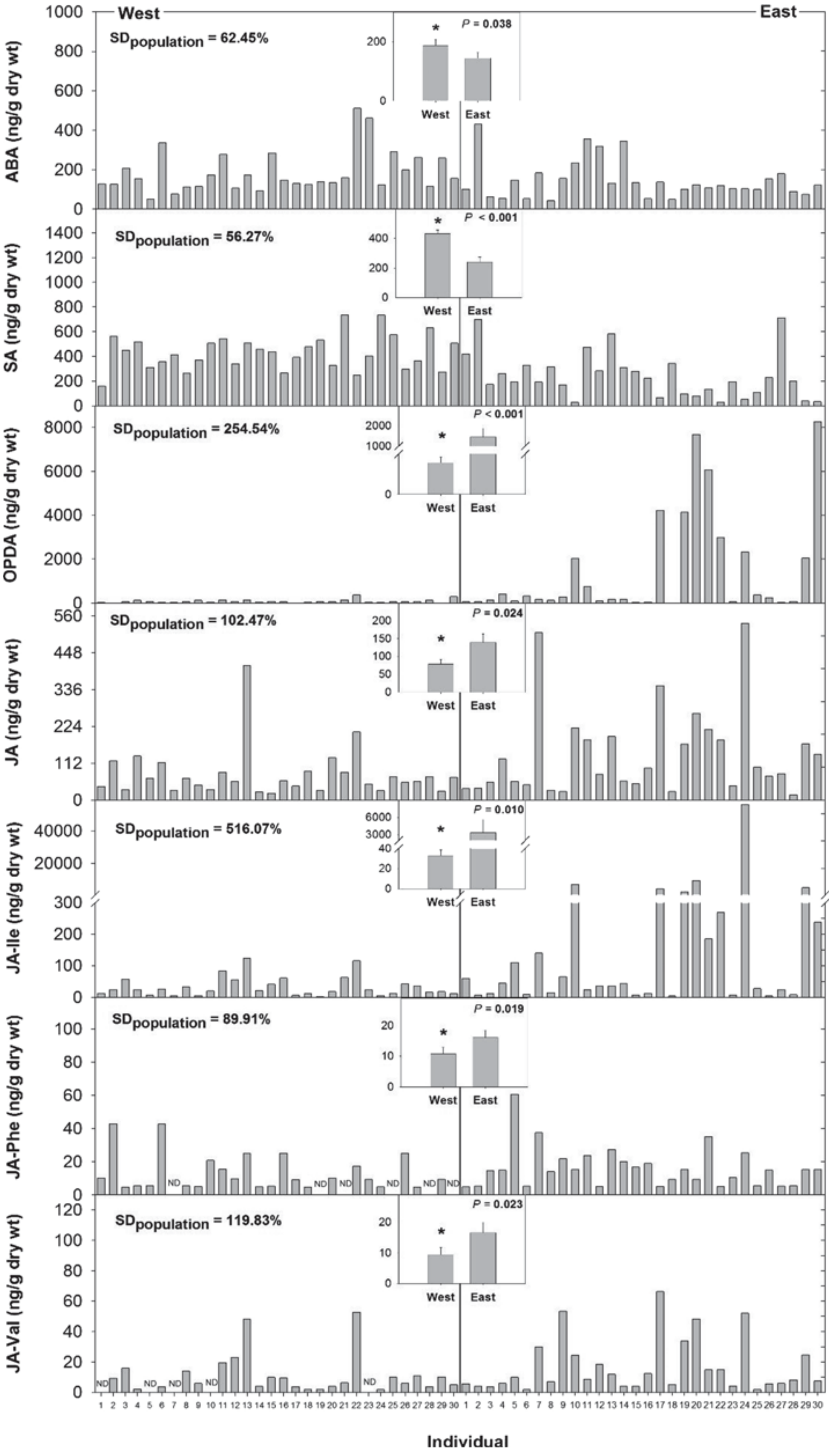


Fig. 2. Effects of sun orientation and inter-individual variability on the endogenous contents of ABA, salicylic acid and jasmonates, including 12-oxo-phytodienoic acid (OPDA), free jasmonic acid (JA) and its conjugated forms JA-Ile and JA-Phe (all expressed as ng/g dry wt) in a *C. albidus* population growing at 1100 m.a.s.l. in the Montserrat Mountains with different sun orientation (East orientation, $n = 30$ individuals, and West orientation, $n = 30$ individuals) during winter (22nd March 2018). Percent deviation for every variable is expressed as $SD_{population.}$ The mean ± SE for each sun orientation are shown in the graphs located in the inlets. Significant differences between groups were tested by one-way analysis of variance (ANOVA, $P \leq 0.05$) and indicated with an asterisk. ND, not detectable; NS, not significant; dry wt, dry matter.

capacity to synthesize JAs, given that ODPA is the precursor of all other JAs. Interestingly, however, the tradeoff between SA and JAs seems not to occur on a diurnal basis, since plants from the experimental garden were able to simultaneously increase both phytohormones at midday during winter. It is tempting to speculate that the occurrence of this tradeoff in the high-mountain population may be specifically due to a high biotic pressure in the East population, thus leading to a forced higher accumulation of JAs (enhanced defense against necrotrophs) that might result in a reduced capacity to accumulate SA (reduced defense against biotrophs, Davies, 2010). Indeed, the negative correlation between SA and OPDA was not observed when the correlation was run with the individuals of the West population only (Spearman's

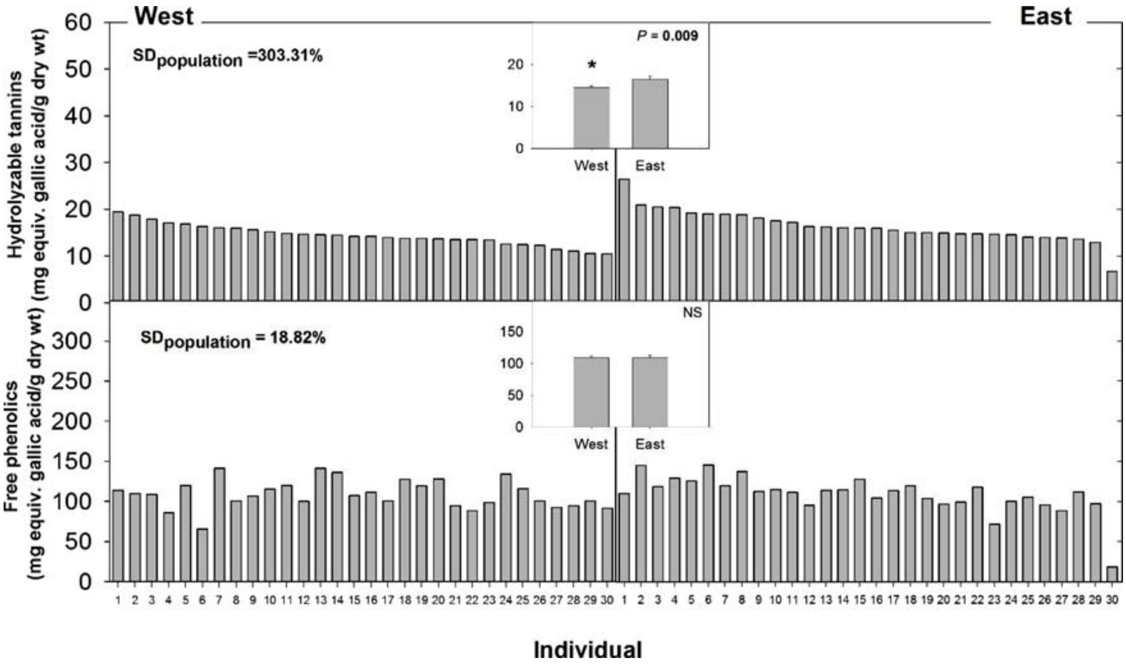


Fig. 3. Effects of sun orientation and inter-individual variability on hydrolyzable tannins and free phenolics contents (both expressed as mg equiv. gallic acid/g dry wt) in a *C. albidus* population growing at 1100 m.a.s.l. in the Natural Park of the Montserrat Mountains with different sun orientation (East orientation, $n = 30$ individuals, and West orientation, $n = 30$ individuals) during winter (22nd March 2018). Percent deviation for every variable is expressed as $SD_{population}$. The mean ± SE for each sun orientation are shown in the graphs located in the inlets. Significant differences between groups were tested by one-way analysis of variance (ANOVA, $P \leq 0.05$) and indicated with an asterisk. NS, not significant; dry wt, dry matter.

rank correlation, $P > 0.05$), or only with those growing in the experimental garden (Spearman's rank correlation, $P > 0.05$). The negative correlation between SA and OPDA was, however, very strong both when the correlation was run with the individuals of the East population only (Spearman's rank correlation, $P < 0.001$) or with all data from all studied individuals in the Montserrat Mountains pooled together (Spearman's rank correlation, $P < 0.001$, Fig. 7). This correlation showed that none of the East individuals accumulated SA above 200 ng/g dw when OPDA reached values above 2 μg/g dw, showing a very strong negative correlation when data were log transformed (ρ = -0.72, $P < 0.001$, Fig. 7). Furthermore, ABA has been reported to act antagonistically to JAs upon wounding (Lee et al., 2018; Proietti et al., 2018), and East-oriented individuals not only showed higher contents of JAs and lower contents of SA compared to the West-oriented ones, but also lower ABA contents. This suggests that SA and ABA may act antagonically to JAs in white-leaved rockrose populations exposed to a high biotic pressure, an aspect that requires further studies.

SA, OPDA, JA and JA-Ile contents showed marked diurnal patterns,

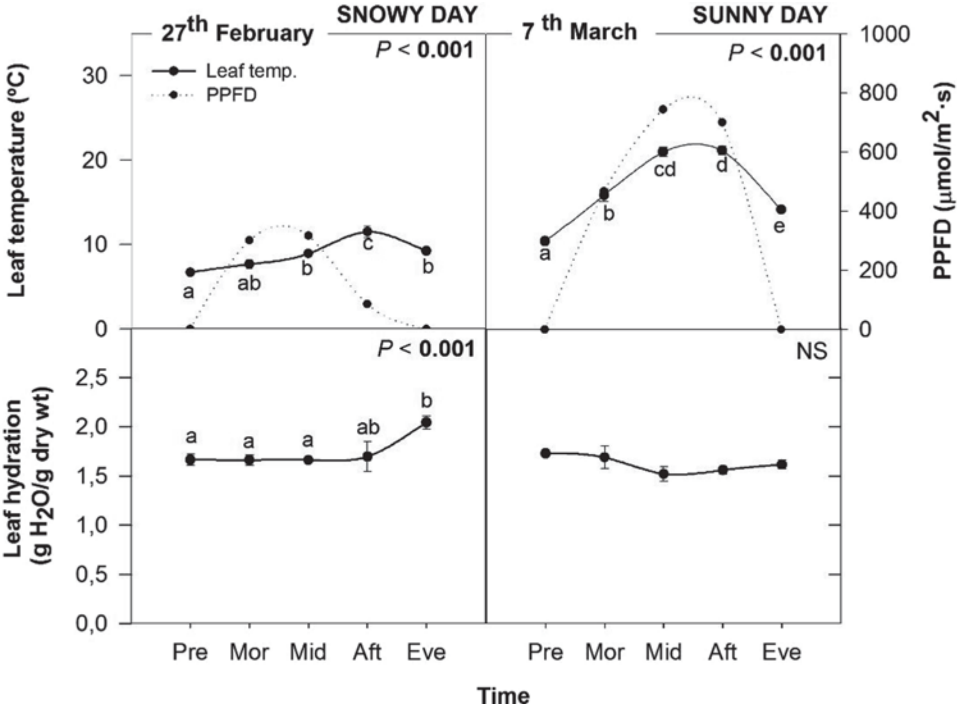


Fig. 4. Diurnal variations in leaf temperature and PPFD, and leaf hydration (H) on a snowy day (27th February 2018) and a cold sunny day (7th March 2018) in *C. albidus* at the experimental garden of the University of Barcelona. Data is the mean ± SE of $n = 6$ individuals. Significant differences between times of the day were tested by conditional Fisher tests ($P \leq 0.05$). Different letters indicate significant differences in Tukey's HSD multiple comparison test ($P \leq 0.05$). NS, not significant; dry wt, dry matter.

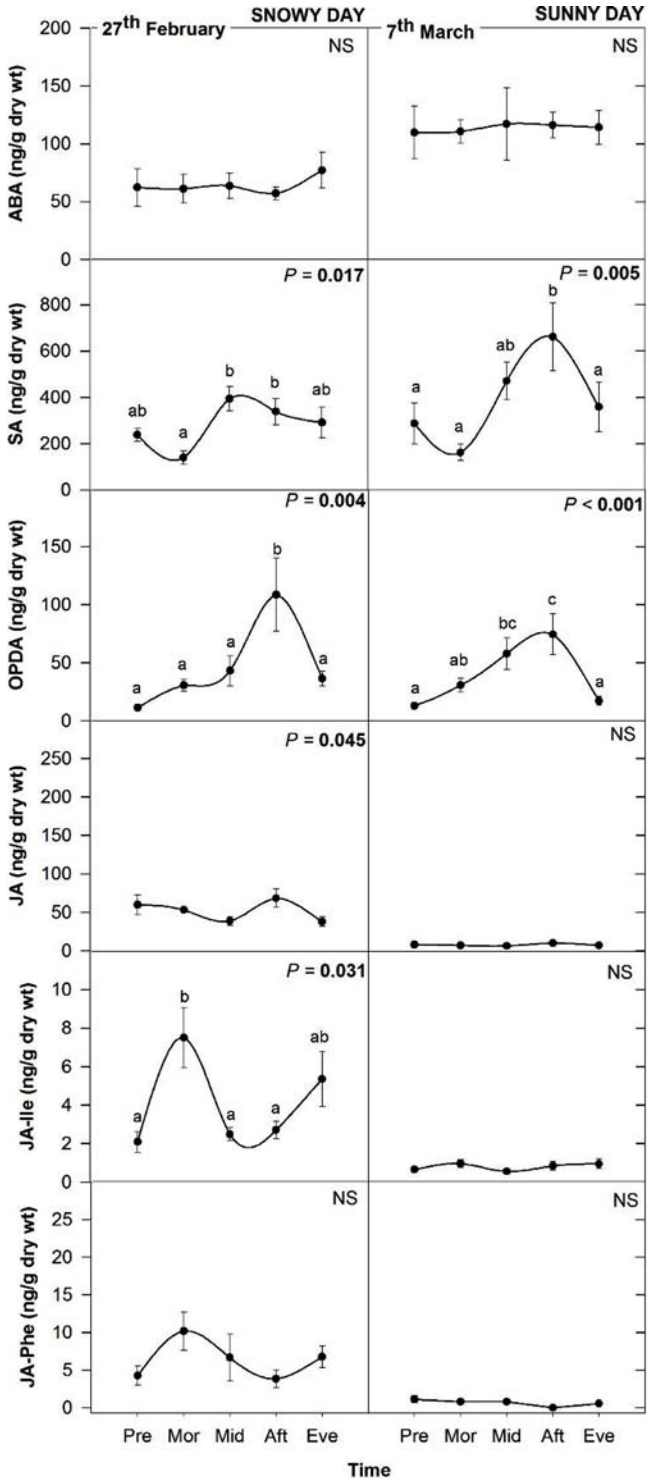


Fig. 5. Diurnal variations in the endogenous contents of abscisic acid (ABA), salicylic acid (SA), and jasmonates, including 12-*oxo*-phytodienoic acid (OPDA), free jasmonic acid (JA) and its conjugated forms JA-Ile and JA-Phe (all expressed as ng/g dry wt) during a snowy day (27th February 2018) and cold sunny day (7th March 2018) in *C. albidus* growing at the experimental garden of the University of Barcelona (Barcelona, NE Spain). Data are mean ± SE of $n = 6$ individuals. Significant differences between times of the day were tested by conditional Fisher tests ($P \leq 0.05$). Different letters indicate significant differences in Tukey's HSD multiple comparison test ($P \leq 0.05$). NS, not significant; dry wt, dry matter.

thus indicating rapid changes in inducible chemical defenses in white-leaved rockrose. Certainly, circadian rhythms have been previously reported in SA- and JA-related genes in model plants (Walley et al., 2007; Mizuno and Yamashino, 2008; Goodspeed et al., 2012). The diurnal pattern of OPDA, however, followed a different trend to that observed for free JA or JA-Ile. While OPDA content was consistently higher in the afternoon in both the snowy and sunny days studied, coinciding with the highest leaf temperature, diurnal variations in JA and JA-Ile were significant on the snowy day only, and differences were very small and with much higher *P* values (note the low amounts JA-Ile). These results suggest that, among JAs, diurnal variations in environmental conditions mostly modulate the contents of OPDA, thus reflecting either (i) a circadian regulation of the capacity to synthesize JAs, since OPDA is the precursor for all other JAs (Wasternack and Song, 2018), and/or (ii) specific functions for OPDA compared to that of other active JAs, as it has been shown that OPDA may play specific functional roles in plants (Maynard et al., 2018; Simancas et al., 2018).

Tannins are important chemical defenses against herbivores, particularly in woody plants, and they have already been previously reported to accumulate in large amounts in *C. albidus* leaves (Qa'dan et al., 2003; Barrajón-Catalán et al., 2011). Sun orientation significantly affected hydrolyzable tannins content, having the East-oriented individuals a greater content of hydrolyzable tannins than the West-oriented ones. Differences in sun orientation on tannins contents have been shown in other studies; having light an enhancing effect on several shrub tannin contents (Baraza et al., 2004; Karolewski et al., 2013). Certain herbivores have also been reported to induce the synthesis of tannins in some woody plants leaves (*e.g.* Arnold and Schultz, 2002; Rossi et al., 2004; Roitto et al., 2009), although responses are very species-dependant and this tannin inducibility upon herbivory does not seem to be universal (Barbehenn and Constabel, 2011). In this manner, the higher hydrolyzable tannins contents in the East-oriented individuals of *C. albidus* could be due to the selectively feeding habits of goats (with *ca.* 250 individuals inhabiting the study site). Indeed, some studies have reported that certain mammals do have a feeding preference for high tannin-content plants in their diet (Marsh et al., 2003; Baraza et al., 2005). Moreover, goats have been observed to have a higher feeding preference for some species of the family *Cistaceae* during winter than for other understory Mediterranean plants (Mancilla-Leytón et al., 2013). With all, the interaction between biotic pressure and sun orientation may determine the high investments in chemical defenses in the natural population, particularly in the East-oriented population.

Free phenolic contents did not vary between the East and West-oriented individuals, despite average leaf temperature differed between them. Concerning the effect of environmental factors such as altitude (Rasmann et al., 2014) or water-related factors (Bautista et al., 2016) on free phenolics, reduced leaf hydration and higher altitude of the mountain population compared to the experimental garden suggests that these abiotic factors affect the investments on chemical defenses, in this case, free phenolics. Furthermore, it has been shown that phenolic compounds play a role in cold-induced acclimation to biotic stress (Hura et al., 2015). In the case of the experimental garden population, free phenolics contents were higher in the sunny day than in the snowy day but there were not significant differences during the day, suggesting a possible long-term effect of light in their accumulation. Free phenolics and hydrolyzable tannins not only differed in their accumulation depending on the sun orientation but also in their inter-individual variability, being tannins the most influenced ones by it. Sun orientation influenced the accumulation of hydrolyzable tannins but not that of total free phenolics, possibly reflecting a protective role of hydrolyzable tannins but not of free phenolics against herbivore pressure.

It is concluded that sun orientation and inter-individual variability strongly influence stress-related phytohormones and chemical defense accumulation in plants, with East-oriented individuals showing higher

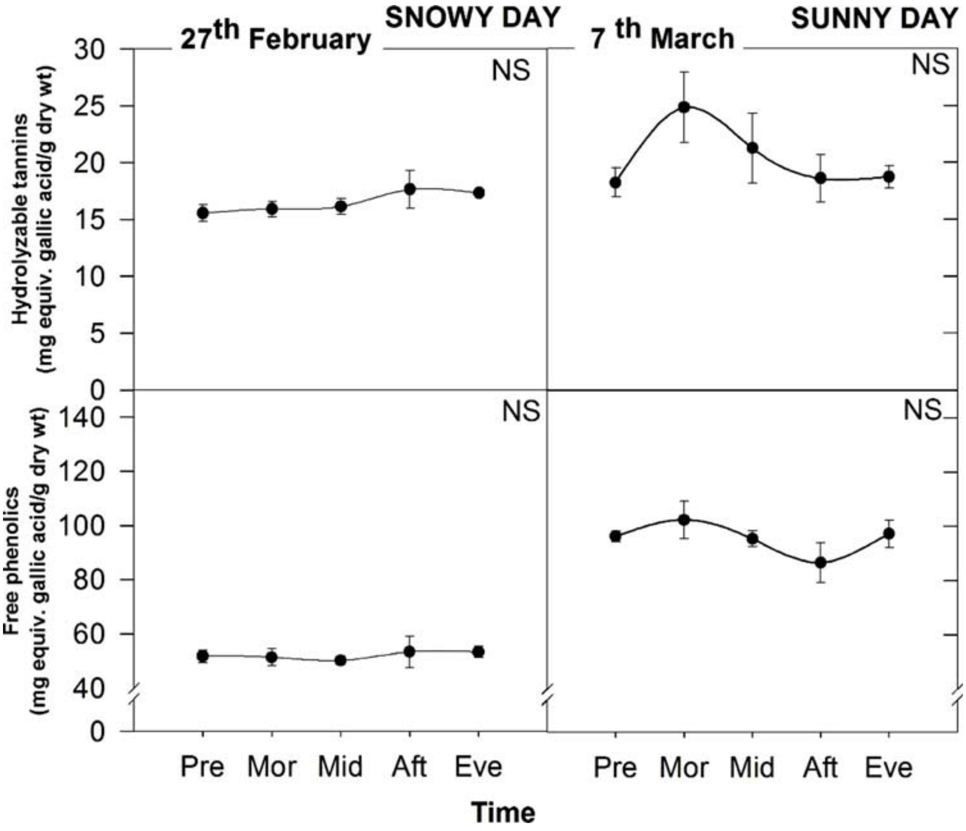
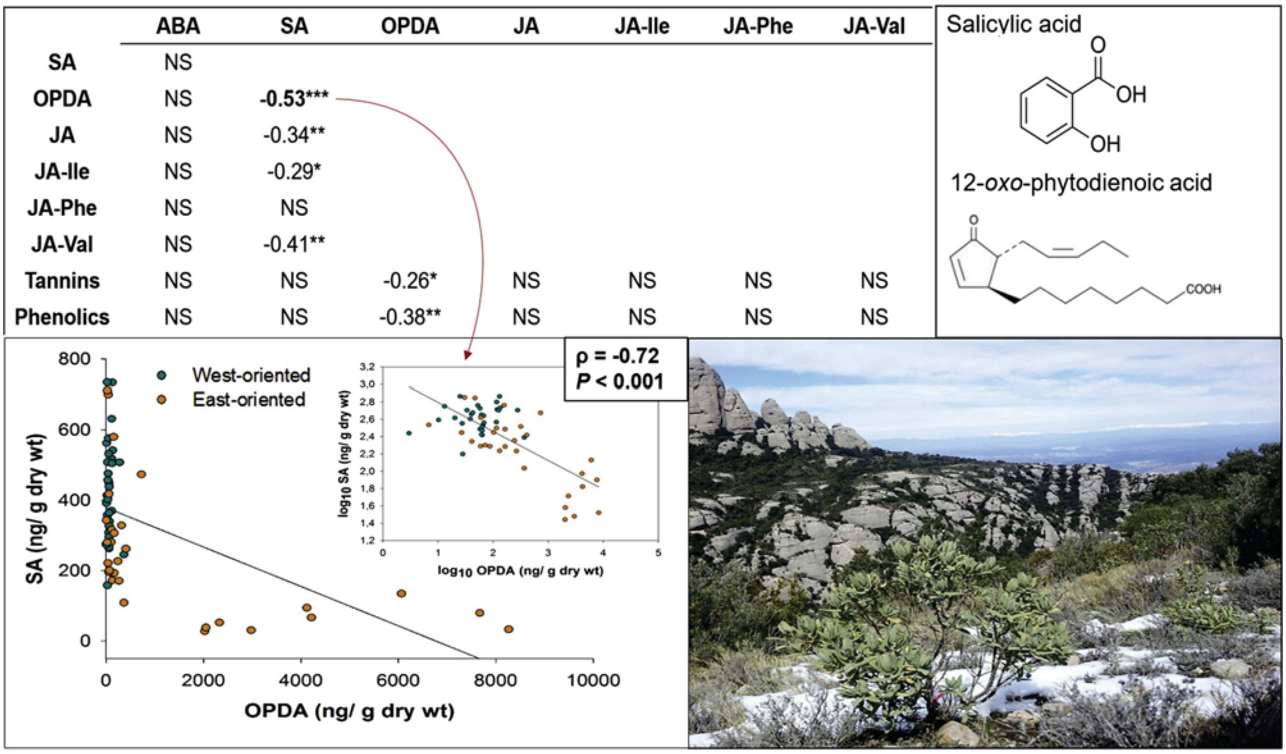


Fig. 6. Diurnal variations in the endogenous contents of hydrolyzable tannins and free phenolics contents (both expressed as mg equiv. gallic acid/g dry wt) on a snowy day (27th February 2018) and on a cold sunny day (7th March 2018) in *C. albidus* at the experimental garden of the University of Barcelona. Data is the mean ± SE of $n = 6$ individuals. Significant differences between times of the day were tested by conditional Fisher tests ($P \leq 0.05$). Different letters indicate significant differences in Tukey's HSD multiple comparison test ($P \leq 0.05$). NS, not significant; dry wt, dry matter.



	ABA	SA	OPDA	JA	JA-Ile	JA-Phe	JA-Val
SA	NS						
OPDA	NS	**-0.53*****					
JA	NS	-0.34**					
JA-Ile	NS	-0.29*					
JA-Phe	NS	NS					
JA-Val	NS	-0.41**					
Tannins	NS	NS	-0.26*	NS	NS	NS	NS
Phenolics	NS	NS	-0.38**	NS	NS	NS	NS

Fig. 7. Results of the Spearman's rank correlation analyses (correlation coefficient and P values are shown) between abscisic acid (ABA), salicylic acid (SA), jasmonates, hydrolyzable tannins and free phenolics in *C. albidus* from a natural population at the Natural Park of the Montserrat Mountains. All data were pooled together for the analyses. Note that a strong negative correlation (correlation coefficient $\rho = -0.53$, $P < 0.001$) was obtained between SA and 12-*oxo*-phytodienoic acid – OPDA – (results shown in bold, *left*), most particularly when SA and OPDA data were log transformed (indicated with a red arrow, correlation coefficient ρ of -0.72, $P < 0.001$). An example of an individual growing in its natural habitat together with the chemical formulas of SA and OPDA are shown (*right*). All correlations were considered stastically significant at $P \leq 0.05$, with one, two and three asterisks indicating P values below 0.05, 0.01 and 0.001, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

amounts of JAs and hydrolyzable tannins, but less ABA and SA than West-oriented ones. Furthermore, results suggest that white-leaved rockrose alters their foliar SA and JAs contents following diurnal variations, while ABA, hydrolyzable tannins and free phenolics contents do not vary during the day. Finally, our results support a tradeoff between SA and JAs accumulation under stress conditions in *C. albidus*. The differential effects of environmental and inter-individual driven variability on ABA, SA and JAs (including OPDA and free JA and conjugated forms), hydrolyzable tannins and free phenolics accumulation illustrate the complex functional diversity of stress-related phytohormones and chemical defenses in white-leaved rockrose and that these defense mechanisms may be influenced to a large extent by both environmental and genetic factors.

Author contribution statement

MPL, AC and SMB contributed to the conception and design of the study; AC, MPL and MM organized the database; MPL performed the statistical analysis; MPL, AC and SMB wrote the manuscript. All authors approved the submitted version.

Conflict of interest statement

The authors declare that they have no conflict of interest in the authorship of this article.

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Chapter 4. The threshold between life and death in *Cistus albidus* L. seedlings: mechanisms underlying drought tolerance and resilience

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Research paper

The threshold between life and death in *Cistus albidus* L. seedlings: mechanisms underlying drought tolerance and resilience

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Drought can lead to important shifts in populations dynamics if occurs during seedling establishment. With the aim of elucidating the underlying mechanisms of drought tolerance and resilience, here we monitored the survival of seedlings of the Mediterranean shrub *Cistus albidus* throughout a year growing in the Natural Park of the Montserrat Mountains (Spain) and, additionally, we studied the response to severe drought and subsequent recovery after re-watering, of seedlings grown in growth chambers. To find possible mechanisms explaining how seedlings respond to drought, growth and survival, together with physiological-related parameters such as chlorophyll contents, vitamin E and stress-related phytohormones were measured. We found that survival decreased by a 30% at the end of summer and that the main proxy of seedling survival was total chlorophyll. Furthermore, modulation of vitamin E and jasmonates contents appeared to be crucial in the drought response of *C. albidus* seedlings. We propose a prediction model of survival with chlorophyll contents being a good long-term predictor of *C. albidus* seedling survival under severe stress, which could help to better foresee populations fluctuations in the field.

Keywords: drought, seedlings, survival, Mediterranean plants, *C. albidus*, tolerance, vitamin E, jasmonates

INTRODUCTION

Drought is one of the main drivers of higher plants' performance and survival. Within the life cycle of a plant, the time between germination and seedling establishment is when plants are most vulnerable (Good and Good 1972) and undergoing a period of water stress during this span can lead to the death of individuals and ultimately, to a shift in populations' dynamics. Mediterranean species have to be in constant acclimation to seasonal environmental changes, including drought events. Due to global warming, these drought events are predicted to be harsher and increase in frequency (Matesanz and Valladares 2014) and, in fact, some of these predicted detrimental effects are already affecting Mediterranean habitats (IPCC 2013), both directly and indirectly (Klein 2015). Therefore, the ability of plants to resist water stress and then return to a pre-drought state (i.e. resilience) during these changing environmental conditions is critical for plant survival. Plants have multiple mechanisms to cope with drought – e.g. carbon allocation (Trugman et al. 2018), shoot and root structural changes (Hartmann 2011; Limousin et al., 2012; Chitra-Tarak et al. 2018), decrease in leaf surface (Wright et al. 2004; Bréda et al. 2006) or the production of antioxidants to tackle cellular damage (Munné-Bosch et al. 2003; Fernández-Marín et al. 2017; Saunier et al. 2018). The concerted action of these mechanisms operating at various levels is what ultimately confers stress tolerance and resilience in natural populations. However, this response is strongly species specific and more studies are necessary to better predict plants' survival and ultimately, populational and successional dynamics in natural populations.

The first physiological response of a plant when it faces water stress is to slow down growth followed by stomatal closure to avoid water loss, which in turn will lead to a decrease in photosynthesis and then, carbon starvation (McDowell 2011). On the other hand, the plant will also undergo hydraulic failure due to cavitation and this will result in the plant's death if stress is severe enough (McDowell et al. 2008). Consequently, in order for the plant to avoid death by water loss, the xylem will have to maintain the water supply and living tissues will need to retain water (Sapes et al. 2019). Interestingly, some studies have proved a close relationship between these two mechanisms (Brodersen et al. 2010; Sevanto et al. 2013; Meir et al. 2015). Drought can also cause a photochemical impairment leading to oxidative stress. Frequently, plants have to suffer water stress combined with high irradiances, especially in the Mediterranean climate and not only in the summer season but also in winter due to low temperatures and scarce rainfall (Martínez-Ferri et al. 2004; Flexas et al. 2014). Mediterranean plants have different mechanisms to avoid this photo-oxidative stress by first avoiding light absorption such as changes in leaf angles (Oliveira and Peñuelas 2002; Pérez-Llorca et al. 2019a), tomentous leaves (Galmés et al. 2007) or decreases in total chlorophyll content (Valladares et al. 2012; Fernández-Marín et al. 2017). If these mechanisms are not enough to avoid excess light absorbance, plants will respond with the accumulation and action of photoprotective pigments and antioxidants to prevent an eventual damaging effect of reactive oxygen species (ROS) production in chooroplasts. Such mechanisms include the xantophyll cycle (Demmig-Adams and Adams 2006; Esteban et al. 2015), hydrophilic antioxidants such as glutathione and ascorbate, which operate in the ascorbate-glutathione cycle as an intrinsic part of the water-water cycle (Szarka et al. 2012; Asada 2006) and lipophilic antioxidants like carotenoids, plastoquinol and tocopherols (Peñuelas and Munné-Bosch 2005; Muñoz and Munné-Bosch 2019; Havaux 2020). Tocopherols (vitamin E) not only are powerful antioxidants to protect plants against abiotic stress, but they have also been proposed to have a key role in cellular signaling (Havaux et al. 2005; Muñoz and Munné-Bosch 2019; Munné-Bosch 2019).

Phytohormones, as signaling regulators, also have a major involvement both in plant development and in the acclimation to environmental stresses. Particularly, abscisic acid (ABA), salicylic acid (SA) and jasmonates (JAs) have been proposed as key stress hormones with a major role in stress tolerance and resilience given their action, activating early responses upon a number of biotic and abiotic stresses (Verma et al. 2016). Under water stress, ABA which is the final product of the MEP-carotenoid pathway (Vranová et al. 2012), is accumulated and triggers a cascade of physiological responses, including stomatal closure and osmotic adjustment, among others (Lee and Luan 2012; Benham et al. 2013). SA, synthesized from either phenylalanine or chorismic acid (Chen et al. 2009), also contributes to drought tolerance (Munné-Bosch and Peñuelas 2003; Bandurska and Stroiński 2005) and it has also been proposed, although controversially, to be involved, together with ABA, in stomatal closure upon drought (He et al. 2007). JAs are fatty acid-derived compounds that have a rapid signaling response to various environmental stresses (Santino et al. 2013; Cotado et al. 2018; Pérez-Llorca et al. 2019b) and, particularly, exogenous application of jasmonates has been reported to improve drought tolerance in some studies (Riemann et al. 2015; Savchenko et al. 2014).

With the aim of better understanding the mechanisms plants have to tolerate and be resilient to drought, we studied the performance of *Cistus albidus* seedlings in their natural habitat. *C. albidus* has been shown to tolerate low water statuses while maintaining a good physiological performance (Pérez-Llorca et al. 2019c); hence, we hypothesised that seedlings of this plant in their natural habitat would survive the harsh Mediterranean climatological conditions, including summer. We followed individuals of *C. albidus* for a year to assess seedling survival. Additionally, given that we found that the extent of a drought episode could be crucial for seedling survival in our study, we performed a complementary study under controlled conditions. We subjected *C. albidus* seedlings to a severe water stress and then followed their recovery to determine the threshold of survival under drought and the underlying mechanisms of drought tolerance and resilience in this Mediterranean species.

MATERIALS AND METHODS

Study species

In this study, *Cistus albidus* L. (Cistaceae), a semi-deciduous

Mediterranean plant, was used as a drought-tolerant model. *C. albidus* or white-leaved rockrose, is a sclerophyllous shrub with tomentous leaves typically found in maquis and garrigue habitats. It is a pioneer shrub in succession, especially after a disturbance (Juhren, 1966), and, due to its strong root system, enables other plants to establish and avoids soil erosion. *C. albidus*'s flowering occurs in March-May; fruits ripen by mid- to late summer; and, seed dispersal (which can last up to months) starts after that (Blasco and Mateu, 1995).

Experimental design

Two complementary experiments were performed in order to study *C. albidus* seedlings response to drought. The first experiment was carried out in *C. albidus* growing at the Natural Parc of the Montserrat Mountains (834 m.a.s.l., Spain) where homogenous seedlings found across a 934 m perimeter were monitored for a year (Figure 1a). We sampled the individuals across four zones (Figure 1a). According to the germination time of the species (see previous section) seedlings were estimated to be < 1 year old and were considered of similar age since there were not any smaller seedlings in the area. Visually healthy seedlings within a range of height between four and twelve centimetres, and within a range of number of leaves between seven and fifteen were selected. The sampling site corresponded to a maquis habitat where the predominant species were *Quercus coccifera, Rosmarinus officinalis* and *Cistus albidus* and all seedlings were growing in open areas. The first sampling was carried out on 27th February 2019 and the last one on 14th March 2020. Sixty individuals were sampled to assess their physiological state on 27th February 2019, which was a clear sunny day, where the average temperature for the day was 16.3ºC and the relative humidity of 24%. Then we followed the growth and survival of the same individuals for a year every two months. This location has a typically Mediterranean weather where winters are mild, and summers are hot and dry. In the case of this study, the beginning of this year of sampling (February 2019 – March 2020) was somewhat dry, with relatively high values of vapour pressure deficit (VPD) and with barely no rains (see Table S1 available as Supplementary Data at *Tree Physiology* Online). During the following months of spring, precipitation was higher and, hence, VPD values decreased. Then, summer started and even though precipitation was higher in the months of June and July compared to February and March of 2019, temperatures were the highest during the experiment, hence, resulting in the driest period for the plants. Mean temperatures kept above 18ºC until October, when they decreased, which coincided with the start of a very humid period during autumn and winter, with an accumulated precipitation from October 2019 until January 2020 of 375 mm. Then, on February 2020, the rain was scarce again. The meteorological data corresponding to the beginning of March 2020 was very similar to the preceding month. Soil in the sampling area was a mixture of conglomerate, sandstone and red shale (Müller et al. 2014).

For the second experiment (hereafter referred as to the growth chamber study) a total of 70 homogenous *C. albidus* seedlings, randomly assigned to the different treatments, that had a height within the range of the seedlings growing in the Montserrat Mountains, were grown in growth chambers from seeds proceeding from a semi-natural population growing in the Experimental Fields of the Faculty of Biology of the University of Barcelona (41.384 N, 2.119E, 59 m.a.s.l., Spain). Conditions in the growth chambers were ca. 100 μmol/m^2/s of photosynthetically active radiation, 16 h light/8h dark photoperiod, 21º C and 65% of humidity. Such low light conditions allowed to assume that the plants were only affected by the stressor imposed (i.e. drought) and not high light, for instance. Fifty seedlings were exposed to drought and 20 were watered during the whole experiment as the control (well-watered treatment, WW). Twenty seedlings were not watered during the whole experiment – i.e. 22 days – (water stress treatment, WS), and two subgroups were re-watered on day 14 and day 16 after the start of the experiment, recovery treatment 1 (R1) and recovery treatment 2 (R2), respectively (see Figure S1a available as Supplementary Data at *Tree Physiology* Online). The performance of R1 and R2 was followed for four days (sampled in days 1, 2 and 4 after re-watering). Both groups were watered *ad libitum* after the first day of re-watering. The well-watered treatment (WW) was sampled at days 0, 14, 18 and 22 of the experiment while the WS treatments was sampled at days 14, 16, 18, 20 and 22 of the experiment (see Figure S1a available as Supplementary Data at *Tree Physiology* Online). For each condition, five plants were sampled.

For the first experiment (hereafter referred to as the field experiment) height, number of leaves, number of branches and seedling survival was assessed every two months in each seedling. For both experiments (for the field experiment during the first sampling and for the growth chamber study during all samplings), one leaf of the seedling was used to assess growth and physiological status-related parameters which included stomatal conductance, relative water content, leaf mass area and maximum PSII efficiency. Then, two to three leaves in the same developmental stage were snap-frozen in liquid nitrogen and then stored at -80ºC at midday - on a clear sunny day for the field experiment – or in the middle of the photoperiod – for the growth chamber study - for biochemical analyses, which included chlorophylls, α-tocopherol and phytohormones (ABA, SA and JAs).

Growth and physiological status-related parameters

Stomatal conductance (g_s), leaf mass area (LMA), relative water content (RWC) and maximum chlorophyll fluorescence (F_v/F_m) were measured on a fully-expanded leaf of *C. albidus* seedlings at midday. g_s was measured on the attached leaf using a leaf porometer (Decagon Devices, WA, USA). LMA was calculated as leaf dry weight (after oven-drying at 65ºC until constant mass) divided by leaf area, which was measured using a flat-bed scanner and subsequently the imageJ software. RWC was assessed using leaf fresh weight (FW), leaf turgid weight (TW) - rehydrated for 24h in darkness at 4ºC - and leaf dry weight (DW). RWC was calculated as a percentage: 100 x (FW-DW)/(TW-DW). F_v/F_m was measured in dark-adapted (at least for 1h) leaves using the Mini-PAM II (Photosynthesis

Yield Analyser, Walz, Germany) fluorimeter.

Chlorophyll contents

Chlorophyll a + b contents were determined spectrophotometrically (Cecil Aquaris CE7400, Cecil Instruments, Cambridge, UK) using the Lichtenthaler (1983) equations. Briefly, 25 mg of freshly ground material per sample were extracted with 250 µL of ice-cold methanol using ultrasonication and vortexing (Branson 2510 ultrasonic cleaner, Bransonic, Danbury, CT, USA) for 30 min, centrifugation for 15 min at 91463 *g* and 4ºC (Universal 32R, Hettich, Tuttlingen, Germany). The supernatant was collected, and the pellet was re-extracted as above. Supernatants were pooled and filtered through a 0.22 µm hydrophobic PTFE filter (Phenomenex Inc, Torrance, CA, USA).

Determination of α-tocopherol contents

Extractions for the determination of α-tocopherol were performed as above. α-Tocopherol contents were determined as described in Cela et al. (2011) by using a normal-phase HPLC system coupled to a fluorescent detector and quantification was made by using a standard curve with authentic standards of α-tocopherol (Sigma-Aldrich, Steinheim, Germany).

Determination of phytohormones

The extractions for the determination of phytohormones were performed as explained in previous sections. ABA, SA and JAs (including JA, 12-*oxo*-phytodienoic acid [OPDA] and jasmonoyl isoleucine [JA-Ile]) were determined by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) as described in Müller and Munné-Bosch (2011). Quantification was made considering recovery rates for each sample by using deuterium-labelled internal standards that were added during the extractions (conducted as above).

Statistical analyses

The effects of “Month” on “Seedling height”, “Number of leaves” and “Number of branches” in *C. albidus* seedlings growing in natural conditions were assessed using a linear mixed model (LMM, function *lme*, package *nlme*) (Pinheiro et al., 2020) where “Month” was fitted as the fixed term and “Plant” and “Zone” were fitted as random terms to account for repeated measures and to account for microsite variability (if any), respectively. The variance structure *varIdent (*package *nlme*) was used to account for different variances within "Zone" and was fitted in the model with the argument "weights". Additionally, restricted maximum likelihood (REML) was used to fit the model. When there were significant differences within Month, multiple comparisons were tested with Tukey's post hoc test, using general linear hypotheses (function *ghlt,* package *multcomp*) (Hothorn et al., 2008). To test for differences between treatment R1 and R2 in *C. albidus* seedlings growing under controlled conditions, we used a linear model (function *lm*) fitting "days after re-watering, DAR" and "Treatment, Treat" as explanatory variables. When there were significant effects, either of the individual factors or their interaction, multiple comparisons were tested using Tukey's post hoc test (function *HSD.test*, package *agricolae*) (de Mendiburu, 2019). All *p* values were obtained using the functions *Anova, Anova* type II or *Anova* type III (package *car*) (Fox and Weisberg, 2019), depending on the number of fixed effects and their interaction. For all models, homoscedasticity and distribution of residuals was visually checked (Zuur et al. 2009). Height was exponentially transformed to the three, number of leaves was log transformed, and number of branches was square-root transformed. g_s, F_v/F_m, OPDA and JA were square-root transformed; LMA, α -tocopherol, α -tocopherol/chlorophylls, JA-Ile, SA and ABA were log-transformed; and, chlorophyll a/b was logit-transformed. All *p* values were considered statistically significant when α = 0.05.

To find proxies of seedling survival in *C. albidus* seedlings growing under natural conditions, we performed a Logistic Regression. To choose the best model predicting seedling survival we compared models based on the Akaike's information criterion (AIC) and model accuracy. We used either generalised linear model (*glm*) or generalised mixed effects model (*glmer,* package *lme4*) (Bates et al., 2015) depending if the model included a random structure or not (see Table S2 available as Supplementary Data at *Tree Physiology*

Online). For all models family was specified to “binomial”. For glm models, survival was fitted as the response factor whereas all the continuous variables were fitted as predictors. For glmer models, a random structure with height (as a covariate) nested in zone was included to account for possible differences within these variables. The data partition was 80% corresponding to the training dataset to perform de logistic regression, and 20% corresponding to the testing dataset, to predict survival and to test for model accuracy. After comparing the full models 1 and 2 with all variables included (see Table S2 available as Supplementary Data at *Tree Physiology* Online), we chose model 2 due to its higher accuracy and then performed a Stepwise Logistic Regression to attain a lower AIC (see Table S2 available as Supplementary Data at *Tree Physiology* Online). Breakpoints in the target models were obtained using the *segmented* function within the *segmented* package (Vito and Muggeo, 2003) with maximum of iterations set to 100 and number of breakpoints to be estimated, 1. All *p* values were considered statistically significant when α = 0.05.

Principal component analyses (PCAs) were performed using the PCA function (*FactoMineR* package) (Lê et al., 2008). For visualisation, a biplot with both individuals and variables (*fviz_pca_biplot, factoextra* package) (Kassambara and Mundt, 2019) and a biplot of variables (*fviz_pca_var*, *factoextra* package) were used for representing *C. albidus* seedlings growing in field conditions data and for seedlings of the growth chambers data, respectively. Contribution of the variables to the factor map was represented by cos2 (calculated as the multiplication of the squared coordinates: var.cos2 = var.coord * var.coord) (Kassambara 2017).

RESULTS

Growth and survival proxies in C. albidus *seedlings growing in the field*

C. albidus seedlings markedly grew from February 2019 until March 2020 in the Natural Park of the Montserrat Mountains (Figure 1). At the beginning of the experiment, *C. albidus* seedlings had a mean height of 6 cm and, at the end, their height reached 11 cm. Leaf number per plant also increased during the experiment, from 10 at the beginning to 23 at the end of the monitoring. Furthermore, plants started to develop more branches, aside from the principal one, particularly after July, going from having only one ramification to three in March 2020 (Figure 1b). Nevertheless, seedlings lost some leaves in July 2019 which coincided with the hottest, and probably most stressful, period of the summer (mean T^e = 22.7ºC and mean VPD = 1.2 kPa, respectively, see Table S1 available as Supplementary Data at *Tree Physiology* Online).

Indeed, this period with the highest temperatures (June 2019 – July 2019) had a big impact on seedling establishment, and this was generally visible on the phenotype of seedlings in July 2019 (Figure 1a) as well as in survival (Figure 1b). In fact, plants started to die in September 2019 and survival further decreased until November 2019, being of about a 70% (Figure 1b). After fitting a logistic regression model with growth and biochemical parameters measured in February 2019 to predict the probability of seedling survival – where the fitted predictors were height, LMA, F_v/F_m and total chlorophyll– we obtained that the statistically significant proxies for survival were total chlorophyll and F_v/F_m (Table 1, Figure 2a). Height and LMA, although being in the final model that best explained survival, had no statistically significant effects. Model accuracy was about 78% with a misclassification error rate of 22%. In the logistic regression, F_v/F_m negatively affected survival, with higher F_v/F_m values accounting for lower survival. Total chlorophyll was the predictor with a higher significance in the predictive model (Table 1); a higher chlorophyll content accounted for higher seedling survival. Additionally, given that we believed that the regression model for total chlorophyll had more biological sense than that of F_v/F_m, we estimated the break-point its logistic regression model. We found that seedlings with 1.87 mg/ g DW of total chlorophyll or more had a higher probability of survival (Figure 2a).

Growth and biochemical parameters in C.albidus *seedlings in the field*

In order to explore the relationships between all the variables measured on February 2019 and their contribution to total variance of *C. albidus* seedlings growing in natural conditions, we performed a principal component analysis (PCA, Figure 2b). The most well-represented variables by the principal components – i.e. with a higher contribution represented by cos2 – were α-tocopherol, height, JA-Ile, JA and $F_v/F_{m.}$ Height and number of leaves were positively correlated as well as height and F_v/F_m and leaves with F_v/F_m (Figure 2b and see Table S3 available as Supplementary Data at *Tree Physiology* Online). α-Tocopherol was negatively correlated with $F_v/F_{m,}$ g_s, LMA and height. OPDA and JA also correlated negatively with α-tocopherol. Alive and dead individuals represented on the PCA did not seem to be divided in any particular pattern. However, there were some differences when comparing photoprotective molecules and hormones. Individuals that died seemed to be stressed at the time of the sampling (February 2019) given their higher values of the α-tocopherol to chlorophylls ratio and their higher ABA levels (see Figure S2 and S3 available as Supplementary Data at *Tree Physiology* Online). On the other hand, alive individuals had higher contents of total chlorophyll at that time (see Figure S2 available as Supplementary Data at *Tree Physiology* Online), further confirming chlorophylls as a good proxy of survival in *C. albidus* seedlings. Furthermore, OPDA levels were higher in individuals that survived compared to those that died (see Figure S3 available as Supplementary Data at *Tree Physiology* Online).

Physiological status-related parameters in* C.albidus *seedlings in the growth chamber study

R1 seedlings were able to recover from 14 days of water stress, as indicated specifically by their g_s, RWC and F_v/F_m values, while R2 seedlings did not fully recover from 16 days of water stress as stress indicators did not reach levels similar to those of the WW plants. After 14 days of withholding water in water-stressed plants, g_s decreased by an 86.4% and then values kept under 8 mol/m^{2}s until day 22 when g_s was 0 mol/m^{2}s. RWC persistently decreased in WS plants until reaching a RWC of 13% after 22 days of water stress, when plants were considered already dead (see Figure S1b available as Supplementary Data at *Tree Physiology*

Online for a phenotype). R1 seedlings recovered from water stress, reaching similar values to those of WW seedlings. R2 seedlings tried to recover their RWC after one day of re-watering, increasing their RWC by a 35% but then, one day after, RWC decreased. When comparing R1 and R2, we found that their RWC significantly differed on day 2 after re-watering (see inlets in Figure 2). All water-stressed treatments increased their leaf thickness as a response to drought (Figure 3 and see Figure S1b for a phenotype comparison between leaves of WW and WS seedlings). WW plants had an average LMA of 28.3 g DW/m^2 while for the rest of the treatments, averages ranged between 47 to 79 g DW/m^2. There were no significant differences between R1 and R2 in LMA (see inlets in Figure 3). All water-stressed treatments presented photoinhibition at least at some point during the experiment (Figure 3). After 14 days of water withholding, WS plants had a F_v/F_m of 0.53 and then there was a progressive reduction until day 18 where values were close to 0. After two days of receiving water, F_v/F_m augmented by a 29% attaining comparable levels to the WW treatment in R1 seedlings, then, levels slightly decreased again. R2 seedlings maintained the same F_v/F_m compared to R1 plants one day after re-watering but then, levels decreased to 0 on days 2 and 4 after receiving water. F_v/F_m in R1 and R2 plants was significantly different on day 2 after-re-watering (see inlets in Figure 3).

Chlorophylls and α-tocopherol in* C.albidus *seedlings in the growth chamber study

WS seedlings suffered a decrease of 69% in their chlorophyll content after 18 days of water stress compared to day 0 in total chlorophyll contents. Then, chlorophyll levels kept constant until day 22 of no water at 1.7 mg/ g DW. Total chlorophylls in treatment R1 slightly increased after four days of re-watering. There was a minor augment in total chlorophyll of R2 seedlings but then, total chlorophyll followed the opposite pattern as R1 plants, decreasing until four days after re-watering (see inlets in Figure 3). The chlorophyll a/b ratio decreased by a two-fold in WS plants compared to the beginning of the experiment after 14 days of water restriction and then kept low for the rest of the experiment. R1 seedlings' ratio increased after two days of re-watering by a 42% whereas the ratio of R2 plants gradually decreased after four days of re-watering. Certainly, there were statistically significant differences between treatments R1 and R2 on day two after re-watering (see inlets in Figure 3).

There was a lot of variation in the levels of α-tocopherol in *C. albidus* seedlings, ranging between 45 μg/ g DW and 722 μg/ g DW corresponding to WS on day 22 and WS on day 16 of the experiment, respectively (Figure 3). For WS seedlings, α-tocopherol decreased after 14 days of water withholding, then there was a steep increase of a two-fold and then levels declined to 45.4 μg/ g DW, when plants were considered already dead and did not seem to be able to synthesize any more compounds. R1 plants, even though presenting a small augment in α-tocopherol levels compared to WW plants, maintained constant levels of the antioxidant after re-watering. R2 plants followed a similar pattern to that of WS plants. Indeed, when comparing R1 and R2 α-tocopherol levels, we found statistically significant differences, particularly on day two after re-watering (see inlets in Figure 3). The tocopherol per chlorophylls ratio was higher in all water-stressed treatments compared to the WW plants but then decreased towards the end of their sampling days (Figure 3). For R1 seedlings, this ratio seemed to be more stable during the first two days after re-watering. When comparing R1 and R2, we did not find significant differences within treatment. We did find, nevertheless, significant effects within days after re-watering (see inlets in Figure 3).

Stress-related hormones in* C. albidus *seedlings growing in the growth chamber

ABA content increased in all treatments upon water stress (Figure 4a), with the lowest levels in R1 seedlings (close to those of the WW plants) and the highest in R2 towards the end of the experiment. When we compared R1 and R2 seedlings' performance we observed that R2 ABA levels were higher on the recovering days compared to R1 (107 ng/ g DW versus 313 ng/ g DW). Certainly, we found significant effects within days after recovery and a significant interaction between days after recovery and treatment, where the levels of ABA in R2 where higher after two days of re-watering than in R1 (see inlets in Figure 4a). SA followed a similar pattern than ABA, occurring the highest value in R2 seedlings after two days of re-watering and the lowest in WW plants. Again, we found a significant interaction between days after re-watering and treatment when we paralleled R1 and R2 seedlings (see inlets Figure 4a). Jasmonates (OPDA, JA and JA-Ile), generally decreased upon drought (Figure 4b). The lowest values of the precursor of JA, were observed in the WS and R2 seedlings whereas the highest in the WW and R1 plants, although in the latter after two days of re-watering. In fact, we found significant differences between R1 and R2 plants, being the levels of OPDA, higher in the R1 treatment on the second day after re-watering (see inlets in Figure 4). JA trailed a very similar pattern as to that of OPDA. Finally, the jasmonate bioactive form (JA-Ile) had a slightly different pattern to that of its jasmonates counterparts. JA-Ile augmented upon water stress and WS and R2 seedlings had the higher levels of the jasmonate conjugate, with a peak on day 18 of water stress (day 2 after re-watering for R2 seedlings). After this increase, there was a decrease of JA-Ile contents. There were not significant differences between R1 and R2 seedlings (see inlets in Figure 4).

Growth and biochemical parameters interactions in **C.albidus** *seedlings in the growth chamber study*

To identify the mechanisms seedlings used to tolerate drought stress and recover from it we performed a PCA with R1 and R2 seedlings data, independently (Figure 5). For R1 seedlings, the two first principal components (PCs) explained a 72.2% of the total variability (Figure 5a). The variables that had a better representation on the factor map and, hence, a higher contribution, were F_v/F_m, RWC, LMA, ABA, SA and JA-Ile. On the other hand, the two first principal components explained a 66.1% of total variability for R2 seedlings and the variables with a higher contribution were RWC, F_v/F_m – these two with a similar contribution than in the R1 PCA -, JA, total chlorophylls, α-tocopherol and JA-Ile (Figure 5b). Even though both R1 and R2 PCAs resulted with a high contribution of JA-Ile for PC2, the contribution of JA-Ile on the R2 factor map seemed to be higher compared to that of R1, given its higher cos2. For both R1 and R2 factor maps, total chlorophyll was positively correlated with JA (Figure 5 and see Table S4 an Table S5 available as Supplementary Data at *Tree Physiology* Online). In R1 seedlings, ABA and SA had a positive correlation given that both of the hormones increased upon the water deficit, whereas in R2 seedlings this positive correlation disappeared. Total chlorophyll was also positively correlated with α-tocopherol in R2 seedlings and the latter correlated positively with JA and negatively with ABA (the last correlation was also observed in R1 seedlings).

DISCUSSION

Growth arrest as one of the first responses to drought

Growth, measured as height, number of leaves and number of branches, did not change during neither spring nor summer and it was not until the end of the summer season that seedlings resumed growth. Higher values of VPD during the summer generally increase evapotranspiration, loss in cell turgor limits cell expansion and plants avoid water loss by closing stomata. Although this is key to prevent water loss and, together with osmotic adjustment prevents cavitation under water stress (Savi et al. 2015), it also lowers carbon fixation and, hence, decreases biomass production. In fact, stomatal conductance of the seedlings growing under natural conditions was a 52% lower compared to the values of WW seedlings under controlled conditions, which implies that seedlings under natural conditions are exposed to constant environmental cues that affect their performance. The low stomatal conductance in water-stressed seedlings in the growth chambers coincided with an increase in ABA contents. Certainly, ABA has long been known to be involved in stomatal closure upon stress (Kim et al., 2010). Furthermore, a recent study proposed ABA to have a direct role in growth arrest, interacting with the SNF1 (sucrose non-fermenting-1)-related protein kinases (Belda-Palazón et al., 2020).

These results suggest that both growth arrest and closure of stomata regulated by ABA are primary responses to drought in *C. albidus* seedlings growing in the Montserrat Mountains. However, summer drought decreased seedling survival by a 30%, thus indicating some degree of stress sensitivity in *C. albidus* seedlings. Abiotic stressors such as drought have been shown indeed to be the main causes of seedling mortality in Mediterranean ecosystems (Matthes and Larson, 2005; Harrison and LaForgia, 2019; López-Sánchez et al., 2019), especially during their first year of life (Šenfeldr and Treml, 2020). Furthermore, the consequences of drought for seedlings can be especially detrimental if there are subsequent periods of stress, such as in our study where late winter and summer entailed two drought events. Interestingly, the fact that some seedlings died after an episode of unfavourable conditions during their first year of life and some did not, besides being the result of an intense summer drought, might be related to microsite heterogeneity along the perimeter sampled. In fact, water microsite differences are likely to be the cause of death of seedlings since young plants have very small root systems that cannot reach deep waters (Battaglia et al., 2000). However, the effects of microsite might not always be so evident. For instance, Šenfeldr and Treml (2020) found that during the first year of life of dwarf pine seedlings, vegetation cover had no significant effect on seedling survival. In our study, all seedlings were sampled in open areas, with barely no vegetation cover to avoid site heterogeneity.

Total chlorophyll as a main proxy of seedling survival

After performing the logistic regression on our data, we found that height, F_v/F_m, LMA and total chlorophyll - although not all of them significant - best explained survival in *C. albidus* seedlings in the field experiment. Particularly, total chlorophyll was the main proxy of seedling survival since it had the most significant *p* value (Table 1). F_v/F_m was also statistically significant, with higher values of the latter implying a lower seedling survival. Although this result might seem contradictory since, generally, the F_v/F_m ratio is correlated with a good physiological status, it appears that a transiently low F_v/F_m ratio during stress may indicate a positive plastic response to stress. In fact, in a study performed in a natural population, this ratio in *C. albidus* plants decreased during summer below 0.5 but then, plants recovered in autumn (Werner et al. 1999), agreeing indeed with the results obtained for R1 plants in the experiment performed in the present study in the growth chamber, where F_v/F_m dropped below 0.5 and then, after 4 days of re-watering, values recovered.

Chlorophyll content is usually used as a physiological trait to characterize drought responses (see Bussotti et al. 2015 and references therein). In our study, we found a threshold for chlorophyll content out of our logistic regression model – 1.87 mg/ g DW – from which seedling probability of survival greatly increased. This agrees with the observed values of chlorophyll under controlled conditions. R1 plants – the group that recovered - presented an average total chlorophyll of 2.87 mg/ g DW while R2 plants – the group that did not recover from the water stress – presented an average total chlorophyll of 1.78 mg/ g DW. Moreover, if we compare seedlings growing in the Montserrat Mountains versus WW seedlings under controlled conditions, the latter had higher total chlorophyll contents, adding to the idea on naturally growing seedlings being generally more stressed, which is also supported by the higher levels of both ABA and the α-tocopherol per chlorophyll ratio in dead individuals for both the first experiment plants and for not-recovered individuals from the second experiment. Indeed, this variability within chlorophyll contents and other functional traits in the same species has been reported to be the result of phenotypic plasticity, which has been shown to better tolerate stress (Bradshaw 2006; Nicotra et al. 2010; Matesanz and Valladares 2014).

Vitamin E and jasmonates as possible mechanisms for drought tolerance and resilience

α-Tocopherol correlated negatively with F_v/F_m and height, which also correlated positively with number of leaves, in *C. albidus* seedlings growing in the field, suggesting that smaller plants were in need of a higher pool of this antioxidant to thrive.

Vitamin E, most particularly α -tocopherol, is one of the most potent non-enzymatic antioxidants that plants have to counteract the detrimental effects of ROS and prevent lipid peroxidation (Azzi 2018), acting not only as a photoprotector but also as a signaling compound (Muñoz and Munné-Bosch 2019; Munné-Bosch 2019). Smaller plants had higher levels of α-tocopherol and a reduced maximum PSII efficiency which indicates a higher vulnerability of smaller plants to stress, which agrees with the findings of Munné-Bosch et al. (2016) in an alpine perennial. In the growth chamber experiment, α-tocopherol notably increased under water stress but then the levels were reduced from day 18, when plants were presumably dead. In R1 seedlings, vitamin E levels were not as high as in the WS or R2 seedlings; particularly there was a significant difference between R1 and R2 after two days of re-watering. Furthermore, levels seemed to be relatively constant after re-watering, suggesting that the action of vitamin E for seedling recovery could be concentration dependent. In other words, if there is too much ROS production that cannot be scavenged by α -tocopherol, like in the case of WS and R2 treatments, the damage will lead to cell death. Another possible explanation for such a strikingly increase in vitamin E levels in WS and R2 seedlings could be explained by the degradation of chlorophylls and subsequent activation of the phytol recycling pathway as a compensatory and protective mechanism due to water stress (Valentin et al. 2006). In fact, we found a positive correlation between α-tocopherol and total chlorophyll in R2 seedlings, where the degradation of chlorophylls was overall higher than in R1 seedlings. In the growth chamber experiment, however, this protective mechanism would not have been enough since R2 seedlings died, and, this correlation could be due to an extensive chlorophyll degradation due to severe stress (Yamburenko et al., 2013).

JAs could be important for survival in *C. albidus* seedlings in the field. Jasmonic acid is generally known for acting as signal for biotic stresses in plants but there is growing evidence that they also have an important role in resilience to abiotic stress (Yang et al. 2019). JAs explained much of the variability in the field data. Furthermore JAs, most particularly OPDA, correlated positively with total chlorophyll, our proxy of survival, hence, plants with higher contents of OPDA could have an advantage in survival. In fact, we found that OPDA was higher in surviving seedlings of the Montserrat Mountains, but there were not any statistically significant differences in neither JA nor JA-Ile. This could seem counterintuitive but, in fact, OPDA has been proven to have a differential role to that of JA and other JA forms under drought. Savchenko et al. (2014) proved by several experiments in different ecotypes of Arabidopsis that OPDA confers enhanced tolerance to drought by among other mechanisms promoting stomatal closure together with ABA.

There was also negative correlation between vitamin E and, OPDA and JA in *C.albidus* seedlings growing in the Montserrat Mountains. α-Tocopherol has been proven to have effects in oxylipins biosynthesis in plants under both abiotic (Munné-Bosch et al. 2007; Maeda et al. 2008; Chen et al. 2018) and biotic stress (Cela et al. 2018). This relationship resides in the relatively new concept of vitamin E acting not only as an antioxidant but also as a signal to different cell processes (Munné-Bosch 2019). α-Tocopherol is thought to modulate the activity of fatty acid desaturases (FADs) (Zhang et al. 2005), changing the linoleic to linolenic acid ratio and, therefore, changing the accumulation of jasmonates, given that linolenic acid is the precursor of their biosynthesis pathway. Munné-Bosch et al. (2007) found that there was a higher accumulation of JA in Arabidopsis mutants lacking tocopherol under high light and low temperature conditions whereas Chen et al. (2018) discovered that high levels of α-tocopherol correlated with a high linoleic to linolenic ratio resulting in decreased levels of JA in *Artemisia sphaerocephala*. The fact that we found a negative relationship between α-tocopherol and jasmonates (except for JA-Ile) in seedlings growing in the Montserrat Mountains suggests that vitamin E could be increasing the contents of linoleic acid as a way of protecting the membranes from oxidative stress, as previously proposed by Chen et al. (2018), which would agree with the hypothesis of JAs having a role in seedling survival. This relationship was however not found in R1 seedlings, suggesting that vitamin E only acts as a modulator of JAs synthesis under specific conditions, an aspect that warrants further investigations.

In regard of the other stress hormones, we found that JAs had a negative correlation with ABA and SA in seedlings growing in the growth chambers. ABA and SA increased upon water stress which agrees with other studies of Mediterranean plants (Abreu and Munné-Bosch 2008) and of the same species (Jubany-Marí et al. 2009; Brossa et al. 2014; Pérez-Llorca et al. 2019c) under drought. The negative relationship between JAs and SA was also reported in *C. albidus* adult plants growing in natural conditions in the same habitat (Pérez-Llorca et al. 2019b) and suggests a trade-off between these two phytohormones and their complementary roles under abiotic stress. While the overall trend for JA and OPDA in water-stressed seedlings growing under controlled conditions was to decrease, JA-Ile, the generally considered bioactive form of JAs (Wasternack and Song 2018), had a different pattern during the experiment. Upon water stress, JA-Ile levels decreased and had a steep increase on day 18 of water restriction and then decreased towards the end of the experiment. Plants that recovered from water stress (R1 seedlings) presented higher contents of both OPDA and JA than WS and R2 plants after two days of re-watering. Even though at the end of the recovery of the two treatments there were not any statistically significant differences in JAs, the variations on day two could have been key for seedlings survival, and, this, agrees with the results of the experiment in natural conditions where surviving seedlings had higher amounts of OPDA. Besides OPDA having a differential role from its other JAs counterparts, the high amounts of JAs could be explained by the signaling role of JAs triggered by singlet oxygen in cell death (Przybyla et al. 2008). Singlet oxygen ($^1O_2$) generated in the chloroplast during photosynthesis is able to produce lipid hydroperoxides, which can lead to OPDA, a precursor of the JA biosynthesis pathway that if accumulated, can lead to higher amounts of JAs in the cell (op den Camp et al. 2004). Compared to the stable levels of JA in R1 seedlings, the levels of OPDA steeply increased after two days of re-watering compared to R2. Interestingly, Przybyla et al. (2008) also proposed that OPDA but not JA could act as a counteractant of the final death phenotype. In this manner, the results of our two experiments and other recent findings of a differential role of OPDA to the other JAs forms (Simancas et al. 2018; Pérez-Llorca et al. 2019b), suggest that the precursor of JA could have an important role in *C. albidus* seedlings' drought resilience and survival.

Overall, our study suggests that drought is the main stressor that causes detrimental effects in *C. albidus* performance and we propose a model where this drought tolerance is a complex response where there are several main photoprotective players (Figure 6). The first signal upon drought is ABA and its accumulation leads to stomatal closure, which in turn, will reduce the carbon fixation and hence, allocation of carbon to growth. Growth arrest in plants upon stress can also be directly promoted by the interplay between ABA and SNF1-related protein kinases (Belda-Palazón et al., 2020). ABA can also reduce the concentration of total chlorophylls by inhibiting chloroplast-related genes (Yamburenko et al., 2013). Drought, both caused by an increase in VPD and scarce rain, provokes excess light in the chloroplast and this increases the amount of ROS, which can oxidise various molecules in the chloroplast including chlorophylls. A controlled reduction in total chlorophylls supposes an advantage for plants under excess light since this reduces the rate of excitation and of charge separation for each reaction centre, and, hence, overproduction of ROS (Walters, 2005). Increments in ROS lead to an increase in α-tocopherol, a potent antioxidant that prevents lipid peroxidation, the accumulation of which is in turn favoured by the degradation of chlorophylls and turnover of phytol through the phytol recycling pathway (Valentin et al. 2006). Finally, JAs, including OPDA, could be having an important role in seedling survival. However, if the stress is too severe, like in the case of R2 seedlings in the growth chambers, ROS overproduction leads to excessive lipid peroxidation and chlorophyll degradation leading to massive cell death and irreversible injuries to leaves.

CONCLUSIONS

In conclusion, we found important thresholds as well as mechanisms for survival in seedlings of the semi-deciduous shrub *C. albidus* in the field and the growth chambers. Total chlorophyll is highlighted as a good proxy of survival, which greatly increases passed a limit of 1.87 mg/ g DW of total chlorophyll in leaves. This threshold is confirmed in seedlings that recovered from severe drought in the growth chambers, although with close values of the non-recovering seedlings. α-Tocopherol and jasmonates appeared to have a major role in the drought response, both under natural and controlled conditions, enabling *C. albidus* to tolerate drought. These mechanisms would allow to resume growth after stress, making *C. albidus* a highly drought adapted species. It is however imperative to perform further studies to better understand the stress response of Mediterranean plants at juvenile stages of their development and its implication in population dynamics since these responses could change in wake of climate change.

SUPPLEMENTARY DATA

All Supplementary Data related to this article can be found at Tree Physiology Online.

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the submitted manuscript.

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while growing the plants in the growth chambers.

AUTHORS' CONTRIBUTIONS

MPL, VC and SMB conceived the experiment. MPL and VC performed the samplings and biochemical analyses. MPL analysed the data. MPL wrote the manuscript with the help of SMB and MM. All authors revised the final manuscript.

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Table 1 Results for the Logistic Regression predicting the probability of survival based on the predictor variables height, LMA, F_v/F_m and total chlorophyll of *Cistus albidus* seedlings growing in the Natural Park of the Montserrat Mountains in February 2019. The model was fit using the *glm* function and specifying the option *family* to *binomial*.

	Coefficients			
	Estimate	**Std. Error**	**z value**	**Pr(>|z|)**
Predictors				
Height	0.464	0.263	1.764	0.078
LMA	0.047	0.031	1.565	0.118
F_v/F_m	-40.488	18.448	-2.195	**0.028**
Total chlorophyll	2.858	1.112	2.570	**0.010**

Differences between *p*-values corresponding to the z-statistic were considered statistically significant at $p \leq 0.05$. LMA, leaf mass area; F_v/F_m, maximum PSII efficiency.

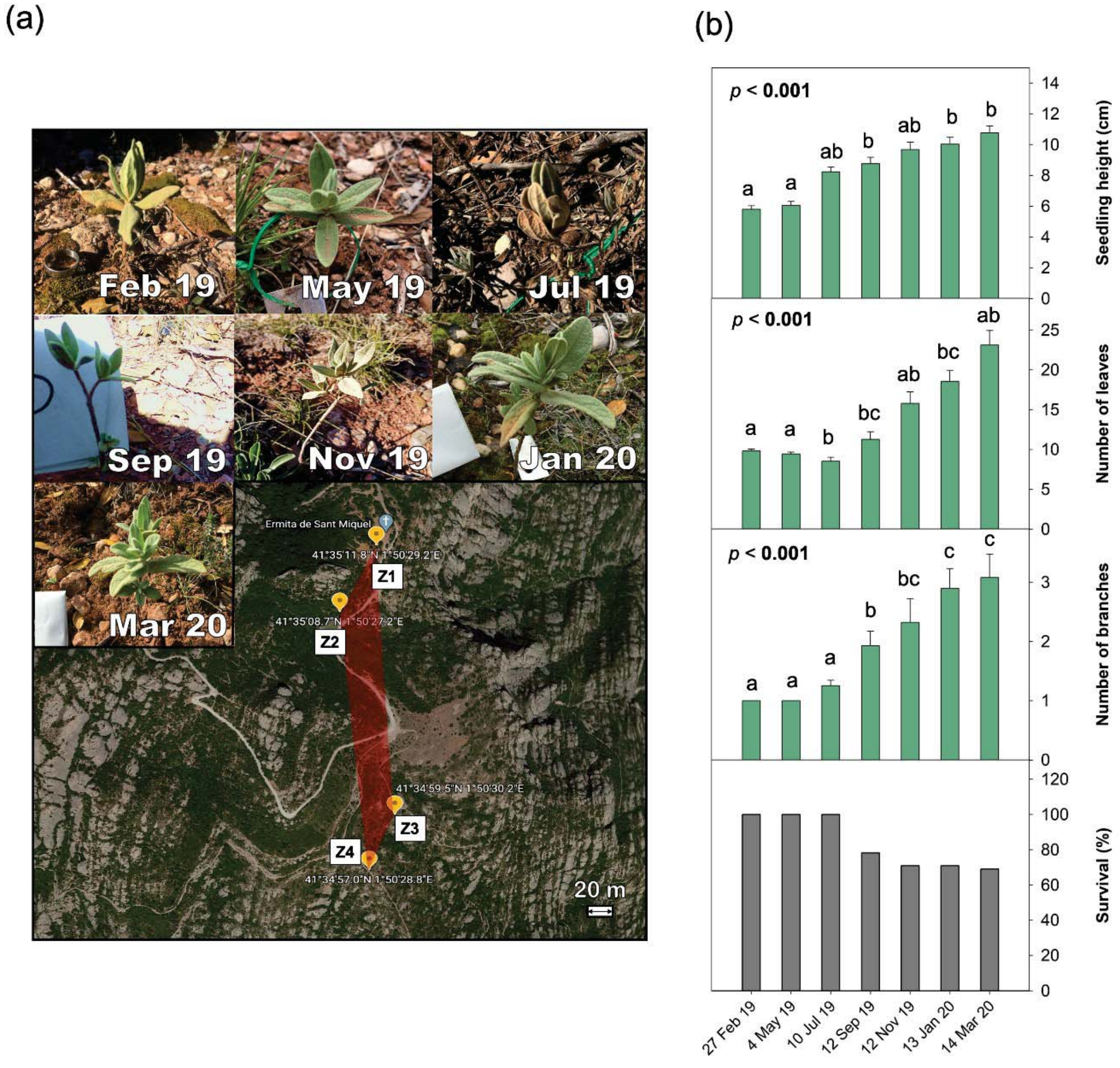


Figure 1 Dynamics of growth of *Cistus albidus* seedlings growing in the Natural Park of the Montserrat Mountains throughout a year. **(a)** Pictures of seedlings representing the general phenological state of the seedlings in every month and a map representing the area sampled in a red shaded polygon. Z1…Z4 correspond to the sampling zones (Z). **(b)** Variations in plant height, number of leaves number of branches and survival from February 2019 to January 2020. For plant height, number of leaves and number of branches only individuals that were alive at the end of the experiment were considered. Data of plant height, number of leaves and number of branches represent the mean ± SE of 39 individuals and differences within monthly samplings were tested using conditional Fisher tests. Different letters indicate differences in Tukey's HSD multiple comparison test. All differences were considered statistically significant at a probability level of α=0.05.

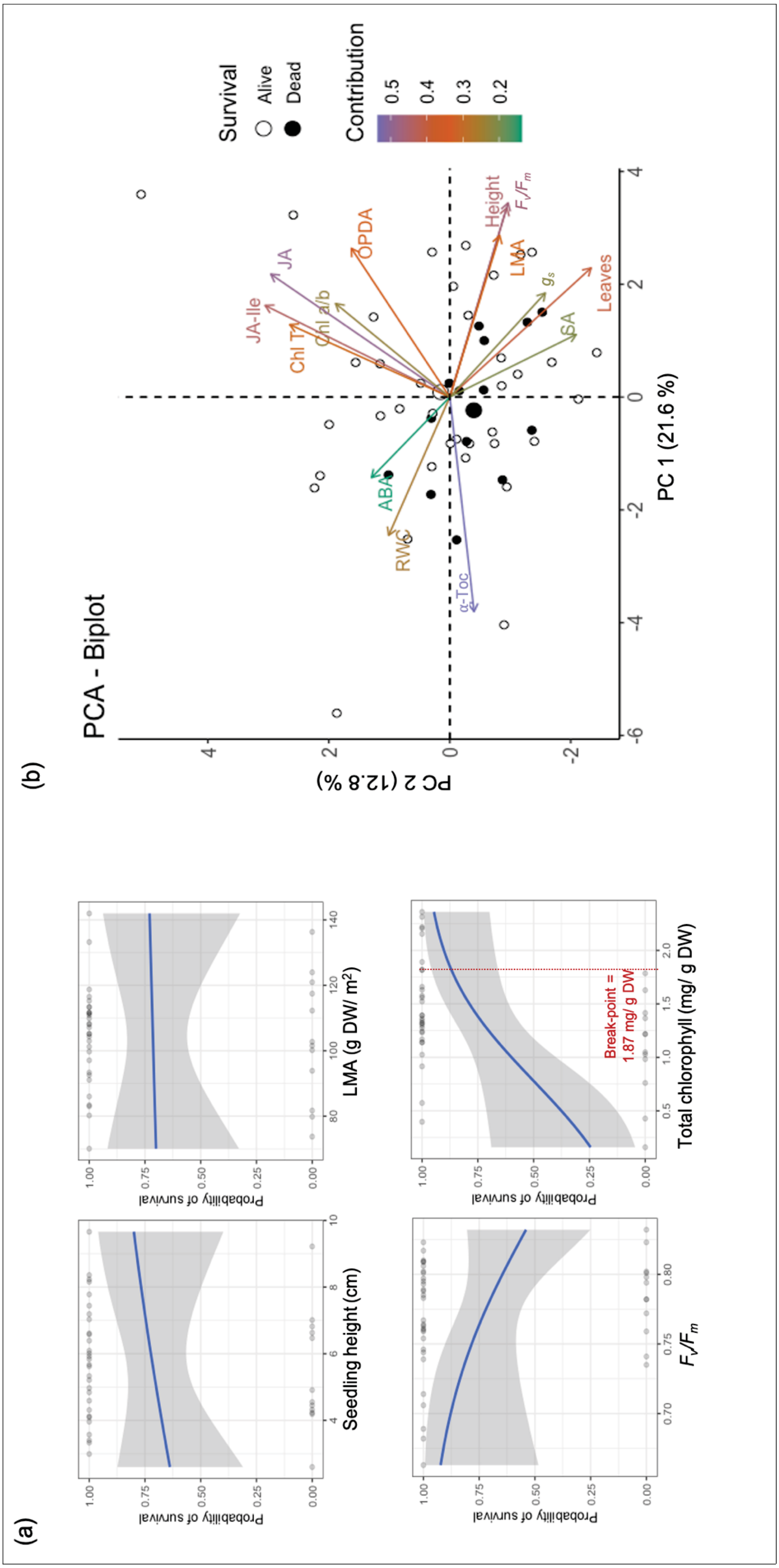

(a)
Probability of survival
Seedling height (cm)
LMA (g DW/ m^2)
F_v/F_m
Total chlorophyll (mg/ g DW)
Break-point = 1.87 mg/ g DW
(b)
PCA - Biplot
PC 1 (21.6 %)
PC 2 (12.8 %)
Survival
Alive
Dead
Contribution
JA-Ile
JA
OPDA
Chl T
Chl a/b
Height
F_v/F_m
LMA
g_s
Leaves
SA
ABA
RWC
α-Toc

Figure 2 a) Logistic regression model representation of probability of *C. albidus* seedling survival for every variable included in the final model (seedling height, LMA, F_v/F_m, total chlorophyll) based on February 2019 data. An estimation of a break-point (change-point) for total chlorophyll is shown in red. **b**) Visualisation of the principal component analysis (PCA) of the Natural Park of the Montserrat Mountains data. PCA represents the relationships between all variables (positively correlated variables are grouped together and negatively correlated variables are positioned on opposite quadrants) and the quality of the variables on the factor map (in this case, represented as cos2). A higher cos2 (violet in the contribution colour scale) indicates a good representation of the variable on the factor map whereas a lower cos2 (green in the contribution colour scale) indicates that the variable is not well represented on the factor map. Additionally, survival of individuals is also represented on the factor map (individuals that were alive at the end of the experiment are represented in white and individuals that were dead are represented in black). PC1 and PC2 are the two principal components along which the variation in the data is maximal.

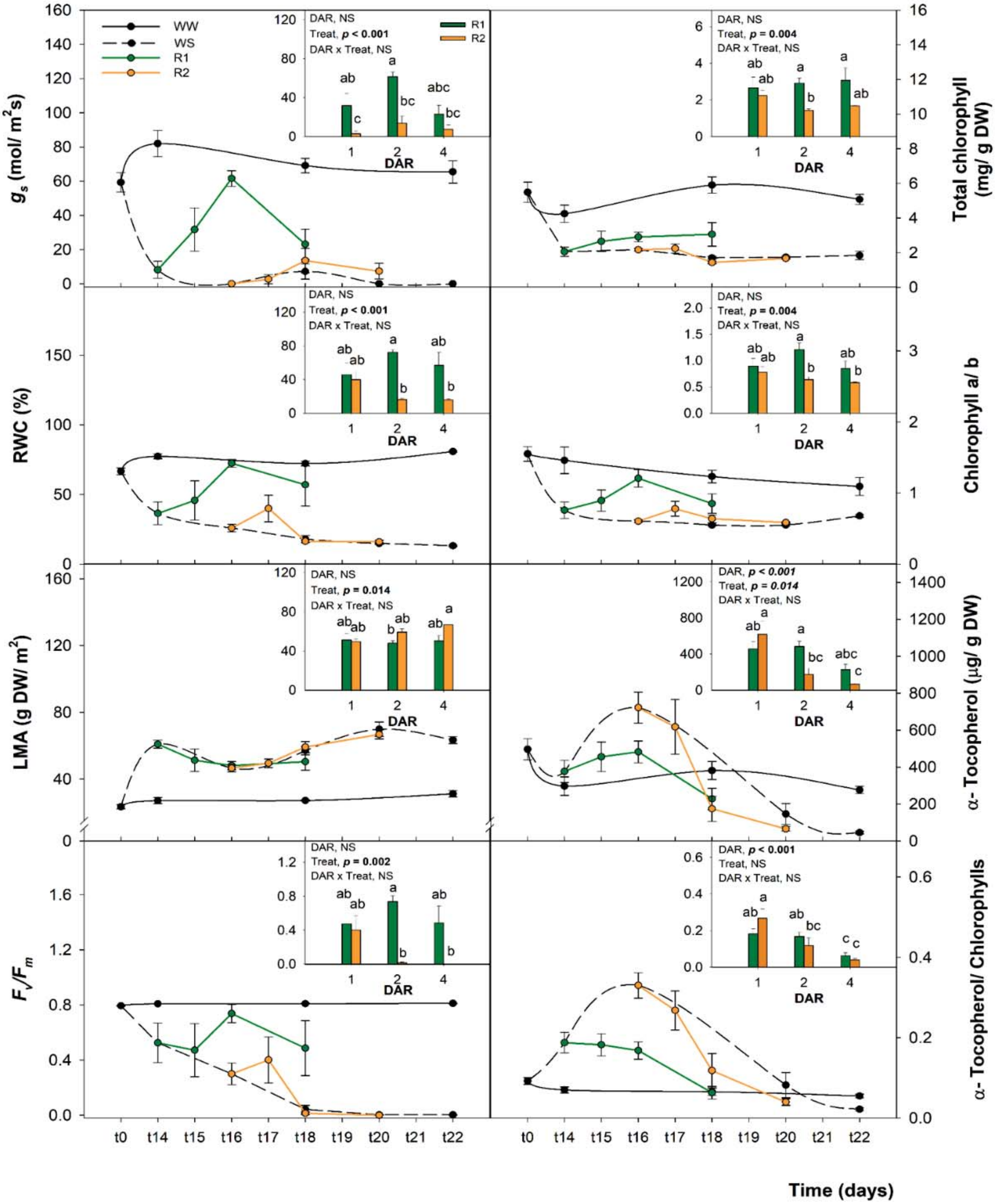

WW
WS
R1
R2
g_s (mol/ m^{2}s)
Total chlorophyll (mg/ g DW)
RWC (%)
Chlorophyll a/ b
LMA (g DW/ m^2)
α- Tocopherol (μg/ g DW)
F_v/F_m
α- Tocopherol/ Chlorophylls
DAR, NS
Treat, $p < 0.001$
DAR x Treat, NS
DAR, NS
Treat, $p = 0.004$
DAR x Treat, NS
DAR, NS
Treat, $p < 0.001$
DAR x Treat, NS
DAR, NS
Treat, $p = 0.004$
DAR x Treat, NS
DAR, NS
Treat, $p = 0.014$
DAR x Treat, NS
DAR, $p < 0.001$
Treat, $p = 0.014$
DAR x Treat, NS
DAR, NS
Treat, $p = 0.002$
DAR x Treat, NS
DAR, $p < 0.001$
Treat, NS
DAR x Treat, NS
DAR
t0
t14
t15
t16
t17
t18
t19
t20
t21
t22
Time (days)

Figure 3 Temporal variations of the different treatments in stomatal conductance (g_s), relative water content (RWC), leaf mass area (LMA), maximum PSII efficiency (F_v/F_m), total chlorophyll, chlorophyll a/b ratio, α-tocopherol and α-tocopherol/chlorophylls ratio throughout the experiment in *Cistus albidus* seedlings exposed to drought under controlled conditions. Inlets show differences after re-watering between seedlings that recovered after drought (treatment R1) and seedlings that did not (treatment R2). Data represent the mean ± SE of 5 individuals and differences within time (days) were tested using conditional Fisher tests. Different letters indicate differences in Tukey's HSD multiple comparison test. All differences were considered statistically significant at a probability level of α=0.05. WS, water stress, WW, well-watered; R1, treatment recovery 1; R2, treatment recovery 2; DAR, days after re-watering; Treat, treatment.

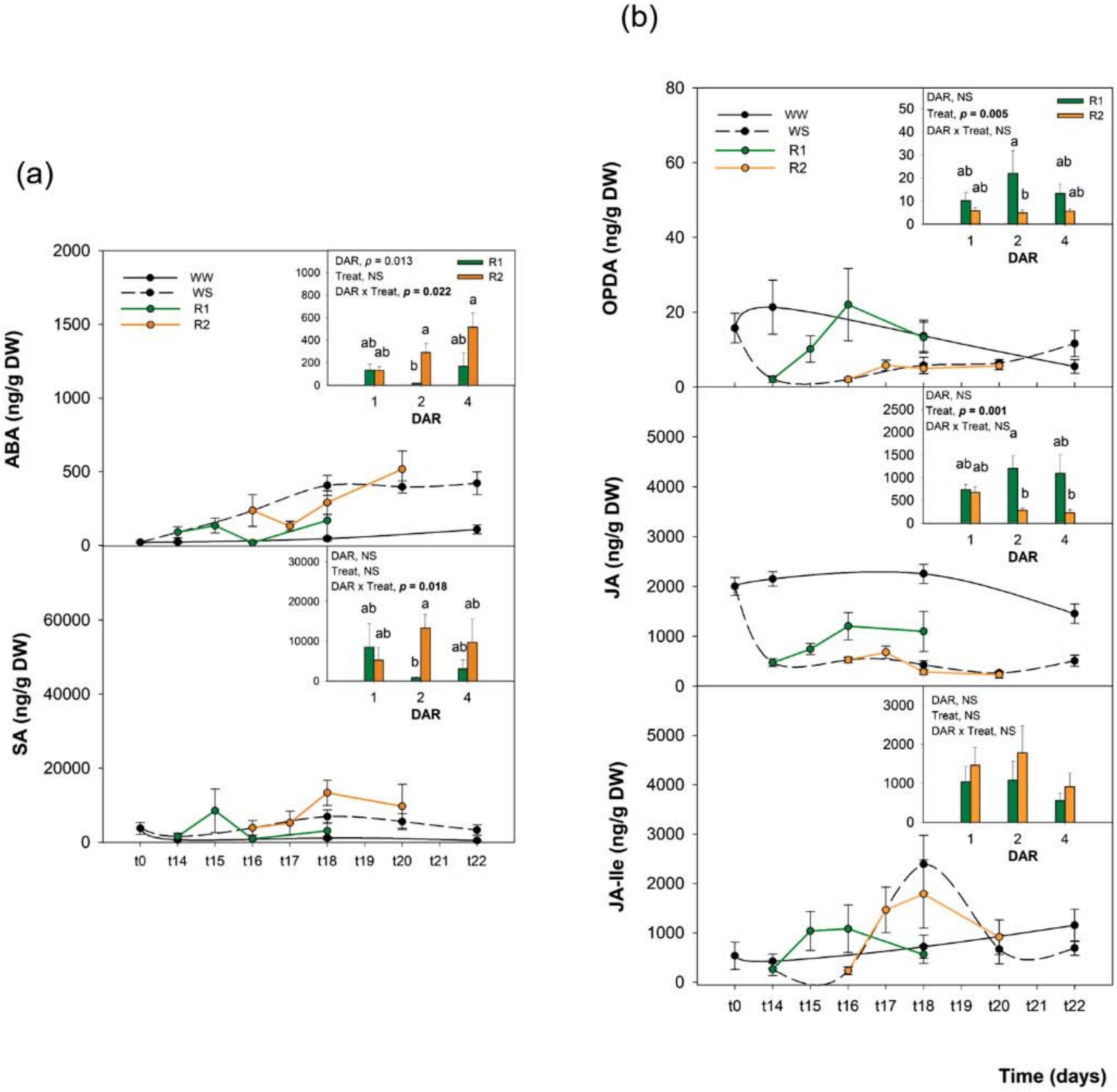


Figure 4 Temporal variations of the different treatments in **(a)** abscisic acid (ABA) and salicylic acid (SA) and **(b)** 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA) and jasmonoyl-isoleucine (JA-Ile) throughout the experiment in *Cistus albidus* seedlings exposed to drought under controlled conditions. Inlets show differences after re-watering between seedlings that recovered after drought (treatment R1) and seedlings that did not (treatment R2). Data represent the mean ± SE of 5 individuals and differences within time (days) were tested using conditional Fisher tests. Different letters indicate differences in Tukey's HSD multiple comparison test. All differences were considered statistically significant at a probability level of α=0.05. WS, water stress, WW, well-watered; R1, treatment recovery 1; DAR, days after re-watering; Treat, treatment.

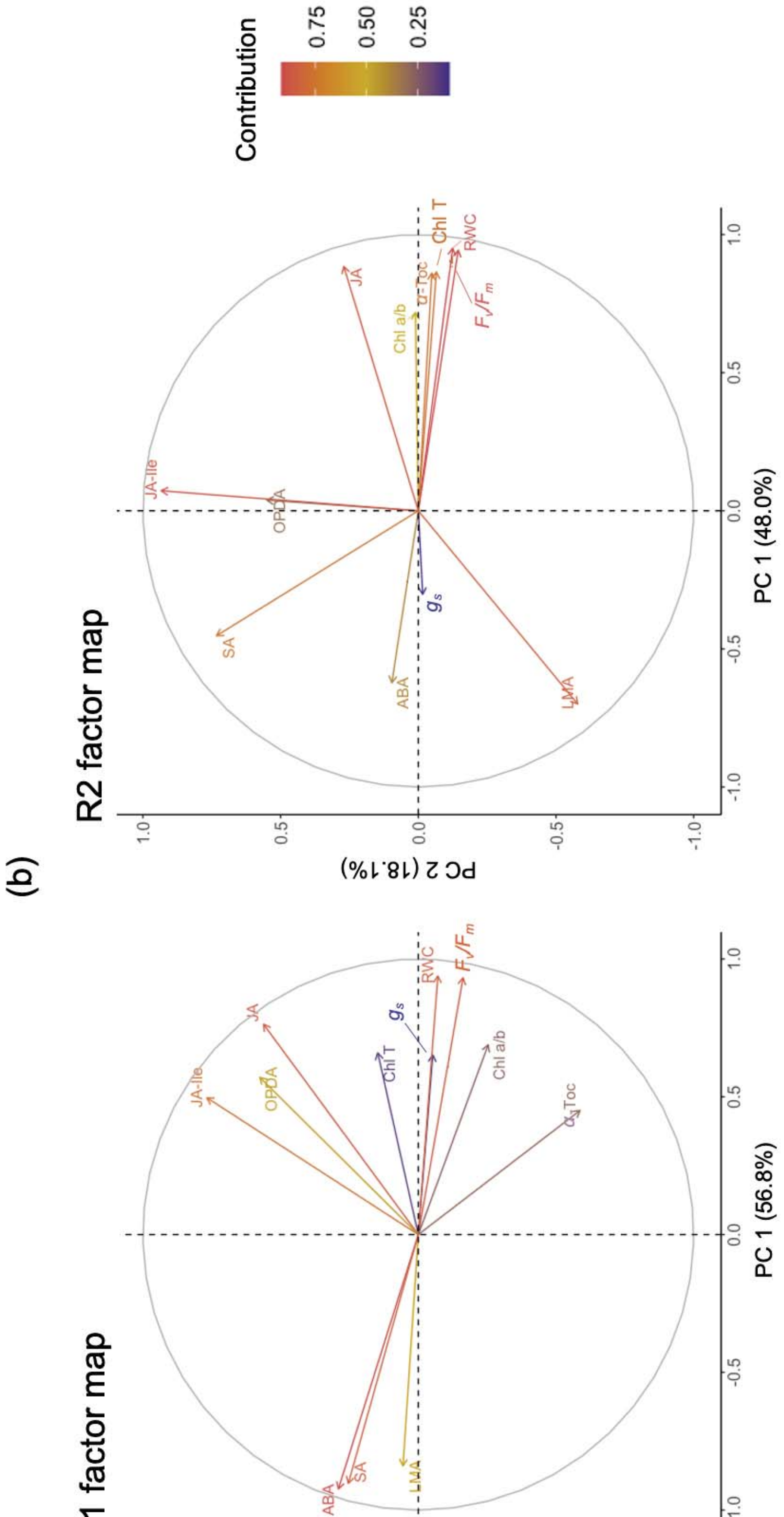

(a)
R1 factor map
PC 1 (56.8%)
PC 2 (15.4%)
-1.0
-0.5
0.0
0.5
1.0
ABA
SA
LMA
JA-Ile
OPDA
JA
Chl T
g_s
RWC
F_v/F_m
Chl a/b
α-Toc
(b)
R2 factor map
PC 1 (48.0%)
PC 2 (18.1%)
JA-Ile
OPDA
SA
ABA
g_s
LMA
JA
Chl a/b
α-Toc
Chl T
RWC
F_v/F_m
Contribution
0.75
0.50
0.25

Figure 5 Visualisation of the principal component analysis (PCA) of the recovery of *C. albidus* seedlings growing under controlled conditions after withstanding **(a)** 14 days of drought (R1 group) and **(b)** 16 days of drought (R2 group). PCA represents the relationships between all variables (positively correlated variables are grouped together and negatively correlated variables are positioned on opposite quadrants) and the quality of the variables on the factor map (in this case, represented as cos2). A higher cos2 (dark orange in the contribution colour scale) indicates a good representation of the variable on the factor map whereas a lower cos2 (purple in the contribution colour scale) indicates that the variable is not well represented on the factor map. Additionally, survival of individuals is also represented on the factor map (individuals that were alive at the end of the experiment are represented in white and individuals that were dead are represented in black). PC1 and PC2 are the two principal components along which the variation in the data is maximal.

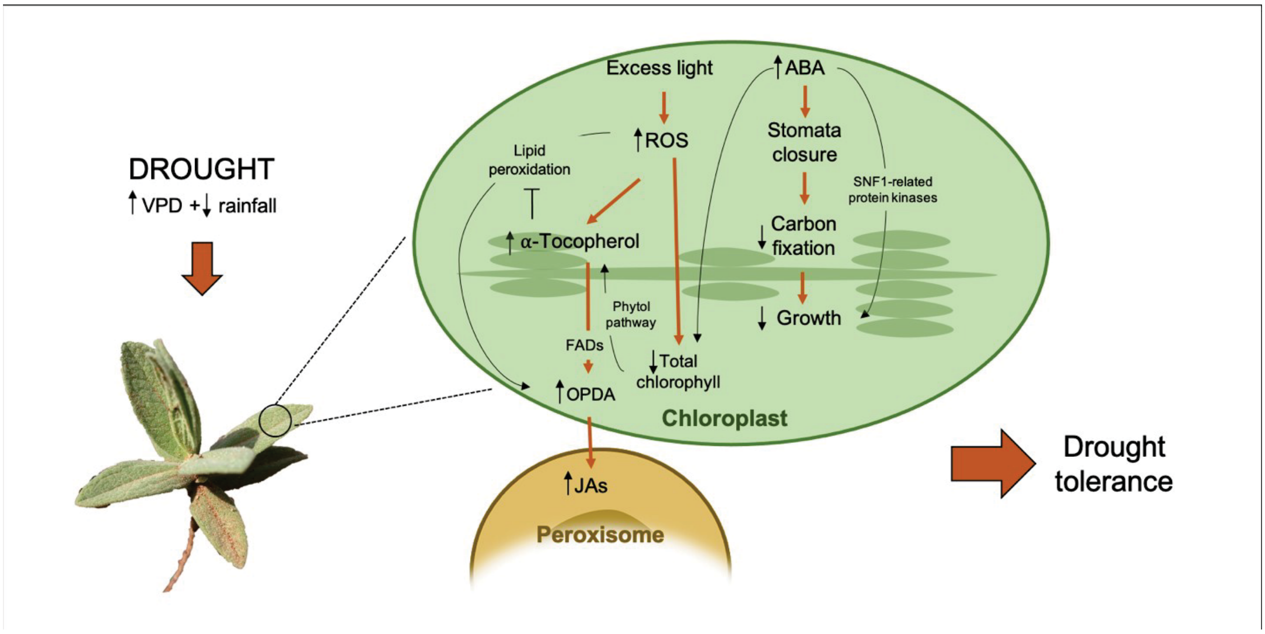


Figure 6 Schematical model of the mechanisms that *C. albidus* seedlings have to be drought tolerant. ABA, abscisic acid; FADs; fatty acid desaturases; JAs, jasmonates; OPDA, 12-*oxo*-phytodienoic acid; ROS, reactive oxygen species; SNF1, sucrose non-fermenting-1; VPD, vapour pressure deficit. *C. albidus* seedling response to drought is a complex network of multiple players (here not all shown) but in our experiment we found that total chlorophylls, vitamin E and JAs were important players. Note that the stop in growth is crucial to allocate carbon to the production of other photoprotective compounds such as α-tocopherol to protect from lipid peroxidation.

Supplementary data

Table S2 Akaike's information criterion (AIC) and accuracy for the models considered for model selection.

Model	Formula	AIC	Accuracy
1	glmer (Survival ~ Leaves+ F_v/F_m + LMA + g_s + RWC + OPDA + JA-Ile + ABA + SA + JA + α-toc + Total chl + α-toc/chl + chl a/b + (1 | Zone/Height))	65.9	0.67
2	glm (Survival ~ Leaves+ F_v/F_m + LMA + g_s + RWC + OPDA + JA-Ile + ABA + SA + JA + α-toc + Total chl + α-toc/chl + chl a/b)	65.65	0.89
3	glm (Survival ~ Height + LMA + F_v/F_m + Total chl + JA	46.48	0.78

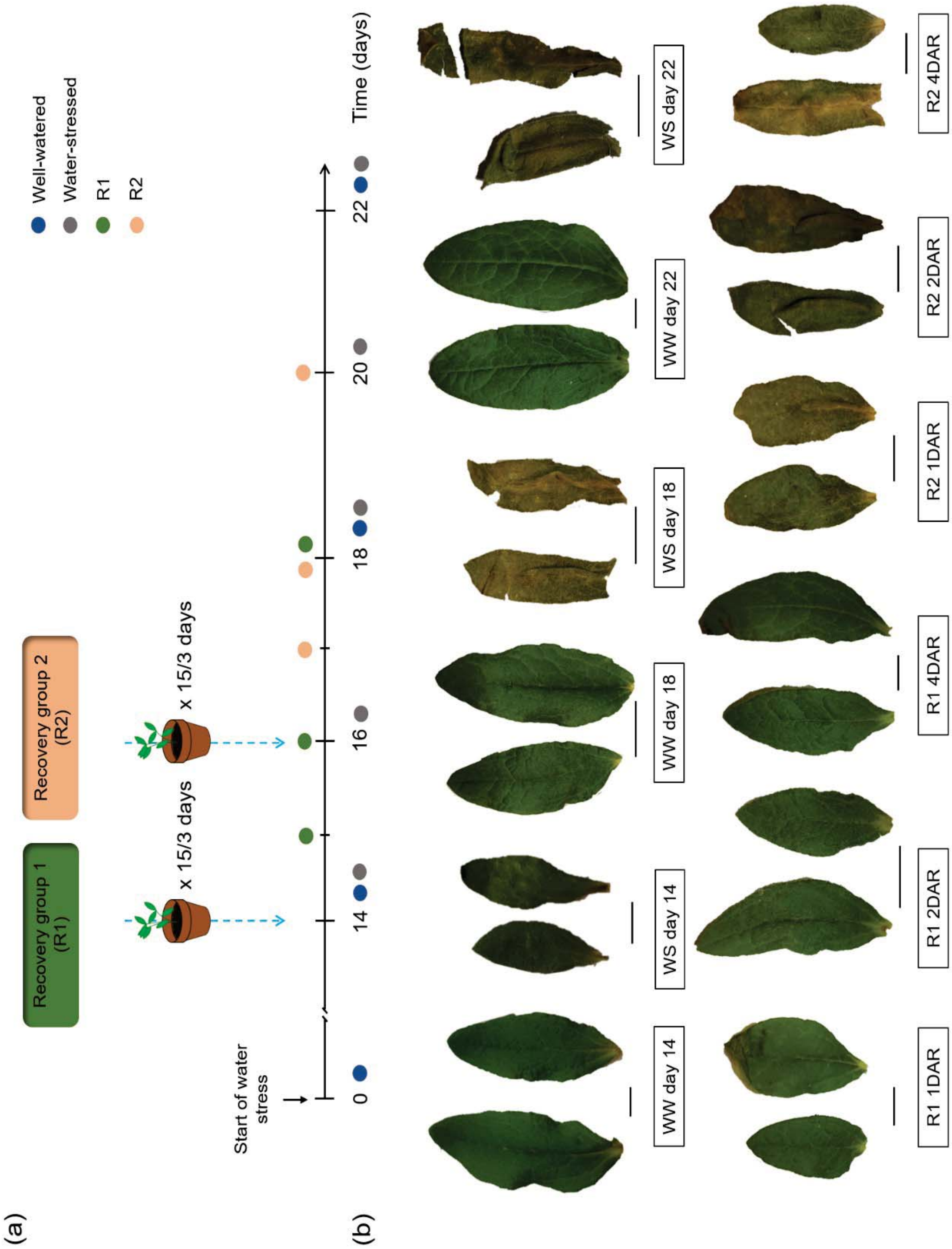

(a)
Recovery group 1 (R1)
Recovery group 2 (R2)
x 15/3 days
x 15/3 days
● Well-watered
● Water-stressed
● R1
● R2
Start of water stress
0
14
16
18
20
22
Time (days)
(b)
WW day 14
WS day 14
WW day 18
WS day 18
WW day 22
WS day 22
R1 1DAR
R1 2DAR
R1 4DAR
R2 1DAR
R2 2DAR
R2 4DAR

Figure S1 (a) Experimental design corresponding to *C. albidus* seedlings growing under controlled conditions during drought and recovery. Plants were exposed to drought withholding water for 22 days and subgroups were re-watered every two days during this period from day 14. Two subgroups were re-watered and their performance was followed for four days (sampled in days 1, 2 and 4 after re-watering) in treatments 1 and 2 (R1 and R2, respectively). Both groups were watered *ad libitum* after the first day of re-watering. Additionally, the performance of a well-watered treatment (watered *ad libitum*) and a water stress treatment (withheld of water during all the experiment) was assessed as positive and negative controls. The well-watered treatment (WW) was sampled at days 0, 14, 18 and 22 of the experiment while the water stressed treatment (WS) was sampled at days 14, 16, 18, 20 and 22 of the experiment. **(b)** Phenotypes of the sampled leaves representative of the well-watered (WW), water-stressed (WS), R1 and R2 treatments during the experiment. Black lines under the leaves represent a 1cm scale. DAR, Days after recovery.

Table S2 Meteorological data during the experiment of *Cistus albidus* seedlings growing in the Natural Park of the Montserrat Mountains.

Month	Mean T^e (ºC)	Mean RH (%)	Mean VPD (kPa)	Ppt (mm)
February 19	8.40 ± 0.63 ab	49.89 ± 3.82 a	0.58 ± 0.06 bcd	3.1
March 19	9.48 ± 0.49 b	59.19 ± 2.59 abc	0.51 ± 0.04 abcd	2.9
April 19	8.82 ± 0.34 ab	70.30 ± 0.40 cd	0.34 ± 0.03 ab	74.4
May 19	12.13 ±0.50 c	66.71 ± 2.56 cd	0.50 ± 0.05 abcd	73.1
June 19	19.36 ± 0.95 de	53.17 ± 3.11 ab	1.19 ± 0.15 f	18.4
July 19	22.66 ± 0.49 f	57.94 ± 2.42 abc	1.22 ± 0.10 f	87.2
August 19	21.73 ± 0.42 ef	64.71 ± 2.64 bcd	0.96 ± 0.09 ef	38.9
September 19	18.31 ± 0.44 d	67.40 ± 2.94 cd	0.73 ± 0.08 de	31.3
October 19	14.50 ± 0.39 c	74.81 ± 3.07 d	0.43 ± 0.06 abc	115.6
November 19	7.63 ± 0.33 ab	68.90 ± 3.09 cd	0.33 ± 0.05 ab	39.8
December 19	8.02 ± 0.37 ab	73.87 ± 2.96 d	0.30 ± 0.04 a	106.2
January 20	6.95 ± 0.36 a	65.16 ± 3.22 bcd	0.36 ± 0.04 a	115.3
February 20	9.51 ± 0.51 b	69.52 ± 2.64 cd	0.38 ± 0.04 abcd	3.2
March 20	9.10 ± 0.59 ab	70.40 ± 2.51cd	0.37 ± 0.05 cdef	5.1

Data represent the mean ± SE (except for Ppt) of either 15 days (March 2020), 28 days (February 2019), 29 days (February 2020), 30 days (April 2019, June 2019, September 2019, November 2019) or 31 days (March 2019, May 2019, July 2019, August 2019, October 2019, December 2019, January 2020). The data corresponding to March 2020 was only considered valuable until one day after the last sampling (15th March) and we considered that the data corresponding to the rest of the month was not relevant for the study. Servei Meteorològic de Catalunya (https://www.meteo.cat/) relinquished meteorological data. Given the absence of some values for RH in Feb 20 and Mar 20 (three and eight, respectively), missing values were imputed from the whole dataframe with the *mice* package where random forest was used as imputing method. Significant differences between months were tested using conditional Fisher tests. Different letters indicate differences in Tukey's HSD multiple comparison test. All differences were considered statistically significant at a probability level of $\alpha=0.05$. T^e, temperature; RH, relative humidity; VPD, vapour pressure deficit, Ppt, accumulated precipitation.

Table S3 Correlation matrix of the field experiment data of *C. albidus* seedlings on February 2019. The Pearson correlation test was used after data was standarised. Values represent the correlation value rho (ρ), which was considered statistically significant at $p < 0.05$. One, two or three asterisks indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

	Leaves	**Height**	**F_v/F_m**	**g_s**	**LMA**	**RWC**	**OPDA**	**JA-Ile**	**ABA**	**JA**	**α-Toc**	**Chl T**	**Chl a/b**
Leaves													
Height	0.37**												
F_v/F_m	0.29*	0.36*											
g_s	0.31*	0.21	0.03										
LMA	0.16	0.26	0.55***	-0.17									
RWC	-0.17	-0.21	-0.34*	-0.22	-0.27								
OPDA	0.09	0.11	0.06	0.2	0.13	-0.09							
JA-Ile	0	0.18	0.08	-0.16	0.1	-0.07	0.18						
ABA	-0.1	-0.14	-0.03	-0.21	-0.14	0.03	-0.15	0.04					
JA	0.01	0.17	0.19	0.08	-0.03	0.01	0.31*	0.43**	-0.03				
α-Toc	-0.11	-0.43**	-0.38**	-0.35*	-0.30*	0.16	-0.39**	-0.11	0.26	-0.33*			
Chl T	0.07	-0.05	0.08	0.06	-0.07	-0.12	0.36*	0.21	0.15	0.24	-0.07		
Chl a/b	-0.13	0.14	0.12	-0.06	0.30*	-0.15	0.14	0.14	0	0.06	-0.26	0.16	
SA	0.23	0.08	0.17	0.12	0.13	-0.12	0.21	-0.04	-0.04	-0.08	0.01	-0.01	-0.2

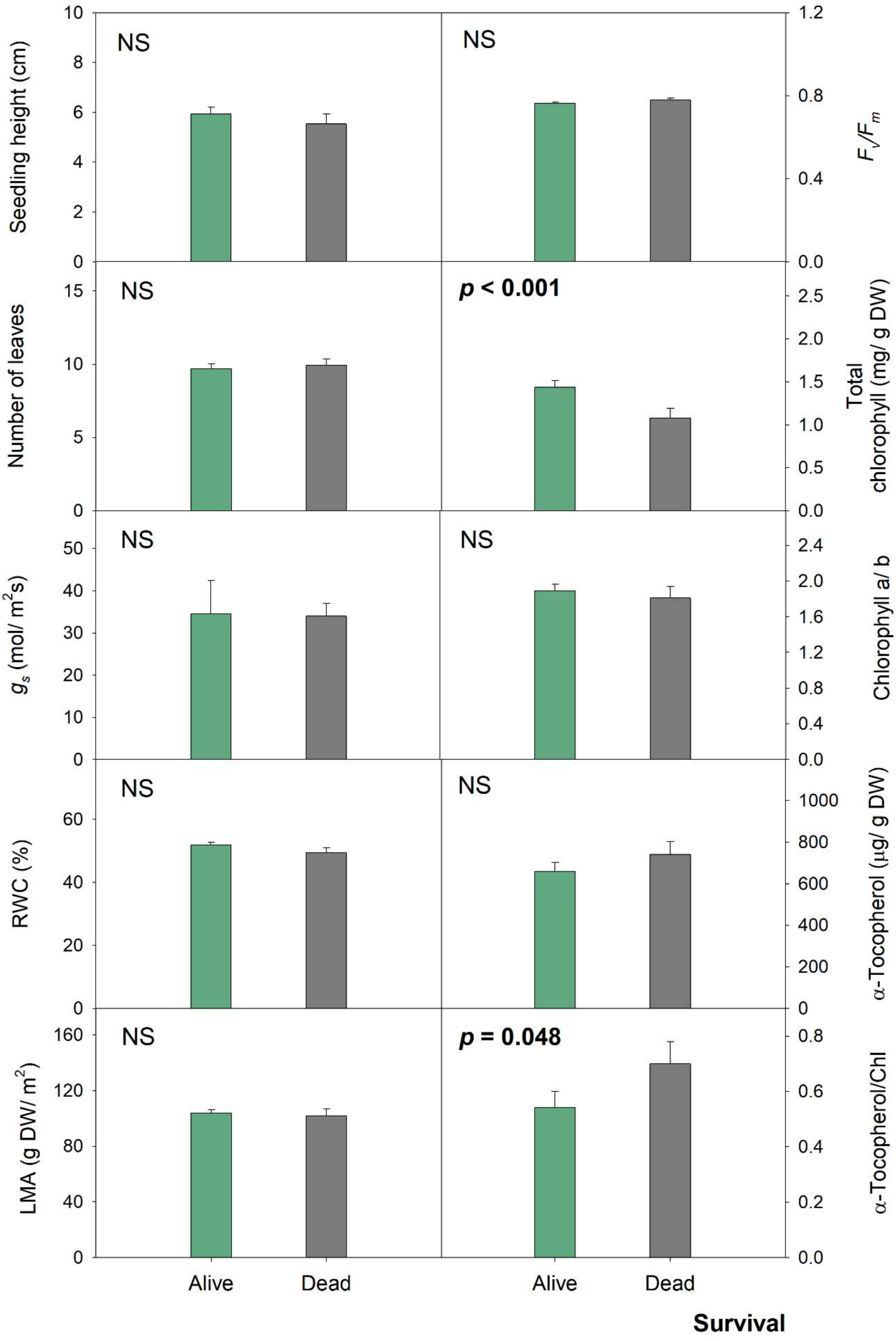

Seedling height (cm)
10
8
6
4
2
0
NS
NS
1.2
0.8
0.4
0.0
F_v/F_m
Number of leaves
15
10
5
0
NS
$p < 0.001$
2.5
2.0
1.5
1.0
0.5
0.0
Total chlorophyll (mg/ g DW)
g_s (mol/ m^{2}s)
50
40
30
20
10
0
NS
NS
2.4
2.0
1.6
1.2
0.8
0.4
0.0
Chlorophyll a/ b
RWC (%)
60
40
20
0
NS
NS
1000
800
600
400
200
0
α-Tocopherol (µg/ g DW)
LMA (g DW/ m^2)
160
120
80
40
0
NS
$p = 0.048$
0.8
0.6
0.4
0.2
0.0
α-Tocopherol/Chl
Alive
Dead
Alive
Dead
Survival

Figure S2 Variations in height, number of leaves, stomatal conductance (g_s), relative water content (RWC), leaf mass area (LMA), maximum PSII efficiency (F_v/F_m), total chlorophyll, chlorophyll a/b ratio, α-tocopherol and α-tocopherol/chlorophylls between surviving (i.e. Alive) and dead seedlings (i.e. Dead) growing growing in the Natural Park of the Montserrat Mountains on February 2019. The distinction between alive and dead individuals corresponds to the data after a year of monitoring (March 2020). Data represent the mean ± SE of 39 individuals for alive seedlings and 16 individuals for dead seedlings. Differences between groups were assessed using a linear mixed model (LMM, function *lme*, package *nlme*) fitting "Survival" as a fixed term and "Zone" as a random term. The variance structure *varIdent* was used to account for different variances within "Zone" and was fitted in the model with the argument "weights". Additionally, restricted maximum likelihood (REML) was used to fit the model. When variances were different within "Survival", the *p* values were obtained using conditional Fisher tests with the function *anova*. When there were significant differences within Month, multiple comparisons were tested with Tukey's post hoc test, using general linear hypotheses (function *ghlt,* package *multcomp*). *p* values were considered statistically significant at a probability level of α = 0.05.

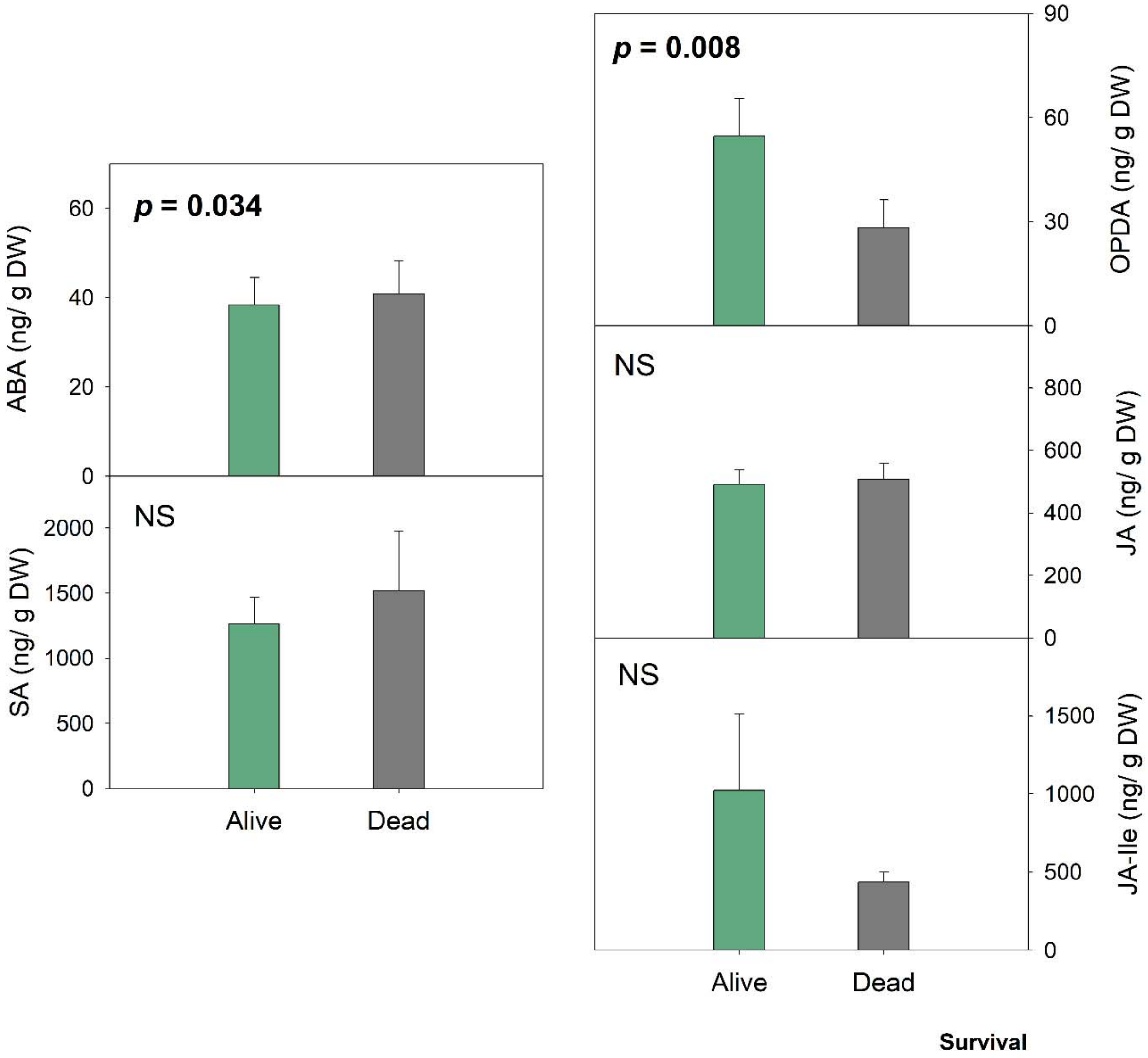
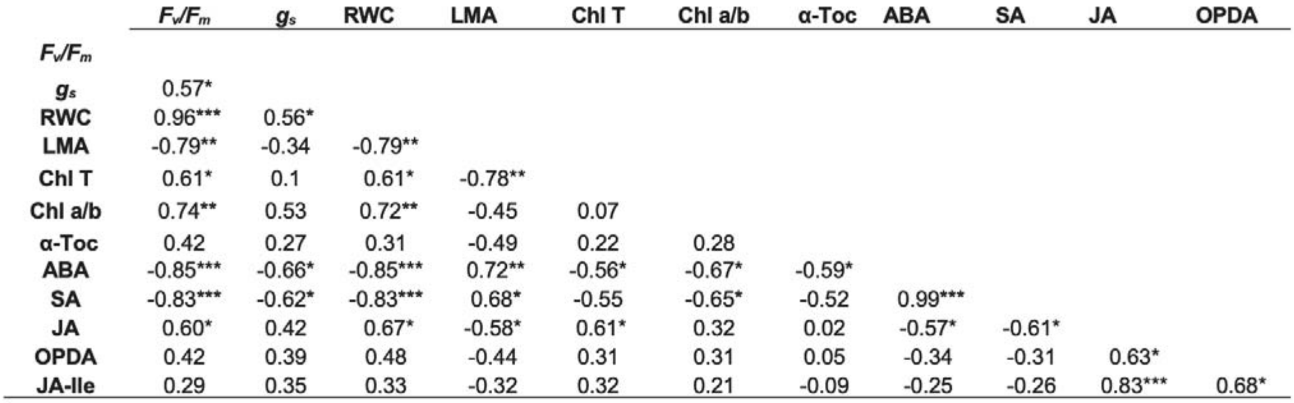


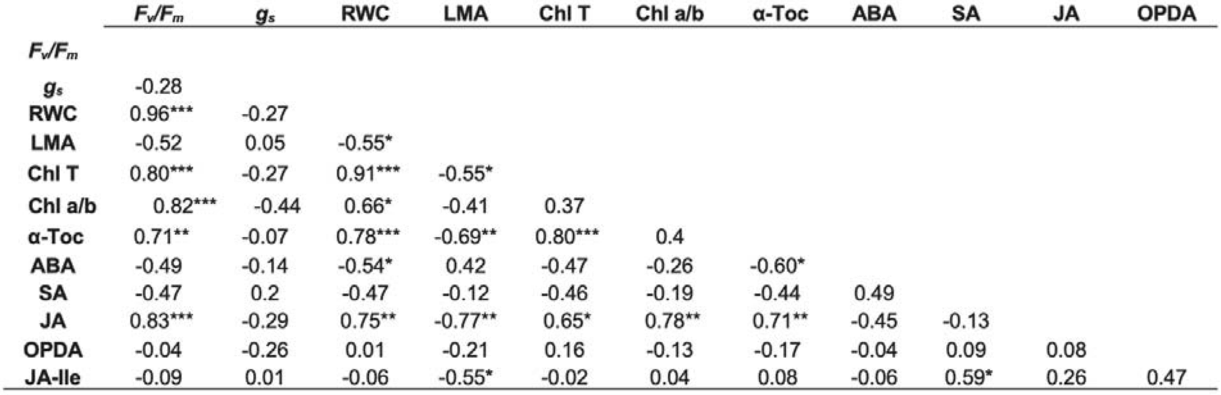
Figure S3 Variations in **(a)** abscisic acid (ABA) and salicylic acid (SA) and **(b)** 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA) and jasmonoyl-isoleucine (JA-Ile) between surviving (i.e. Alive) and dead seedlings (i.e. Dead) growing growing in the Natural Park of the Montserrat Mountains on February 2019. The distinction between alive and dead individuals corresponds to the data after a year of monitoring (March 2020). Data represent the mean ± SE of 39 individuals for alive seedlings and 16 individuals for dead seedlings. Differences between groups were assessed using a linear mixed model (LMM, function *lme*, package *nlme*) fitting "Survival" as a fixed term and "Zone" as a random term. The variance structure *varIdent* was used to account for different variances within "Zone" and was fitted in the model with the argument "weights". Additionally, restricted maximum likelihood (REML) was used to fit the model. *p* values were obtained using conditional Fisher tests with the function *anova*. When there were significant differences within Month, multiple comparisons were tested with Tukey's post hoc test, using general linear hypotheses (function *ghlt,* package *multcomp*). *p* values were considered statistically significant at a probability level of α = 0.05.

Table S4 Correlation matrix of the growth chambers experiment data of R1 *C. albidus* seedlings. The Pearson correlation test was used after data was standarised. Values represent the correlation value rho (ρ), which was considered statistically significant at $p < 0.05$. One, two or three asterisks indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.



	F_v/F_m	g_s	RWC	LMA	Chl T	Chl a/b	α-Toc	ABA	SA	JA	OPDA
F_v/F_m											
g_s	0.57*										
RWC	0.96***	0.56*									
LMA	-0.79**	-0.34	-0.79**								
Chl T	0.61*	0.1	0.61*	-0.78**							
Chl a/b	0.74**	0.53	0.72**	-0.45	0.07						
α-Toc	0.42	0.27	0.31	-0.49	0.22	0.28					
ABA	-0.85***	-0.66*	-0.85***	0.72**	-0.56*	-0.67*	-0.59*				
SA	-0.83***	-0.62*	-0.83***	0.68*	-0.55	-0.65*	-0.52	0.99***			
JA	0.60*	0.42	0.67*	-0.58*	0.61*	0.32	0.02	-0.57*	-0.61*		
OPDA	0.42	0.39	0.48	-0.44	0.31	0.31	0.05	-0.34	-0.31	0.63*	
JA-Ile	0.29	0.35	0.33	-0.32	0.32	0.21	-0.09	-0.25	-0.26	0.83***	0.68*

Table S5 Correlation matrix of the growth chambers experiment data of R2 *C. albidus* seedlings. The Pearson correlation test was used after data was standarised. Values represent the correlation value rho (ρ), which was considered statistically significant at $p < 0.05$. One, two or three asterisks indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.



	F_v/F_m	g_s	RWC	LMA	Chl T	Chl a/b	α-Toc	ABA	SA	JA	OPDA
F_v/F_m											
g_s	-0.28										
RWC	0.96***	-0.27									
LMA	-0.52	0.05	-0.55*								
Chl T	0.80***	-0.27	0.91***	-0.55*							
Chl a/b	0.82***	-0.44	0.66*	-0.41	0.37						
α-Toc	0.71**	-0.07	0.78***	-0.69**	0.80***	0.4					
ABA	-0.49	-0.14	-0.54*	0.42	-0.47	-0.26	-0.60*				
SA	-0.47	0.2	-0.47	-0.12	-0.46	-0.19	-0.44	0.49			
JA	0.83***	-0.29	0.75**	-0.77**	0.65*	0.78**	0.71**	-0.45	-0.13		
OPDA	-0.04	-0.26	0.01	-0.21	0.16	-0.13	-0.17	-0.04	0.09	0.08	
JA-Ile	-0.09	0.01	-0.06	-0.55*	-0.02	0.04	0.08	-0.06	0.59*	0.26	0.47

DISCUSSION

1. *C. albidus* mechanisms under abiotic stress: a spatiotemporal approach

1.1. *C. albidus* faces multiple stresses seasonally and diurnally

As sessile organisms, plants have to be adapted and acclimate to constant changing environmental conditions. In the Mediterranean basin, we find four seasons each one with different ranges of temperatures and precipitation. To this variability among seasons (*i.e.* winter, spring, summer and autumn), we have to add variability within a day. For instance, temperatures and solar radiation are normally higher at the middle of the day. Plants are not homogenous individuals either and light, for instance, influences the plant differently depending on the position of the leaf on the plant and what cardinal direction faces. In this manner, plants are exposed to different gradients and types of stress during not only throughout the year but also during the day. Furthermore, the current global change is already affecting local climate dynamics; hence, plants also need to acclimate to these unpredicted circumstances. In this dissertation, the structural and physiological responses to abiotic stress of a well-stress adapted Mediterranean plant were studied during the Mediterranean seasons as well as during a day.

Plants were investigated during all **seasons** that take place during the year. In **Chapter 1** and **2** we assessed the effects of autumn and winter (and the environmental variability that these comprise). The main stressors present during these studies were **light stress** (given the high photosynthetic active radiations in both studies) and **water stress** (given the scarce rain during autumn **in Chapter 2**). In **Chapter 3**, the experiments of which were performed in winter, light stress was also intrinsic since, like in **Chapter 1**, a part of the experiment was performed at a high altitude for the study species (Guzmán and Vargas, 2010). Apart from the harsh conditions of the dry Mediterranean summer, **winter** is also a stressful period for Mediterranean plants since clear sunny days are abundant during winter, with consequently high solar radiations, and this together with relatively low temperatures can potentially lead to an excess light and, ultimately, to photo-oxidative damage.

Indeed, photoinhibition of PSII has been reported in several Mediterranean species such as holm-oak (Oliveira and Peñuelas, 2000; Oliveira and Peñuelas, 2004), dwarf palm (Morales et al., 2016), common box (Silva-Cancino et al., 2011) and white-leaved rockrose (Oliveira and Peñuelas, 2000). **Cold stress** related to the low temperatures during Mediterranean winter has been seen to affect *C. albidus* survival (Oliveira and Peñuelas, 2004), the model of study. In this manner, in **Chapter 4**, *C. albidus* seedlings growing in their natural habitat were monitored throughout a year to assess survival in every season. Again, in winter, seedlings underwent a stressful period, particularly in February. However, this stressful phase was not characterised by low temperatures; it was a period where plants suffered from **water stress** given the scarce rain during the whole month (Table S1, **Chapter 4**). Despite there was a high accumulation of rain, it was in the summer when plants experienced a more intense **drought**, as shown by the vapour pressure deficit (VPD) values.

The maximum efficiency of PSII (F_v/F_m) has been long used to assess the overall physiological state of plants. Primarily, it is high light stress that triggers an imbalance in photochemical energy conversion, potentially photo-oxidating the reaction centres and/or the pigments and proteins of the light-harvesting complexes, modulating chlorophyll fluorescence and leading to PSII photoinhibition (Ohnishi et al., 2005; Demmig-Adams et al., 2012). However, any abiotic stress can cause a depression in PSII activity (Melis, 1999; Adir et al., 2003; Murata et al., 2007) since environmental stressors normally reduce the photosynthetic fixation of CO_2 by stomatal closure, creating an energy imbalance.

During autumn and winter, *C. albidus* plants did not experience any decreases in their F_v/F_m ratio (**Chapter 1** and **Chapter 2**), despite the dry conditions in November and the low temperatures in December accompanied by the high PAR (photosynthetically active radiation) values (Table 1, **Chapter 1**; Table S1, **Chapter 2**). These findings agree with other studies in *C. albidus* under winter stress, where F_v/F_m values were always above 0.75 (Oliveira and Peñuelas, 2000; Silva-Cancino et al., 2011) – a well-known threshold for the absence of photoinhibition –, indicating

C. albidus tolerance to relatively low temperatures. However, in the study of Silva-Cancino et al. (2011), when plants were exposed to mean temperatures below 0ºC, there was photoinhibition. Additionally, Oliveira and Peñuelas (2002) also found that *C. albidus* plants experienced photoinhibition after a cold event with minimum air temperatures of -4.2ºC which was also accompanied by plant mortality. In other *Cistus* species, Puglielli et al., (2017b) found that there was photoinhibition during December and January, when rain had been scarce since November and mean temperatures were *ca.* 8ºC, a value well above the threshold where *C. albidus* was found to be photoinhibited in the studies aforementioned. Photoinhibition appears to be negatively correlated with temperature and this relationship seems to be species-dependent. Indeed, Oliveira and Peñuelas (2000) confirmed this point in their study. These findings suggest that *C. albidus* might be well-adapted to respond to mild cold temperatures together with high solar radiation; however, if the low temperatures are more extreme, *C. albidus* might not be able to cope, becoming sensitive to cold stress.

During the day, there are several environmental changes that affect plant performance such as changes in light intensity, light quality, or temperature extremes. In the Mediterranean basin, compared to other temperate regions, winter photoperiods are relatively long, with > 9h of light. Clear sunny days are abundant and at solar midday, light intensities are also moderately high, with PAR values near 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ near sea level but with radiations over 2000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at about 1,100 m.a.s.l. (Figure 1, **Chapter 3**). Furthermore, maximum temperatures at solar midday usually surpass 15ºC (*e.g.* Table 1, **Chapter 1**). The F_v/F_m ratio did not significantly vary comparing predawn *versus* midday measurements in autumn nor winter (Figure 1, **Chapter 1**). In a more thorough diurnal study, F_v/F_m values did not vary throughout a (particularly mild) day in January either (Table 2, **Chapter 2**). This coincides with other findings in *C. albidus*, where F_v/F_m remained above 0.75 during winter, with the exception of south-facing leaves (Oliveira and Peñuelas, 2000).

The absence of winter photoinhibition in *C. albidus* contrasts with other evergreen species that coexist with it. For instance, holm-oak (*Quercus ilex*), an evergreen sclerophyll, has intrinsically lower values of F_v/F_m in winter compared to *C. albidus*, particularly under extremely low temperatures (Oliveira and Peñuelas, 2004). It is not uncommon for drought and chilling/cold stress to occur simultaneously during the Mediterranean winter and the combination of the two can have additive effects. Cold stress, apart from limiting the kinetics of various physiological processes (Ruelland et al., 2009), directly affects root water uptake (Stewart et al.,1990). There was a decrease in the water status of *C. albidus* plants in January after two months of scarce rain and low temperatures in December (Figure S1, **Chapter 2**), which translated into other biochemical modulations that will be discussed in the following subsection.

Water status, measured as relative water content (RWC) is also an indicator of plants experiencing abiotic stress, particularly drought. RWC explained much of the variation of the seasonal data (Figure 6a, **Chapter 2**). RWC decreased to relatively low levels (<50%) for a plant that is not reducing its photochemistry (as shown by F_v/F_m, values). After the decrease in RWC, there was a recovery in water status as the season progressed. RWC has been reported to recover as soon as three days after receiving water in *Cistus* spp. (Puglielli et al., 2017c). This agrees with the results obtained in **Chapter 4**, where seedlings exposed to severe water deficit recovered their RWC to initial values after 2 days of re-watering (Figure 3, **Chapter 4**). Whether this decrease in RWC is a consequence of rain scarcity or the cold event in December is difficult to discern, but it is probably a consequence of the two stresses combined. Certainly, in natural conditions plants have to cope with several abiotic stresses and assessing the effects of multiple stresses is important, since a stress alone can have certain effects but when combined with another one, the consequences will be different (Mittler, 2006).

1.2. Structural and morphological mechanisms avoid excess of light

The position of leaves in *C. albidus* is an important feature of this species to face excess light as shown in **Chapter 1**. Although having overlapping leaves has been reported to reduce the light intercepted by leaves in an opposite phyllotaxis (Brites and Valladares, 2005), it could also represent an advantage depending on the habitat. Reductions in light interception could be detrimental for small life forms that live in the understory of a forest because the light that reaches these species is already scarce. In the case of Mediterranean plants inhabiting the machis, this could be an advantage because most of these plants are fully exposed to the sun, which makes these species more vulnerable to the effects of light stress. The case of *C. albidus* is particular, given that it not only has an opposite phyllotaxis but its leaves are also dispositioned in a decussate manner along the stem (Figure 2). In this manner, all leaves are receiving the same light. If this functional adaptation is the result or the cause of the specific features of its habitat is unclear because excess light is common in Mediterranean-type ecosystems (Werner et al., 2002). Nevertheless, it is noteworthy the fact that *C. albidus* keeps high photosynthetic rates compared to other co-occurring evergreen species, even under drought, since they avoid loss of water by partially closing stomata but keeping a good mesophyll conductance (Medrano et al., 2009; Galle et al., 2011) and high values of F_v/F_m (**Chapter 1, 2 and 3**).

Auxin was found to have a differential accumulation depending on the whorl along the stem (Figure 4, **Chapter 1**). Auxin is known for having a crucial role in phyllotaxis, forming gradients that promote the growth of leaf primordia (Reinhardt et al., 2003). Decussate phyllotaxis have been proven to have mirror asymmetrical auxin concentrations, with pairs of leaves of the same whorl having similar concentrations and *vice versa* (Martinez et al., 2016), a fact that was confirmed in **Chapter 1**. However, it is important to note that differential auxin patterns might be affected by other factors such as light besides leaf development alone.

Phyllotaxis is not an adaptation the effects of which can be interpreted alone but there are also other leaf structural mechanisms that prevent photoinhibition in *C. albidus.* Certainly, Brites and Valladares (2005) found that light absorption, besides being dependent on leaf disposition, also depended on leaf inclination and distance between internodes. Steeper leaf inclinations in Mediterranean species have been proven to be beneficial to avoid photoinhibition (Comstock and Mahall, 1985, Valladares and Pearcy, 1999). In *C. albidus* leaf inclination has been reported to change seasonally (Werner et al., 1999), with leaf angles being steeper in summer, when environmental conditions are normally harsher (Jubany-Marí et al., 2009). While in the study of Werner et al. (1999) leaf inclination in *C. albidus* was not sufficient to avoid chronic photoinhibition – *i.e.* the situation when F_v/F_m does not recover overnight and predawn values are still low – in summer, other studies confirm the photoprotective role of steeper leaf angles in winter (Oliveira and Peñuelas, 2000; Oliveira and Peñuelas, 2002). The fact that summer leaves are more prone to photoinhibition is probably due to the fact that there are other environmental stressors such as summer drought that combined with high light intensities lead to photoinhibition, regardless of having steep leaf angles, suggesting the higher sensitivity of summer leaves. Some authors have indeed claimed that summer and winter leaves of *Cistus* spp. are physiologically and morphologically different (Catoni et al., 2012), with summer leaves being thicker and having lower values of photosynthesis, stomatal conductance and chlorophyll contents. This could be the result of the effects of environmental stressors, intrinsic genetic differences or a combination of both but what is clear, however, is that this has implications for stress tolerance in *Cistus* spp. In **Chapter 1**, not only were there more open leaf angles found from apex to more distal leaf positions of the shoot (Figure 3, **Chapter 1**), but also diurnal variations were observed, with steeper angles of leaves at midday compared to predawn in winter (Figure 1, **Chapter 1**). Leaves with wider angles presented slightly lower values of F_v/F_m as well as higher values of the antioxidant α-tocopherol, evidencing the photoprotective role of having steeper leaf angles. It is noteworthy, however, that leaves at more distal positions from the apex, even though having lower values of F_v/F_m, these were always above 0.8. These findings suggest that *C. albidus* has different photoprotection strategies within an individual, and, therefore, that photoprotection mechanisms should be assessed in different tissues of individuals to have a better picture at whole-plant level.

In this manner, leaf angle is an important mechanism of photoprotection. There are several studies that define this erectophylly (*i.e.* steep angles) as an avoidance mechanism (*e.g.* Comstock and Mahall, 1985; Werner et al 1999; Oliveira and Peñuelas, 2000). Oliveira and Peñuelas (2000) discuss that the steep angles in

Cistus spp. are a product of the developmental program, with younger leaves in the apex of the stem having steeper angles and older leaves, that are in more distal positions from the apex, having more open angles. This is an advantage for younger leaves since these are normally more sensitive to photo-oxidative stress (see Juvany et al., 2013). This coincides with the results in **Chapter 1**, but diurnal variations were also found in leaf angle, suggesting an active movement of the leaf during the day, which agrees with what is proposed in *C. salviifolius* and *C. monspeliensis* (Puglielli et al., 2017c). Oliveira and Peñuelas (2000) argue that maybe some of the differences they found relative to leaf inclination were a result of not measuring the same leaves throughout their experiment, which is one of the constraints of **Chapter 1** too, since in order to quantify certain biochemical compounds, the measurements needed to be destructive. However, in the same year, Gratani and Bombelli (2000) showed leaf angle variations in *C. incanus* during the day and, in this case, they followed the same leaves for the experimental period. As such, there is no mechanism described yet that explains how these leaves move during the day but what is clear is that is an essential mechanism of photoprotection of *Cistus* spp.

1.3. Pigments and antioxidants are key for *C. albidus* photoprotection

A lack of photoinhibition in *C. albidus* does not necessarily mean that the plant is not physiologically coping with stress. For instance, *C. albidus* plants experienced a decrease in their chlorophyll contents in December (Figure 1, **Chapter 1**; Figure 2, **Chapter 2**), one of the typically coldest months of winter. **Chlorophylls** are part of the light harvesting complexes and the reactions centres of photosystems (in the case of chlorophyll *a*), and since they absorb excitation energy, they are key for protecting the photosynthetic apparatus from photo-oxidative damage (Fernández-Marín et al., 2017). Decreases in chlorophylls, especially in the chlorophyll *a* + *b* pool, are generally associated with cold and water stress (Esteban et al., 2015). In the case of this dissertation, the decreases in total chlorophyll coincided with minimum temperatures in December that were below 7ºC, but also the sampling concurred with a cold event of a temperature drop of about 10ºC, reaching minimum temperatures below 5ºC (Figure S1, **Chapter 2**). Chlorophyll synthesis is indeed affected by low temperatures and drought, and a reduction in its synthesis could be due to (1) the inhibition of the enzyme 3,8-divinyl protochlorophyllide (an 8-vinyl-reductase) caused by either low or high temperatures (Nagata et al., 2005); (2) or by a reduction in the transport of nutrients (particularly N, which is essential for chlorophyll synthesis) caused by a decline in water potential under drought. This, together with chlorophyll degradation due to the action of ROS, leads to decreases in the chlorophyll *a* + *b* pool. However, decreases in chlorophylls, if not too severe, can result in an acclimation to stress since a lower amount of chlorophylls reduces the rate of excitation and of charge separation for each reaction centre, and, hence a reduction in the (over)production of ROS (Walters, 2005). Oliveira and Peñuelas (2000) also observed a decrease in total chlorophylls in response to winter stress in *C. albidus* in their natural habitat. The fact that *C. albidus* recovered its chlorophyll contents after December (Figure 1, **Chapter 1**; Figure 2, **Chapter 2**), suggests that *C. albidus* acclimated to the cold event successfully, being resilient to these specific winter conditions. The importance of chlorophylls in stress tolerance and resilience was further confirmed in **Chapter 4**, where after a logistic regression analysis it was found that total chlorophylls were a proxy of *C. albidus* seedling survival (Table 1, **Chapter 4**). In this case, however, mortality occurred after six months after the chlorophyll measurement, so most probably it was simply reflecting the physiological status of the plants (Figure 1**, Chapter 4**). In fact, in **Chapter 1**, a positive correlation between leaf hydration and total chlorophyll was found (Figure 7, **Chapter 1**).

The decrease in total chlorophyll coincided with a peak of the cytokinin *trans*-zeatin after a period of scarce rainfall and a cold event (Figure 4, **Chapter 2**). Cytokinins (CKs) are known to be inhibitors of senescence, and they are believed to achieve this by indirectly inhibiting the senescence promoter SnRK1 (Sucrose nonfermenting-Related Kinase 1) (Lara et al., 2004; Jongebloed et al., 2004). Furthermore, CKs have been reported to maintain N and C assimilation during stress which was paired with high photosynthesis rates (Reguera et al., 2013). If these are the mechanisms through which CKs protect the photosynthetic apparatus under stress is still not well elucidated, but what it is clear is that more and more studies are emerging supporting this protective role of CKs, where they have been reported to play a role in acclimation to abiotic stresses such as cold (Jeon et al., 2016), drought (Zhang et al., 2010; Prerostova et al., 2018) or high light (Cortleven et al., 2014). In **Chapter 2**, this increase in *trans*-zeatin was transient and concentrations rapidly went back to basal levels in January. This agrees with the findings of Zwack et al. (2016), where they propose that the cytokinin response factor 4 (CRF4) could have a role in short-term acclimation to cold stress.

The decrease in total chlorophyll also coincided with the highest levels of plastochromanol-8 (**PC-8**) during the experiment (Figure 3, **Chapter 2**). PC-8 is a tocochromanol derivative from plastoquinone-9, both with a long prenyl chain that quenches $^1O_2$ (Gruszka et al., 2008). This makes PC-8 a potentially good antioxidant and, in fact, it has been suggested that it could have higher quenching activity than tocopherols (Kruk et al., 2014). Particularly it has been proven to accumulate in leaves under drought (Fleta-Soriano and Munné-Bosch, 2017) and high light stress (Ksas et al., 2015). Despite being a potent antioxidant, PC-8 accumulates in lower concentrations than α-tocopherol and it has in fact been recently proposed to have a secondary role in photoprotection since its levels greatly increased in the absence of α-tocopherol (Kumar et al., 2020). This differential accumulation of tocopherols and PC-8 could be due to the fact that both tocochromanols are synthesised by the same enzyme (VTE1) (Szymańska and Kruk, 2010), and a higher need for the accumulation of α-tocopherol, could limit the accumulation of PC-8. This agrees with the results in **Chapter 2**, since, first, the levels of α-tocopherol were fourfold higher than the levels of PC-8, and, second, this higher accumulation of PC-8 in autumn coincided with a period of drought. After this period of water stress, PC-8 decreased, and, interestingly, this decrease coincided with an increase in total carotenoids during the months of winter (Figure 2, **Chapter 2**). In fact, carotenoids not only had a significant role seasonally but also diurnally (Figure 2, **Chapter 2**). The pool of xanthophylls together with lutein and β-carotene increased in the morning, keeping at high levels at midday and then decreased towards the evening (with the exception of lutein, β-carotene and neoxanthin the levels of which remained high). This not only confirms the important role of the VAZ cycle in energy dissipation under high light (Jahns and Holzwarth, 2012) but also that the activation of this mechanism in *C. albidus* is an anticipation to the most stressful conditions that occur at solar midday, suggesting that these photoprotective compounds are controlled by circadian rhythms. Certainly, several genes involved in the synthesis of carotenoids have been found to be overexpressed under circadian regulation (Covington et al., 2008). In **Chapter 2**, the levels of carotenoids and PC-8 negatively correlated, suggesting a possible trade-off between these two photoprotective responses, with carotenoids being key under winter stress and to face excess light from high light intensities during the day, and with PC-8 as a secondary sentinel to α-tocopherol under severe drought. Carotenoids (lutein, β-carotene and the VAZ pool) have also recently been shown to have an important role in photoprotection during winter under high light conditions in high-mountain shrubs (González-Rodríguez et al., 2020).

α-Tocopherol, despite its levels not varying during autumn nor winter, kept at high concentrations (*ca.* 250 μg/g DW) during this period (Figure 3, **Chapter 2**). In **Chapter 1**, autumn and winter levels of α-tocopherol were similar (Figure 6, **Chapter 1**), but in **Chapter 4**, seedlings had even higher amounts of the antioxidant (*ca.* 650 μg/g DW) in February, evidencing the higher need of younger plants for photoprotection. Such high levels of α-tocopherol in *C. albidus* suggest that cold temperatures during winter could be as stressful as summer drought. α-Tocopherol also increased as soon as 3h before midday in a day in January (Figure 3, **Chapter 2**), confirming its crucial function under excess light (Munné-Bosch, 2005) and overall, as a primary potent antioxidant in *C. albidus*.

1.4. Phytohormones as a regulatory hub for stress responses

Phytohormones are signalling molecules that are found in very low concentrations in plants. One of the first responses to abiotic stress is the increase in **ABA**. ABA's most known job is to intervene in the closing of stomata (Kim et al., 2010). This is particularly important for abiotic stress tolerance since several abiotic stresses usually imply a water stress (Beck et al., 2007; Uddin et al., 2016; Hussain et al., 2019), and closing stomata prevents water loss. Furthermore, ABA starts a signalling cascade that regulates different developmental processes and the acclimation to stress. Among these processes there is growth inhibition (Belda-

Palazón et al., 2020), which is crucial for the allocation of carbon to stress defence molecules.

In **Chapter 4** we found that ABA increased upon drought in seedlings of *C. albidus* growing in growth chambers, which coincided with a steep increase in stomatal closure (Figures 3 and 4, **Chapter 4**). ABA concentration in seedlings growing in the field was very low compared to that of seedlings in the growth chambers (Figure S3, **Chapter 4**), which could be indicating a higher stress in the latter. However, stomatal conductance in the field was way lower than in the growth chambers (Figure S2, **Chapter 4**), below a threshold from which plants are believed to be under severe drought (Medrano et al., 2002). Then, in which situation seedlings were under a higher drought pressure? This is a tricky question to answer since phytohormone levels can vary within minutes (Wasternack and Hause, 2013; Kollist et al., 2019) and setting thresholds is difficult. Furthermore, ABA levels might differ between species, developmental stage and, of course, the combination of environmental stressors. During a relatively wet autumn, ABA contents were quite low (~ 10 pmol/g DW) in 2-year-old *C. clusii* plants (Munné-Bosch and Lalueza, 2007) but under severe summer drought, Brossa et al., (2015) found higher levels of ABA (*ca.* 600 ng/g FW) in 1-year-old *C. albidus* seedlings, levels that are comparable to the ones found in seedlings with a water deprivation of 18 days (**Chapter 4**). In **Chapter 3**, ABA was higher in plants with low leaf temperatures (*ca.* 10ºC) compared to plants with higher leaf temperatures on a cold day in March (Figures 1 and 2, **Chapter 3**), which suggests that ABA has an important role in cold stress response even though its implication might not be direct in response to cold stress but rather to osmotic stress (Xiong et al., 2001). ABA concentration in seedlings in the field in February was similar to that of *C. albidus* 14-year-old plants in autumn and in February in Chapter 2 (Figure 4, **Chapter 2**). Before that, ABA levels had increased to ~ 150 ng/g DW after two months of barely any rain, which is attributed to the combination of a cold event and drought. However, this increase in ABA was in January, a month later from where we observed the decrease in chlorophylls and the increase in ***t*-Z**, both indicators that plants were coping with stress. Is ABA accumulation then, a more “long-term” response? A possible explanation for this “delay” in ABA synthesis upon stress is that this increase in ABA in January is independent from the stress events from the previous months. The sampling on January was performed on a particularly mild day in winter (Table 2, **Chapter 2**), and in fact, RWC of plants was below 50% (Table 1, **Chapter 2**), which could have prompted the synthesis of ABA and would agree with the study of Suzuki et al. (2015), where they found that ABA transcripts peaked under light stress within 0-90s. This poses an important aspect to take into account when designing experiments in ecophysiology: can past meteorological events be discussed as the cause of certain modulations in the phytohormone pool? Again, the answer is not easy, and it is essential to consider complementary measurements to have a wider picture of the action of hormones since the modulation of one hormone will depend on the action of others (*i.e.* cross-talk, Weiss and Ori, 2007).

ABA had a positive correlation with the cytokinin isopentenyl-adenosine (iPA). iPA followed a similar pattern as that of ABA, decreasing from January to February (Figure 4**, Chapter 2**). The role of **CKs** as senescence inhibitors is well known as well as ABA’s in promoting it. ABA and CKs signalling pathways have been reported to interact, the regulation of one inhibiting the regulation of the other (Nishiyama et al., 2011; Maruyama et al., 2014). This agrees with a recent work studying the fate of reproductive and non-reproductive ramets in a clonal plant where reproductive ramets, which presented signs of senescence, seemed to recover their chlorophyll levels by increasing their contents of CKs (Fenollosa and Munné-Bosch, 2020), which contrasts with the positive correlation between ABA and iPA found in **Chapter 2**. However, Nishiyama et al. (2011) also proposed that ABA could be maintaining CKs homeostasis as a stress-tolerance response. This could be a more plausible explanation for our results since the variations in iPA concentrations were of ~ 2 ng/g DW. In fact, iPA is not generally considered an active CK since it is the free CK forms that normally have a high active and direct role in development (see Mok and Mok, 2001). However, iPA, by hydroxylation, can yield more active CKs such as *t*-Z (Letham and Palni, 1983), which could explain iPA’s relatively constant levels and could imply its role in stress acclimation. Furthermore, sustained levels of CK have been proven to confer protection of the photosynthetic apparatus under stress preventing the degradation of photosynthetic protein complexes (Rivero et al., 2010).

Another growth promoting hormone that seemed to respond to light was auxin, with higher levels of indole-3-acetic acid – **IAA**, the main auxin – under light (Figure 2, **Chapter 1**). Auxins are key for nutrient transport since their asymmetrical distribution in different tissues creates gradients that enable plant growth and development (Petrásek and Friml, 2009). The fact that in **Chapter 1** higher contents of IAA were found at midday compared to predawn agrees with the findings of Sairanen et al. (2012), where they found that IAA synthesis was under the control of the circadian clock. Furthermore, the auxin signalling pathway is believed to be an ancestral pathway that evolved in land plants, since they needed more high light and desiccation tolerance mechanisms compared to aquatic plants (Yue et al., 2012; Bowman et al., 2017). The levels of IAA decreased when the winter progressed and differences between light and no-light faded (Figure 2, **Chapter 1**). These findings not only suggest that IAA could be key under light but also that they might have an important role in acclimation to abiotic stress in *C. albidus*.

Light also seemed to affect the accumulation of **melatonin**, with a higher concentration at predawn and lower concentrations at solar midday (Figure 2, **Chapter 1**). Melatonin is a relatively new-discovered plant hormone and their functions are controversial since it has been reported to have a dual role; either as a regulator or an antioxidant (Arnao and Hernández-Ruiz, 2019). Melatonin role under abiotic stress has been widely reported so far (*e.g.* Zhang et al., 2015), and particularly, it has been observed to increase under light (Tan et al., 2007). Certainly, ROS induce the synthesis of melatonin and the latter is able to scavenge $OH^{\cdot}$ and $^1O_2$, among other ROS (Ianăş et al., 1991). This would disagree with the results in **Chapter 1** but melatonin has been also proven to be involved in the protection of the photosynthetic apparatus by both preventing the degradation of photosynthetic pigments and by regulating the expression of photosynthesis-related genes (see Arnao and Hernández-Ruiz, 2015), and the higher levels at predawn could be a signal to accomplish these protecting functions that are not directly related to its antioxidant function but rather to its role as a hormone.

However, it is still not well elucidated if melatonin accumulation is governed by the internal clock since studies are contradictory (Kolář et al., 1997; Beilby et al., 2015). Interestingly, auxin levels had an opposite pattern to that of melatonin in **Chapter 1**. Auxin and melatonin share biosynthetic pathways (see Pérez-Llorca et al., 2019) and, in fact, the synthesis of auxin has been reported to be heightened at certain concentrations of melatonin, promoting root growth (Chen et al., 2009; Wen et al., 2016). Even though melatonin has auxin-like functions, it is not a substitute of it and their concerted action is believed to be more of a cross-talk between the two (Wang et al., 2016). However, most of the studies reporting this cross-talk between auxin and melatonin have been reported in root development and whether these synergies occur also in shoots and under stress, is still yet to be determined. Altogether this data, suggests that melatonin could be part of the protective network of *C. albidus,* probably in a concerted action with auxin, although more studies would be necessary to give light to the specific role of melatonin in Mediterranean plants.

The other hormone involved in plant development that presented diurnal variations was **GA_1** – as a main active form in *C. albidus* together with GA_4. GA_1 levels were high at predawn and then decreased for the rest of the day (Figure 4, **Chapter 2**). GAs are governed by the internal clock, with higher levels at night than during the day, which contrasts with the pattern of DELLA proteins (negative regulators of GAs) as a way to control rhythmic growth (Arana et al., 2011). DELLA proteins accumulate under abiotic stress, which would imply a reduction in GAs pool and hence, in growth (Colebrook et al., 2014). January is not the growth season for *C. albidus* but such high levels of GA_1 at night – levels were increased by a tenfold compared to daylight conditions – suggest that GAs are actively regulating a process despite the stress. A possible explanation is the progressively longer photoperiods in January, which would trigger flowering with GAs implicated (Xu et al., 1997; Gocal et al., 2000). In fact, *C. albidus* plants show the first signs of flowering in January due to an increase in photoperiod, and the creation of the flowering structures could justify the GA_1 high levels (Blázquez et al., 2002). On the other hand, lower levels of GAs could imply higher levels of DELLA due to a more stressful environment during the day. From these findings, what is evident is that *C. albidus* was able to cope with winter stress while continuing with its phenological program thanks, in part, to GAs.

Salicylic acid (**SA**) peaked between midday and afternoon in *C. albidus* and its concentrations were higher on a clear day with higher solar radiation compared to a cloudy/snowy day (Figure 3, **Chapter 3**). This indicates, on one hand, that SA accumulation might be governed by the internal clock and on the other, that SA is responsive to light, given that its levels kept high until the afternoon but decreased in the evening, maintaining low levels until the morning. SA, also referred as a stress-hormone just as ABA and JA, has an essential role in the response of plants to abiotic stress (Khan et al., 2015). Particularly, SA has been reported to have an antioxidant role against H_2O_2 together with glutathione (Han et al., 2013) in maintaining photosynthesis (Mateo et al., 2006), and also a possible function in stomatal closure (Mateo et al., 2004), being essential in this manner during excess light. SA synthesis has been observed to be regulated to some extent by circadian rhythms (Covington et al., 2008), however studies are not conclusive and SA accumulation during the day in **Chapter 3** is probably rather a result of the effect of environmental cues. In **Chapter 3**, higher levels of SA were found in leaves that had a lower leaf temperature. These higher levels of SA were found in plants growing at high elevation and high solar radiations during a particularly cold day (Figure 1, **Chapter 3**). Certainly, SA is involved in the cold stress response of plants (see Miura and Tada, 2014) and, most recently, it has been directly related to the regulation of the NAC transcription factors (Hou et al., 2020), which are key for abiotic stress tolerance (Jensen et al., 2010). Cold/freezing tolerance mediated by SA has been proven to be achieved by the synthesis of compatible solutes, antioxidants and osmolytes (Min et al., 2018). Supporting the roles in abiotic stress tolerance of SA, strikingly high levels of the phytohormone (*ca.* 10 mg/g DW) were found under severe stress in *C. albidus* seedlings (Figure 4, **Chapter 4**). Such high levels of SA might also have an additional function of limiting cell death (Straus et al., 2010). After rewatering, the levels of SA decreased, indicating stress alleviation.

SA correlated negatively with **JAs**, which was attributed to a trade-off in functions (Figure 7, **Chapter 3**). This negative correlation was also found in **Chapter 4** but in *C. albidus* seedlings (Table S2, **Chapter 4**), supporting the complimentary roles of these defence mechanisms under abiotic stress regardless of developmental stage. A full JAs profile revealed that JA, its precursor OPDA, the active form JA-Ile and other conjugated forms accumulated in *C. albidus* leaves during a cold day in a population growing at high elevation. This trade-off seemed to be concentration-dependent and one possible explanation for a high variability in JAs contents is a high biotic pressure (see Bari and Jones, 2009). JA has been proven to be a negative regulator of SA in the presence of biotic stress by the action of the transcription factor MYC2 (Laurie-Berry et al., 2006). In this manner, under abiotic stress alone, this cross-talk might change. JAs were also strongly affected by light in *C. albidus* growing under semi-controlled conditions (Figure 5, **Chapter 3**), with OPDA peaking in the afternoon and JA-Ile increasing in the morning. Similar to SA, JAs have also been reported to be partially regulated by the circadian clock, although it seemed more like a cross-talk between JAs and the circadian clock than a unilateral regulation by the internal clock (Zhang et al., 2019). In fact, the regulation of JAs synthesis has been proven to be more affected by the circadian clock of pathogens than the circadian clock of plants itself (Godspeed et al., 2012). Particularly, **OPDA** seemed to be under the influence of circadian rhythms since its peak in the afternoon occurred during both a cloudy/snowy day and a sunny day. However, this peak coincided with the highest leaf temperature during the day. Therefore, OPDA accumulation could be influenced by both the circadian clock and environmental cues. It is noteworthy the differential modulation of OPDA to its other JAs counterparts. While JA and JA-Ile only varied significantly during the cloudy/snowy day, OPDA showed a marked pattern during both days. OPDA is the precursor of JAs and variations in its contents could signify modulations in JAs synthesis, but OPDA alone has been also reported to have a differential role to the other JAs under abiotic stress (Maynard et al., 2018; Simancas et al., 2018). In **Chapter 4**, surviving seedlings of *C. albidus* had higher amounts of OPDA (Figure S3, **Chapter 4**). Mortality in seedlings was mainly attributed to drought, which agrees with the extensive work of Savchenko et al. (2014) where they prove that OPDA confers more drought tolerance than other JAs. Moreover, OPDA has been reported to counteract the death phenotype (Przybyla et al., 2008). All considered, JAs seem to have a very important role in the drought tolerance of *C. albidus,* tolerance that is thought to be conferred by a cross-talk between GAs, ABA, JAs and the transcription factor MYC2 (see Reimann et al., 2015 and Figure 3).

Overall, it becomes clear that *C. albidus* response to stress is modulated by an intricate network of hormones. Figure 3 shows a model of the response of *C. albidus* to drought and cold stress (the two main stressors that affect *C. albidus* performance), with the hormone cross-talks and their physiological implications that are probably occurring in the cell conferring *C. albidus* with stress tolerance. Drought or cold stress trigger the accumulation of ABA, SA and JAs. JAs together with ABA and GAs conform a cross-talk that has been described to confer osmotic tolerance in plants (Dombrecht et al., 2007; Fu et al., 2020). JAs degrade the protein repressors JAZ (JA ZIM-domain proteins), which enable the action of the transcription factor MYC2, which, in turn, encodes for different secondary metabolites such as osmolytes that confer stress tolerance. GAs, at the same time also play a role in the degradation of JAZ: GAs are inhibited by stress and then stop repressing DELLA proteins, which, in turn, degrade JAZ, also prompting the action of MYC2 (Wild et al., 2012). ABA directly induces MYC2 (Aleman et al., 2016). JAs and auxins have been proven to have a cross-talk (Tiryaki and Staswick, 2002), which has been shown to promote lateral root development through the transcription factor ERF109, with the responsive factor increasing auxin biosynthesis in both shoot and root tissues (Cai et al., 2014). At the same time, accumulation of ABA triggers the release of the α subunit of SnRK1, inhibiting growth and prompting other stress responses (Belda-Palazón et al., 2020). Under stress, CKs might be playing a dual role: by inhibiting SnRK1 and avoiding senescence (Lara et al., 2004); and by protecting the proteome of the photosynthetic apparatus (Reguera et al., 2013). This contrasts with the role of ABA, which is able to degrade chlorophylls by repressing the transcription of chloroplast-related genes (Yamburenko et al., 2013). ABA has also been reported to promote the homeostasis of CKs (Nishiyama et al., 2011). In this manner, ABA and CKs seem to have a clear cross-talk with opposite functions. However, the concerted action of the two is probably facilitating *C. albidus* stress response. Finally, high levels of SA can induce the action of antioxidants to counteract ROS (Min et al., 2018).

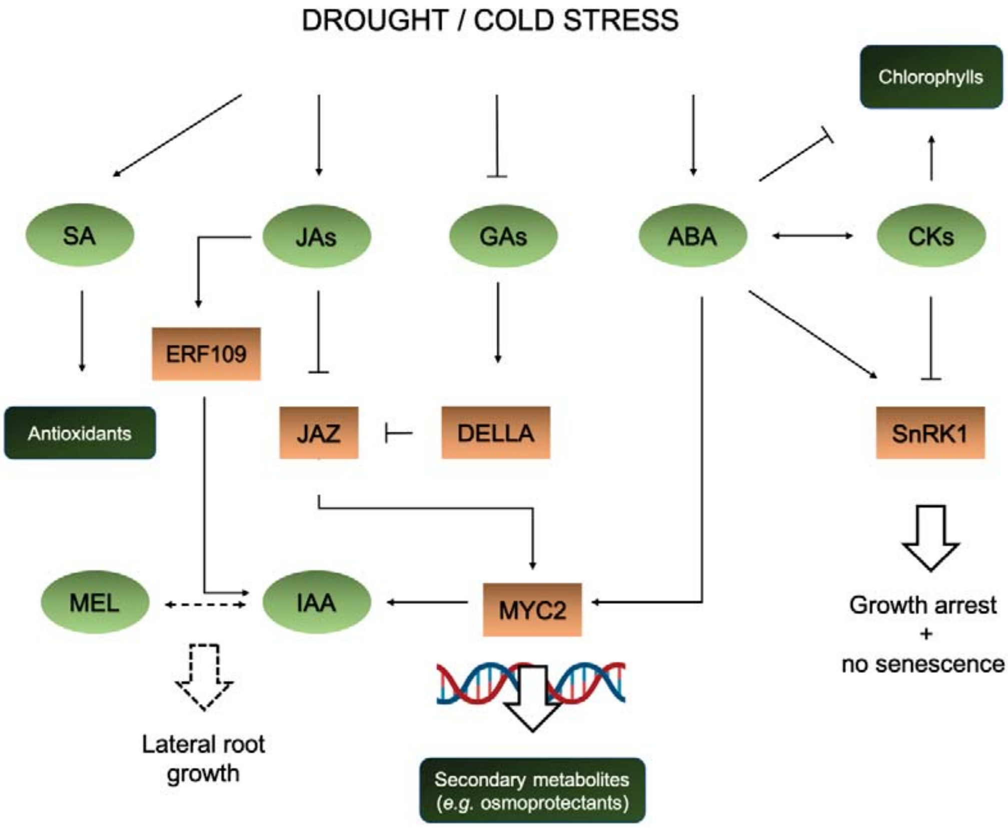


Figure 3 Hormonal cross-talks of *C. albidus* under drought and cold stress. Hormones are shown in green and regulators in orange. Discontinuous lines indicate not very well established cross-talks. ABA, abscisic acid; CKs, cytokinins; DELLA, aspartic acid-glutamic acid-leucine-leucine-alanine repressor proteins; ERF109, JA-responsive ethylene response factor 109; GAs, gibberellins; IAA, indole-3-acetic acid; JAs, jasmonates; JAZ, jasmonic acid ZIM-domain proteins; MEL, melatonin; MYC2, basic-helix-loop-helix transcription factor; SA, salicylic acid; SnRK1, sucrose nonfermenting-related protein kinase 1. Part of this figure was created with BioRender (BioRender.com).

1.5. Drought appears to be the main environmental stressor affecting *C. albidus*

Plants have to face multiple stresses at once during their life-history and discerning the absolute effects of one stressor alone is rather difficult. However, to better understand plant performance and find simpler explanations on the variations of certain compounds, it might be useful to separate the effects of a single stressor. Furthermore, in nature, the effects of multiple stressors will always be dominated by one stressor at a given point. Here, a simplification of the effects of abiotic stressors was aimed, knowing that there was overlapping between stressors.

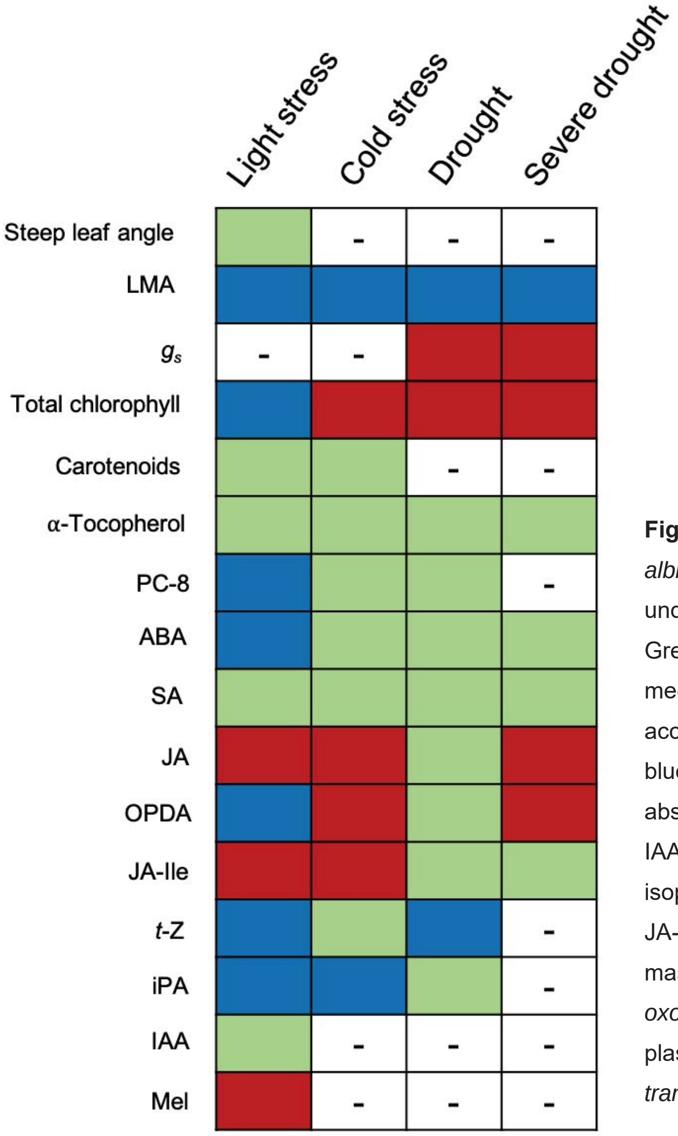
Drought is one of the main threats for plant survival and productivity. After considering all the stressors that *C. albidus* faced in all experiments of this dissertation, **drought** was the stress that resulted in the activation of more photooxidative protection mechanisms (Figure 4). Furthermore, severe drought resulted in seedling death (**Chapter 4**). It was considered that *C. albidus* plants coped with drought during autumn and the beginning of winter in **Chapter 2** – due to scarce rainfall and increasing levels of ABA – and in **Chapter 4** in February in natural conditions as well as in the seedlings that recovered from drought stress in the growth chambers – indicated by the scant rain accumulated in winter and the low values of stomatal conductance (Medrano et al., 2002). It was observed a reduction in g_s and total chlorophyll as mechanisms of avoiding water loss and to reduce excess light. This coincided with an accumulation of the antioxidants α-tocopherol and PC-8, evidencing the need for counteracting the effects of ROS. At the same time, ABA and SA also increased, indicating that *C. albidus* is undergoing a stress, and, in the case of SA, that its accumulation could be serving as a signal to activate other antioxidant responses. JAs also accumulated under drought as well as the cytokinin iPA. LMA kept constant under the levels of drought the plants experienced as well as *t*-Z, which seemed to have a more important role under cold stress.

Cold stress came to a close second as a main stress that *C. albidus* has to endure. Under cold stress, *C. albidus* activated the same amount of protective mechanisms (Figure 4) but these differed between the two stresses, despite cold stress and water stress having been proposed to have common mechanisms (Hussain et al., 2018). Cold or chilling stress was considered to occur in **Chapter 1** in *C. albidus* plants growing at high altitude in winter; in **Chapter 2** in December after the cold event; and in **Chapter 3** during the cloudy/snowy day as well as in *C. albidus* plants that had lower leaf temperatures in the field. While they agreed in the decrease in total chlorophyll and the increase of α-tocopherol, PC-8, ABA and SA, they differed in the accumulation of JAs, with JAs being higher under low temperatures. The cytokinin *t*-Z peaked after the cold event, probably protecting from the extensive degradation of chlorophylls, but iPA, which increased under drought, did not suffer any variations, just as LMA. The contrasting effects of *t*-Z and iPA are noteworthy in the response to cold stress. *t*-Z peaked after a cold event while there was iPA homeostasis. As it has been mentioned in the previous section, free CKs such as *t*-Z are usually the active CKs in plants, but nucleosilated forms derivative from isopentenyladenosine- 5′-monophosphate (iPMP), such as iPA, can produce zeatin-type cytokinins (Letham and Palni, 1983). This would suggest that iPA homeostasis could have enabled the cold response by *t*-Z, hence, implying a cross-talk between different types of CKs.

Under **light stress**, *C. albidus* increased the steepness of its leaves as a way of protection from excess light (**Chapter 1**). Even though the levels of α-tocopherol were not as high in steeper leaves compared to more open leaves in the stem, α-tocopherol still was an important antioxidant counteracting the effects of excess light since it was crucial in the diurnal response of *C. albidus*. Carotenoids, especially the xanthophyll pool, increased under light stress, acting as key energy dissipators. PC-8, ABA and CKs remained unchanged during daylight while SA and IAA accumulated under light. OPDA presented variations during the day but the pattern was very similar between a day with low PAR values and a day with higher PAR (cloudy *versus* sunny day), which could be attributed to circadian rhythms rather than to the direct effect of high light. This contrasts with the decline of JA and JA-Ile, suggesting a differential role between the diverse JAs. Melatonin decreased under light. This could imply either that melatonin is being oxidised, thus acting as an antioxidant and having an important protective role under light, which agrees with Simopoulos et al.

(2005); or that is having a signalling function since its contents are at low concentrations. However, melatonin has been shown to have growth-regulating functions at concentrations of 25 ng/g FW in lupin (Hernández-Ruiz et al., 2005), and the fact that in **Chapter 1** levels did not surpass these concentrations suggests that melatonin is having a hormone/regulatory role rather than acting as an antioxidant. Furthermore, in another study with lupin that was exposed to high levels of ROS (Arnao and Hernández-Ruiz, 2013), melatonin levels were three times higher than the concentrations found in **Chapter 1.**

Finally, **severe drought**, considered when *C. albidus* individuals died (**Chapter 4**), differed with the responses to drought mainly in the accumulation of JAs. JA and OPDA levels were low while JA-Ile increased in seedlings that were exposed to more days of drought. The fact that under severe drought OPDA and JA decreased might be explained by cell death and the cessation of metabolism, just as α-tocopherol decreased on the last days of the experiment. Extensive lipid peroxidation by ROS triggers the synthesis of oxylipins (Mosblech et al., 2009) and these act as cell death promoters together with ethylene (Rao et al., 2000; Kim et al., 2015). By day 18 of water restriction, most of *C. albidus* plants were dead (see a leaf phenotype in Supplementary data of **Chapter 4**) and a peak in JA-Ile, as the main active form of JA, could be showing a final death signal.



	Light stress	Cold stress	Drought	Severe drought
Steep leaf angle	🟩	-	-	-
LMA	🟦	🟦	🟦	🟦
g_s	-	-	🟥	🟥
Total chlorophyll	🟦	🟥	🟥	🟥
Carotenoids	🟩	🟩	-	-
α-Tocopherol	🟩	🟩	🟩	🟩
PC-8	🟦	🟩	🟩	-
ABA	🟦	🟩	🟩	🟩
SA	🟩	🟩	🟩	🟩
JA	🟥	🟥	🟩	🟥
OPDA	🟦	🟥	🟩	🟥
JA-Ile	🟥	🟥	🟩	🟩
t-Z	🟦	🟩	🟦	-
iPA	🟦	🟦	🟩	-
IAA	🟩	-	-	-
Mel	🟥	-	-	-

Figure 4 Protective response of *C. albidus* to the different stressors it underwent in **Chapter 1, 2, 3** and **4.** Green means when a mechanism/compound was accumulated; red when it decreased; and blue, when it remained unchanged. ABA, abscisic acid; g_s stomatal conductance; IAA, indole-3-acetic acid; iPA, isopentenyladenosine; JA, jasmonic acid; JA-Ile, jasmonoyl isoleucine; LMA, leaf mass area; Mel, melatonin; OPDA; 12-*oxo*-phytodienoic acid; PC-8, plastochromanol-8; SA, salicylic acid; *t*-Z, *trans*-zeatin.

2. Inter-individual variation in *C. albidus*

2.1. Implications of inter-individual variability in plant stress response

It is clear that environmental variability such as variations in season and day result in differential responses in *C. albidus*. Within an individual, variability was also found in **Chapter 1**. Leaf angle varied along the stem and with it other physiological and biochemical parameters. Phyllotaxis also affected the contents of certain compounds such as auxin. In this manner, within a plant exist multiple microhabitats that all together define the whole plant response to stimuli. These differences within an individual are defined as intra-individual variability and it is important at all levels of the scientific process: from the experimental design to drawing conclusions. Intra-individual variability is primarily driven by phylogeny (Marks, 2007) and avoiding its effects is almost impossible. However, we can consider it in our experimental designs; for instance, focusing in one plant tissue.

Another source of variability that is almost unavoidable when performing studies is the inter-individual variability. Within a population every individual behaves slightly different due to both microhabitat, which leads to trait plasticity, and genetic variation (Matesanz et al., 2012). Inter-individual variability has been found to be comparable to the variation that exists between species (Messier et al., 2010; Auger and Shipley, 2013), hence performing studies that consider this variability is essential for the trueness of our measurements and ultimately of our conclusions (Bean et al., 2012; **Annex 1**). However, if included in the hypothesis, inter-individual variability can provide a lot of information. The standard error of a given measured parameter in a group (*i.e.* standard deviation divided by the square root of the sample size) classically represents process variability, which is the sum of inter-individual, intra-individual and methodological variability (Boitani et al., 2000). If intra-individual variability and methodological variability are considered to some extent in the experimental design – which is not always easy (see **Annex 1**) –, it can be assumed that the standard error mainly represents inter-individual variability.

In **Chapters 1** and **3** inter-individual variability was obtained as the percentage deviation (SD_{pop}) – *pop* meaning population as in a data population not a genetic population – or coefficient of variation (Pélabon et al., 2011, 2020) of a given trait (standard deviation divided by the mean of the population multiplied by 100) in order to better understand how individual performance affected the stress response of *C. albidus* in winter. Expressing inter-individual variability as SD allows to compare parameters and to see to some extent how variable these parameters are in natural conditions since it is assumed that the mean of the population is the optimal environmental value. The most striking results were when comparing physiological parameters such as F_v/F_m and leaf hydration with phytohormones. Taking together the results of **Chapter 1** and **3**, while F_v/F_m and leaf hydration had very little inter-individual variability, phytohormones presented a great variability (8% in F_v/F_m and leaf hydration *versus* 170% in phytohormones). First, that F_v/F_m and leaf hydration had such low variability in *C. albidus* is indicating that all plants considered in the experiment had a similar physiological status (in terms of a functioning photosynthetic apparatus and a high water status, see discussion of **Chapters 1** and **2)**. Second, that the accumulation of phytohormones is rapidly induced, as discussed in section 1.2, which is reasonable since hormones are the signals that regulate plant responses to stress. Pigments and antioxidants also had a relatively low inter-individual variability compared to that of phytohormones (an average of 34% from **Chapters 1** and **3**).

In **Chapter 4**, *C. albidus* individuals were also studied in their natural habitat (at a close location to the individuals in **Chapters 1** and **3**). Environmental conditions were quite different, being the day of sampling in **Chapters 1** and **3** a very cold day in winter after a cold event, and the day of sampling in **Chapter 4** a relatively mild day in winter after a couple of months of very scarce rain. Again, phytohormones had a higher inter-individual variability compared to F_v/F_m and leaf hydration, with the first having a percentage deviation of 136% and the second of 8% (Table 2). Total chlorophyll and α-tocopherol also had a similar inter-individual variability to that of the other experiments in late winter. The fact that inter-individual variability was similar for certain parameters in different situations suggests that the measured parameters are good traits to compare stress responses in plant communities.

Traits related to chemical compounds have been found to have higher variability compared to morphological traits including species all around the globe (Siefert et al., 2015). The variation of (functional) traits related to morphology and the implications of this in species distributions have been fairly studied (*e.g.* He et al., 2018), but studies on the variation of physiological traits related to biochemical compounds are scarce (but see Rozendaal et al., 2006; Siefert et al., 2015; Vilà-Cabrera et al., 2015). He et al. (2018) suggested that species with low trait variabilities have a more important role in structuring communities since a low variability is related to more stable habitats. This would agree with *C. albidus* ecology since it is a pioneer species in maquis, which are habitats with high environmental heterogeneity (Cowling et al., 2015). However, *C. albidus* only presented a low variability in traits like LMA or F_v/F_m while there was a high variability in phytohormones, and a relatively high variability in pigments and antioxidants (**Chapters 1, 3** and **4**). Then, what type of traits drive *C. albidus* distribution? Is distribution determined only by the less variable traits and the high variable traits are the ones that confer the plant with stress tolerance? Indeed, being highly plastic has been related to plant acclimation and adaptation (Valladares et al., 2006; Lande, 2009; Nicotra et al., 2010) but also to distribution in invasive plants (Higgins and Richardson, 2014). Therefore, it seems that a combination of low and high variable traits might be the key of success of highly stress tolerant plants such as *C. albidus*.

Trait	SD_{pop} (%)
F_v/F_m	5.2
g_s	34.3
RWC	71.0
H	10.8
LMA	15.3
α-Tocopherol	39.9
Total chlorophyll	36.8
ABA	87.1
SA	106.0
OPDA	132.7
JA	52.1
JA-Ile	303.1

Table 2 Inter-individual variability measured as SD_{pop} in *C. albidus* seedlings growing at the Montserrat Mountains in February 2019. ABA, abscisic acid; F_v/F_m, maximum PSII efficiency; g_s, stomatal conductance; H, leaf hydration; JA, jasmonic acid; JA-Ile, jasmonoyl isoleucine; LMA, leaf mass area; OPDA, 12-*oxo*-phytodienoic acid; RWC, relative water content; SDpop, percentage deviation of the data population.

Altogether, from this intra-individual high variability, it can be inferred that individuals in **Chapters 1, 3** and **4** were responding very differently to the environmental conditions, a result probably due to microhabitat differences between plants. How variable is a trait or a compound in an individual can affect its fitness (Valladares et al., 2014) because it suggests that this individual is not able to reach certain extremes in trait variability that might lead to its survival. In **Chapter 4**, total chlorophyll was an indicator of plant survival, with increasing seedling survival passed a threshold of 1.87 mg/g DW. The inter-individual variability of total chlorophyll in seedlings was intermediate (compared to that of leaf hydration and phytohormones), with values ranging from 0.1 mg/g DW to 2.4 mg/g DW. Individuals that were not able to reach these 1.87 mg/g DW had lower fitness, which lead to plant death. It seems that the combination of low, intermediate and high inter-individual variability provides *C. albidus* with the tools necessary to respond to a high heterogenous environment, particularly one with a high variation in the accumulation of phytohormones. Trait plasticity is specifically important in the current global change scenario, where environmental conditions are less predictable for plants and traits that might not have needed to be as variable as before, they might need to in a near future.

2.2. How does variability affect ecophysiological studies?

Different types of variability can affect the trueness of our results and hence drawing the appropriate conclusions. In the previous section, intra-individual variability (*i.e.* spatiotemporal variability) and inter-individual variability have been proposed as sources of variability that modify the precision of our estimates. Environmental variability – which has been shown in this dissertation to be very high – and methodological variability need to be taken into account as well.

An example of methodological variability is provided by Table 2. It was remarkable the difference between the two measures of water status in **Chapter 4**: RWC and leaf hydration (H). While H presented a low inter-individual variability, RWC had relatively high values of percentage deviation, comparable to those of ABA, for instance. Discussing the results of the water status of a given experiment based on H, one could conclude that all plants had a relatively stable water status but if the discussions are based on RWC, the conclusions might slightly change. Leaf hydration represents the quantity of water that a tissue presents in a given moment while RWC is the quantity of water a tissue has in a given moment relative to the maximum it could hold (*i.e.* a 100%). In this manner, these two parameters provide slightly different information and depending on your hypothesis it would be suitable to use one or the other, or the two of them. Both measurements have their advantages and drawbacks. While RWC might be a good indicator of stress, the time to perform this measure is longer than in the case of H since the tissue to be assessed needs to rehydrate for at least 24h (Barrs and Weatherley, 1962). This could be a time limitation in ecophysiological studies where sample size is big and leaf hydration could be a more feasible measure. Furthermore, although RWC might seem to have an easy methodology it has some constraints because the rehydration step is species-dependent, and variations in the methodology (*i.e.* method accuracy) can add to the standard error, and hence, to misleading results. For instance, very pubescent species retain more air on their surface and if the leaf is not fully submerged in water during the rehydration process, the turgid weight after 24h might not be accurate.

To counteract the effects of this variability and to improve accuracy, generally, sample size is increased. In **Chapters 1** and **3**, the aim was to find differences between sun orientations and 30 plants for each condition were sampled. Phytohormones presented a very high inter-individual variability, with some individuals having as little as 2 ng/g DW of OPDA and others having as much as 8 µg/g DW. What would have happened if we had reduced the sample size in the experimental design? That we would have probably included more individuals at either of the extremes, not showing the existing inter-individual variability and leading to "wrong" conclusions about the sampled population, thinking that our sample size was representative of a biological process. On the other hand, for other parameters such as α-tocopherol, the range of which was between 120 µg/g DW and 830 µg/g DW, the consequences of sampling a smaller number of plants would not be "as bad" as in the case of other parameters with higher inter-individual variabilities. From this, it is clear that the improvement in accuracy given by the sample size is trait-dependent, which agrees with Harmon and Losos (2005). But, what sample size to choose when measuring different traits?

In **Annex 1** it was illustrated how the sources of variability influence sample size in an ecophysiological study with *C. albidus,* taking RWC as an example of a functional trait. It was found that a sample size of four was enough to guarantee representativeness of the population. However, if the aim of the study was to compare groups with different means, which is usual in ecology and physiology studies, the sample size needed was n=26. These results would be different if dealing with other parameters, since different traits have different intraspecific (inter-individual and intra-individual) and methodological variability. This paper offered a list of recommendations to improve the accuracy of our estimates, among which there was the performing of preliminary studies to run jack-knife techniques to estimate the minimal optimal sample size based on variance. This would be ideal, but science is generally limited by two very important things: time and funding. These external factors limit the amount and the length of experiments and it is not always possible to perform pilot experiments because of the lack of time and resources. Furthermore, to these limitations, it needs to be added the fact that in an ecophysiological experiment, several traits will be measured to answer our questions. Considering that every trait has a different variability, an analysis should be implemented for each trait, which would on one hand multiply the factor time and on the other hand, result in different minimal optimal sample sizes. Should the highest sample size be chosen? Certainly, the answer to this question is not easy and making decisions on what sample size to choose would depend on too many factors. Doing preliminary studies and analyses might be an advantage for certain experiments such as studies with only one species and where there is a low number of parameters to be measured as well as having only one or two questions to answer. Despite the fact that doing preliminary studies might not be always possible, there are other practices that can reduce non-desired variability (Box 1, **Annex 1**); for instance, quantifying as many environmental and spatiotemporal variables that might influence inter and intra-individual variability, respectively.

In this dissertation all types of variability were evaluated to have the best and most accurate picture possible of *C. albidus* stress response – considering that methodological variability was minimal. Environmental variability (*i.e.* temperature, precipitation, radiation, relative humidity) was considered for all chapters. Inter-individual variability was measured for **Chapters 1, 3** and **4** and intra-individual variability was assessed on a spatiotemporal basis considering (i) different developmental stages (*i.e.* mature plants and seedlings, **Chapters 1, 2, 3** *versus* **4**); (ii) seasonal and diurnal variations (**Chapters 1, 2, 3** and **4**) (iii) a gradient on the stem (*i.e.* leaf positional effects, **Chapter 1**).

3. The threat of severe stress in *C. albidus*

3.1. How tolerant and resilient is *C. albidus* to stress?

Throughout this dissertation different thresholds in the performance of *C. albidus* have been shown. These thresholds are important to define how plastic a species is and to determine its tolerance, resilience, avoidance or sensitivity to a given stress. As described in the introduction of this dissertation, these terms overlap in their occurrence, since, for instance, a plant cannot be an avoidant to all stressors and neither tolerant nor resilient. Furthermore, a species can be an avoidant during a certain period to a certain stress and then tolerant to other environmental changes, such as annual plants, which normally avoid winter stress but then have to face warm summers.

Mechanisms of **tolerance** and **resilience** but also **avoidance** have been found in *C. albidus*. Depending on when the protective response to stress occurs, different lines of defences have been defined for *C. albidus* stress response (Figure 5). Leaf angle together with leaf disposition were mechanisms to avoid excess light (**Chapter 1**), hence a first line of defence in *C. albidus.* As a second line of defence, once ^{1}Chl* has been formed, carotenoids dissipate the excitation energy in the light harvesting complexes to avoid ROS formation by ^{3}Chl* and cell damage (*i.e.* xanthophyll cycle, **Chapter 2**). If ROS are formed, chlorophylls will degrade as a way of reducing the light absorbed, and hence, excess light (**Chapters 1, 2** and **4**). Carotenoids also act as antioxidants scavenging ROS, namely singlet oxygen and the superoxide anion (Dall'Osto et al., 2007; Hauvax et al., 2007; Ramel et al., 2012), which is considered a third line of defence already and a tolerance mechanism to repair damage (**Chapter 2**). In this same line we found other antioxidants such as α-tocopherol that increases in the presence of ROS to scavenge singlet oxygen and that has been found to accumulate under all types of stress (**Chapters 1, 2, 3** and **4**). Scavenging singlet oxygen, we also find PC-8 (**Chapter 2**), which has been shown to act as a complementary antioxidant to α-tocopherol (Kumar et al., 2020), hence acting in the third line of defence but with a secondary role when perhaps the stress is too intense. All these lines of defence are regulated by phytohormones (**Chapters 1, 2, 3** and **4**; Figure 3). All phytohormones have a role in stress tolerance, which allows to define them as tolerance mechanisms. However, attributing their action to one of the lines of defence would be difficult because phytohormones interact between them and form a complex signalling network with other factors that enables plant stress response.

To illustrate the complexion of defining this network of antioxidants and phytohormones and their role as mechanisms of avoidance or tolerance, it is noteworthy the particular case of total chlorophylls as a mechanism of photoprotection. A decrease in chlorophylls could be defined either as a tolerance or as an avoidance mechanism. On one hand, decreasing total chlorophylls reduces the antenna size, therefore reducing the rate of excitation in reaction centres (Walters, 2005), and hence, **avoiding** excess excitation energy, and hence, photo-oxidative damage. This decrease in chlorophylls in response to stress can be triggered either by ABA signalling (Yamburenko et al., 2013) or by direct degradation by ROS, which indicates that the plant is already coping with and **tolerating** excess light and stress, making it harder to discern between avoidance and tolerance. Furthermore, ABA and ROS could also act simultaneously under a sustained stress degrading chlorophyll. Then, altogether, one could conclude that chlorophyll decrease is both an avoidance and tolerance mechanism.

Altogether, it seems that *C. albidus* has a myriad of mechanisms to tolerate abiotic stress but how **resilient** to stress is this Mediterranean shrub? The ability to return to a pre-stress state after a disturbance (*i.e.* stress) is defined as resilience. The method to assess resilience in a species is to see its recovery after stress. Recovery was observed in *C. albidus* mature plants and seedlings in **Chapters 2** and **4**, respectively. In **Chapter 2**, after the cold and drought events in December, RWC, chlorophylls, ABA and CKs returned to their initial values in November. Carotenoids, after the increase in February related to prolonged winter stress, also recovered. Diurnally, α-tocopherol increased during the morning-midday and then, by the evening, levels returned to similar values at predawn. The VAZ pool followed a parallel pattern as vitamin E, but other carotenoids such as lutein and β-carotene increased during the day and did not recover their predawn values. Similarly, GA_1 did not return to its initial values in the evening and PC-8 accumulation remained low at the end of winter and did not return to the high values of late autumn. This seasonal recovery in protective mechanisms in *C. albidus* agrees with the resilience of *C. salviifolius* after severe summer drought (Grant et al., 2014). Total chlorophyll, vitamin E and carotenoids, including β-carotene, lutein and VAZ recovered their initial values after a month of abundant rain. In this study, it was noteworthy that even though undergoing a severe stress, *C.salviifolius* plants always kept their F_v/F_m above 0.75, even though values after recovery did not return to the initial F_v/F_m. Given that in **Chapter 2** most of the protection compounds returned to initial conditions after a combination of cold and water stress, and that during the experiments there was no photoinhibition, it can be suggested that *C. albidus* was also a resilient species under winter stress.

In **Chapter 4**, the recovery of *C. albidus* seedlings to severe drought was tested. *C. albidus* seedlings after 14 days of water restriction survived and presented different drought tolerant mechanisms to achieve this. To further test whether or not in this case seedlings were resilient to severe drought, comparisons between control conditions and the recovered plants were made (Table 3).

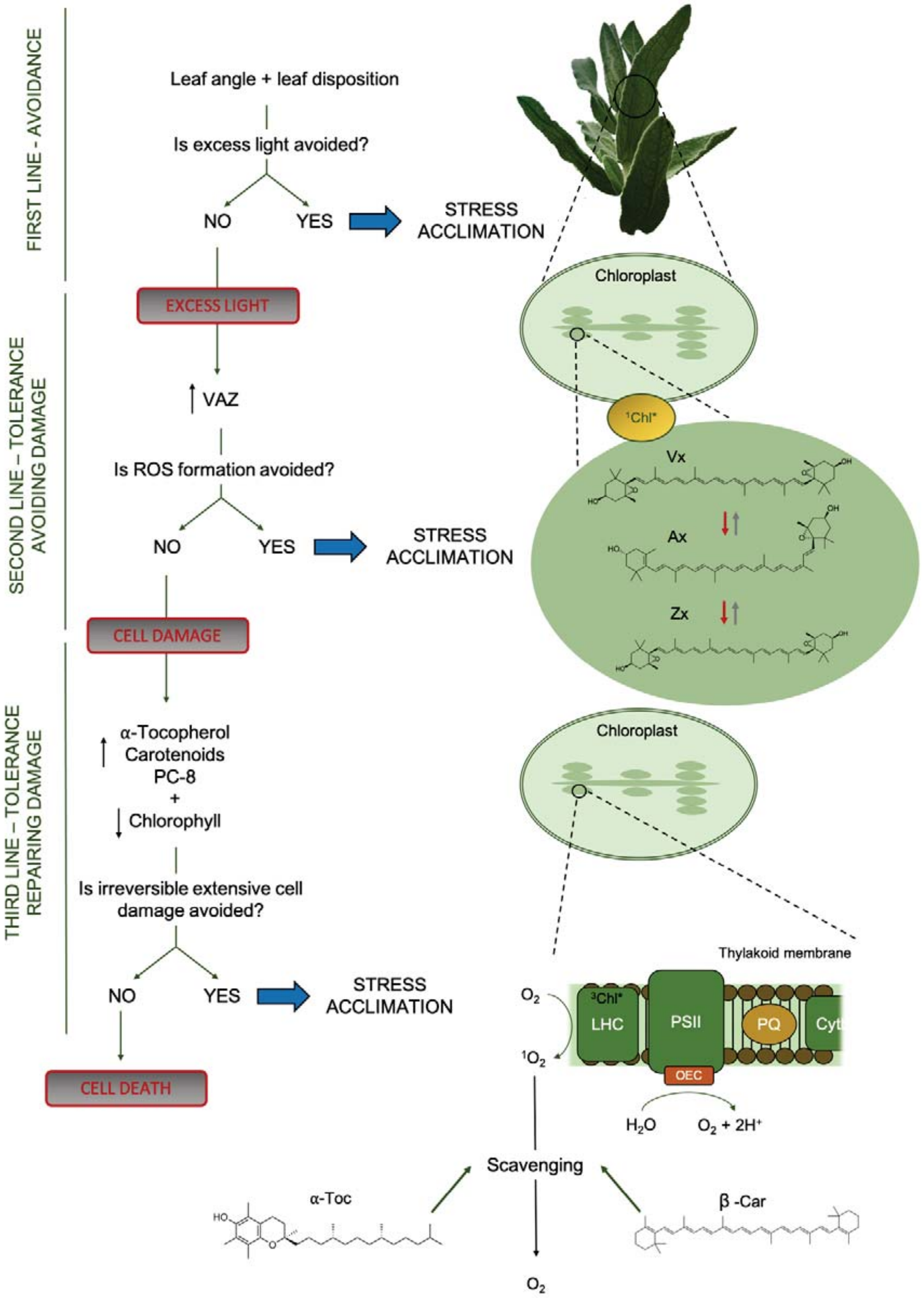

FIRST LINE - AVOIDANCE
Leaf angle + leaf disposition
Is excess light avoided?
NO
YES
STRESS ACCLIMATION
EXCESS LIGHT
SECOND LINE – TOLERANCE AVOIDING DAMAGE
↑ VAZ
Is ROS formation avoided?
NO
YES
STRESS ACCLIMATION
CELL DAMAGE
THIRD LINE – TOLERANCE REPAIRING DAMAGE
↑ α-Tocopherol
Carotenoids
PC-8
+
↓ Chlorophyll
Is irreversible extensive cell damage avoided?
NO
YES
STRESS ACCLIMATION
CELL DEATH
Chloroplast
¹Chl*
Vx
Ax
Zx
Chloroplast
Thylakoid membrane
O₂
³Chl*
LHC
PSII
PQ
Cyt
¹O₂
OEC
H₂O
O₂ + 2H⁺
Scavenging
α-Toc
β -Car
O₂

Figure 5 Protection mechanisms in *C. albidus* in a spatiotemporal scale. The first line comprises structural mechanisms such as leaf angle and leaf disposition. If the first line of defence is not enough, there will be excess light in the chloroplasts. The second line of defence includes the VAZ cycle dissipating thermal excess energy from ^{1}Chl*. If the second line of defence is not enough, ROS will be formed producing cell damage. The third line of defence involves the action of antioxidants such as α-tocopherol and β-carotene, which scavenge singlet oxygen ($^1O_2$), one of the most abundant and easily formed ROS by ^{3}Chl*. ROS will cause membrane damage and the degradation of molecules such as chlorophylls. However, in this case, chlorophyll breakdown will avoid more excess light. If all these mechanisms are not enough, cell death will occur. α-Toc, α-tocopherol; β-Car, β-carotene; ^{1}Chl*, singlet excited chlorophyll; ^{3}Chl*, triplet excited chlorophyll; Ax, antheraxanthin; LHC, light harvesting complex; OEC, oxygen-evolving centre; PC-8, plastochromanol-8; PSII, photosystem II; PQ, plastoquinone; ROS, reactive oxygen species; VAZ, violaxanthin + antheraxanthin + zeaxanthin pool; Vx, violaxanthin; Zx, zeaxanthin.

Parameter	***t***	**df**	***P***
g_s	4.691	5.694	**0.004**
RWC	0.993	4.103	0.376
LMA	-4.514	4.044	**0.010**
F_v/F_m	1.626	4.001	0.179
Total chlorophyll	3.448	7.024	**0.010**
Chlorophyll a/b	2.325	6.937	0.053
α-tocopherol	1.891	5.974	0.108
α-tocopherol/chl	0.122	4.775	0.908
ABA	-1.055	4.131	0.349
SA	-0.871	4.312	0.430
OPDA	0.078	7.999	0.940
JA	2.599	5.694	**0.043**
JA-Ile	-0.860	4.217	0.436

Table 3 Results of the Student's t-Test between well-watered and recovered seedlings of Chapter 4. g_s, stomatal conductance; RWC, relative water content; LMA, leaf mass area; F_v/F_m, máximum PSII efficiency; chl, total chlorophyll; ABA, abscisic acid; SA, salicylic acid; OPDA, 12-*oxo*-phytodienoic acid; JA, jasmonic acid; JA-Ile, jasmonoyl isoleucine.

From all the parameters measured, only four – g_s, LMA, total chlorophyll and JA – did not return to their initial values after four days of rewatering. RWC and F_v/F_m recovered, which indicates that the plants recovered their good physiological status. This agrees with the study of Puglielli et al (2017c) where they show that *C. monspeliensis* recovers its RWC and photosynthetic rates from a 10-day water restriction after three days of rewatering. However, in the other species they tested, *C. salviifolius*, the recovery of these traits took six days instead of three. All phytohormones with the exception of JA also recovered in *C. albidus* after a 14-day water restriction – although the *p*-value of JA was very close to 0.05. This contrasts with Brossa et al. (2015) where they observed that the values of ABA did not recover until after one month of rewatering in *C. albidus*. LMA values did not recover, which is not surprising since LMA is a structural change that takes more than three days to return to well-watered values, particularly around a month (Brossa et al., 2015). Total chlorophyll and g_s did not recover entirely in four days which entices to suggest that these plants kept these parameters at these lower values as a result of stress memory (Savvides et al., 2016), but to test this hypothesis repeated droughts should have been applied. Given the few days after rewatering that were tested and based on the other studies aforementioned, this is probably a result of not having enough days to recover, and these plants were presenting normal instead of an enhanced tolerance. However, Galle et al. (2011) discovered that, after one drought cycle, *C. albidus* plants already presented certain acclimation to stress with higher water use efficiency values. They propose that they achieved this by maintaining a low stomatal conductance together with a high mesophyll conductance, which still enables high rates of photosynthesis. Photosynthesis recovered in this study and in others (Brossa et al., 2015; Puglielli et al., 2017c). This unique mechanism would not only confer drought tolerance but also resilience in *C. albidus*.

From these results, it can be concluded that *C. albidus* is a **tolerant and resilient** species since it presents different tolerance mechanisms that enable its survival and, in most instances, it recovered from this stress returning to a pre-stress state. However, defining a species as tolerant or resilient is not that arbitrary since this resilience depends first, on the type and intensity of stress, and second, on the behaviour of different traits that have different responses: some of them presenting a tolerant response and others a resilience response.

3.2. Could severe stress imposed by global change threaten *C. albidus*?

Shrubs protect maquis habitats from soil erosion. However, desertification caused by the increase in temperatures and drought events in the Mediterranean regions is predicted to increase, potentially threatening these shrublands – particularly seedling recruitment (Lloret et al., 2009) – which are already suffering the effects of global change (see section 1.1, Introduction). An essential question that plant biologists have been trying to answer is "will Mediterranean communities survive?". Mediterranean plants are very plastic in responding to changing environmental conditions (Nicotra et al., 2010) but this plasticity has its limits and tipping points. This plasticity in different traits related to plant morphology and physiology enables the plant to acclimate to stress (Valladares et al., 2014).

Despite *C. albidus* being a tolerant and resilient species, as it has been shown in the previous section, it has limits, since traits are not infinitely plastic and have certain ranges that depend on genetic factors but also on resource partition. In **Chapter 4** the survival of *C. albidus* seedlings throughout a year was monitored to test its vulnerability in natural conditions as well as the thresholds of its trait plasticity. It was found that *C. albidus* seedling survival decreased by a 30% after summer drought (Figure 1, **Chapter 4**). Furthermore, during that year in winter, they had experienced a period of very scarce rains, which was considered a mild drought event followed by a more intense drought in summer. Even though subsequent drought events have been reported to provide *C. albidus* with tolerance and acclimation (Galle et al., 2011), some *C. albidus* seedlings were not able to withstand the harsh dry conditions of the Mediterranean summer. Certainly, summer is a very stressful period for *C. albidus* (Jubany-Marí et al., 2009). Bongers et al. (2017) found, in a study with species from the families Fagaceae, Ericaceae, Cistaceae and Adoxaceae, that Cistaceae species presented a 12-25% decrease in survival after summer drought, with *Cistus albidus* survival decreasing by a 12%.

In **Chapter 4**, it was found that chlorophyll contents in winter were a good proxy of survival after the summer, with seedlings that presented levels below a threshold of 1.87 mg/g DW of chlorophyll dying (Table 1, **Chapter 4**). This threshold was further confirmed for seedlings that survived an intense drought and recovered (Figure 3, **Chapter 4**). It would seem, then, that seedlings that died were not as plastic as seedlings that survived. This plasticity seemed to be conferred by the availability of resources, in this case water, a plasticity that is known as non-adaptive or passive plasticity (van Kleunen and Fischer, 2005; Nicotra et al., 2010). In surviving seedlings in the growth chambers, it was notable the high variability explained by JAs, ABA and SA (Figure 5a, **Chapter 4**), which agrees with the high inter-individual variability of phytohormones found in **Chapters 1** and **3**. While plasticity has been studied for physiological traits such as chlorophyll fluorescence or water use efficiency, there are no studies including antioxidant and/or phytohormone profiles in models predicting Mediterranean species distributions in a changing climate – with the exception of the study of Fenollosa and Munné-Bosch (2019) in the invasive plant *Carpobrotus edulis*. This could be a result of the fact that traits with low variability are the ones that determine species distribution since they are more stable than high-variable traits (He et al., 2018). With the already occurring effects of global change and given that a high trait plasticity is the key to stress acclimation (Valladares et al., 2006), including these traits in predictive models could help to better understand species' behaviour to global change.

In **Chapter 4**, physiological traits of *C. albidus* seedlings showed very wide ranges under drought. The cases of RWC, F_v/F_m, α-tocopherol and phytohormones were especially noteworthy. RWC in surviving seedlings decreased to 36% and then, reached values of 57% after four days of rewatering. These very low values of RWC contrast with the study of Cotado et al. (2020) where they found a RWC death threshold of ~60% in an alpine perennial plant under severe drought stress. Our results agree with Bongers et al. (2017) where RWC in *C. albidus* but also in *C. salviifolius* reached values below 40% and then recovered to control values after rewatering. Seedlings with a F_v/F_m around 0.5 under drought survived but seedlings with values below 0.4 did not. In this case, Cotado et al. (2020) found a higher threshold for their species (0.45) but it is important to note that F_v/F_m *in C. albidus* is quite constant regardless of drought (Jubany-Marí et al., 2009; **Chapter 1, 2,** and **3**). Possibly, the fact that in **Chapter 4** plants had a lower threshold of F_v/F_m might rely on plant age, with seedlings being more sensitive to stress than mature plants (Lloret et al., 2009). α-Tocopherol varied from 45 µg/g DW to 720 µg/g DW in water-stressed seedlings (which did not survive). These wide ranges, besides suggesting a high trait plasticity under these particular circumstances, set thresholds of survival. For instance, reaching the maximum concentration of α-tocopherol did not imply survival.

In fact, seedlings that survived presented a barely variable concentration of α-tocopherol, being the mean 388 µg/g DW, which was very similar to the concentrations found in **Chapters 1** and **2**. These results further suggest that *C. albidus* has a high trait plasticity that allows recovery and acclimation after stress. Certainly, Puglielli et al. (2017a) found a high physiological plasticity in *Cistus* spp. plants that recovered in a short period of time.

From the studies included in this dissertation, it seems that *C. albidus* has a fine-tuned network of **tolerance and resilience mechanisms** to counteract the detrimental effects of chilling stress, light stress and drought. However, due to the limits of its plasticity in some traits, it might not be able to withstand certain intensities of stress. Mortality in *C. albidus* and other congeneric species has been also observed in an especially dry summer, with a rain accumulation of 4.3 mm (Bongers et al., 2017). In the study of Jubany-Marí et al. (2009), even though being a relatively dry summer, *C. albidus* plants did not experience photoinhibition and survived with an accumulated rain of 39.3 mm. Hence, the precipitation threshold for *C. albidus* survival probably lies within the range of 4 and 40 mm during the summer. It is also noteworthy the difference between years, with a 40% decrease in precipitation in 2017 compared to 2009. Certainly, predictions since the beginning of the 21st century stated that precipitation was going to decrease in the Mediterranean basin along with a higher frequency of temperature extremes and, indeed, these predictions are being met (Hanel et al., 2018). Moreover, the last IPCC report (IPCC, 2013) shows that in the next decades dry climates are going to become drier. However, in 2019, when the experimental work of **Chapter 4** was performed, accumulated rain during the summer was 175.8 mm and plants still died, which contrasts with the findings of Bongers et al. (2017). Again, this is probably a result of differences in age since **Chapter 4** was performed in seedlings. Therefore, if summers become drier and drier it is very likely that *C. albidus* survival, especially that of seedlings, experiences a decrease because the tipping points of plasticity will be surpassed. *C. albidus* mortality does not only occur during summer: Oliveira and Peñuelas (2005) also reported mortality in *C. albidus* under cold stress, which indicates that *C. albidus* is not only threatened by summer drought but also by cold events and winter stress. If cold temperatures are extreme and these are coupled with scarce rain, as it has been predicted to occur in the near future (Rojas et al., 2013), *C. albidus* could be threatened by global change, especially seedlings. If recruitment in *C. albidus* populations fails because of seedling mortality, shifts in *C. albidus* populations and plant communities are very likely to occur. In this manner, being *C. albidus* an essential species in Mediterranean shrubland communities, studies of its responses to extreme environmental conditions, either under controlled conditions or in natural conditions under the already occurring effects of climate change (*e.g.* Alderotti et al., 2020 in *C. incanus*), are needed in order to find thresholds of survival and generate data to fit in both survival and distribution models, including morphological and physiological traits.

CONCLUSIONS

- *C. albidus* modulates leaf angle on a diurnal and seasonal basis as a first line of defence, preventing photoinhibition.
- Enhanced α-tocopherol contents help withstand combined high light with low temperature stress in *C. albidus* growing at high elevation.
- Leaf angle and α-tocopherol form an effective fine-regulated photoprotection mechanism together with an adequate orientation of decussate leaves as part of the developmental program to face high light conditions.
- α-Tocopherol and carotenoids are key photoprotective compounds in *C. albidus,* avoiding oxidative damage under cold and water stress.
- ABA has an important role in stress responses together with *trans*-zeatin, both protecting the photosynthetic apparatus from extensive damage.
- α-Tocopherol and xanthophylls seem to be governed in part by diurnal rhythms, hence having a main role under high light, accumulating in the morning to face the most stressful conditions during the day at midday.
- Sun-orientation, even though not being different in light intensity at midday, results in a differential accumulation of stress hormones, including jasmonates, and other chemical defences.
- A trade-off between salicylic acid and jasmonates accumulation exists under certain stress conditions.
- Inter-individual variability, especially that of jasmonates, is high in *C. albidus* growing at high elevation.
- *C. albidus* seedlings are vulnerable to summer drought, presenting a decrease in survival of 30% in natural conditions.
- Total chlorophyll is a good long-term proxy of seedling survival in natural conditions.
- The modulation of vitamin E and jasmonates seems to be key for *C. albidus* drought response.

- *C. albidus* faces cold, light and drought stress during the Mediterranean winter and, even though it experiences all these stressors at once, activates different lines of defences to avoid photoinhibition.
- Cold stress and drought are the abiotic stresses that activate more protective mechanisms in *C. albidus.*
- A decrease in chlorophyll contents is a photoprotection mechanism normally activated by *C. albidus* to prevent too much excess light under cold stress and drought.
- OPDA seems to have a differential function under abiotic stress in *C. albidus* to that of other jasmonates, having a strong diurnal rhythm and accumulating in surviving seedlings.
- *C. albidus* has a great inter-individual variability in both seedlings and mature plants, particularly that of phytohormones, which is key to understand plant responses to stress.
- The combination of high, intermediate and low variable traits allows *C. albidus* to respond to a high heterogenous environment.
- *C. albidus* is highly abiotic stress tolerant and resilient, since it can return to a pre-stress state, thanks to its big fine-tuned network of protective mechanisms that are activated in a spatiotemporal manner. However, not all protective mechanisms present the same resilience and under some instances, *C. albidus* is a sensitive plant.
- Seedling survival, and hence populations of *C. albidus*, could be threaten by global change if temperatures become more extreme and more intense drought events occur.

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ANNEX

Annex 1. What is the minimal optimal sample size for plant ecophysiological studies?

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Letter to the Editor

What Is the Minimal Optimal Sample Size for Plant Ecophysiological Studies?

Dear Editor,

Although significant advances have been made toward elucidating the most appropriate sample size for studies in many fields (Meere and Mulchrone, 2003; Fiske et al., 2008; McDonald, 2008; Pérez-Harguindeguy et al., 2013; Hajian-Tilaki, 2014), choosing the minimal optimal sample size in plant physiology remains a challenge. This is particularly true for ecophysiology (i.e. research carried out in the field using physiological techniques), where both top-down and bottom-up approaches are required to understand not only the responses of individuals but also those of populations and ecosystems to a variety of environmental factors such as high temperature, drought, or salinity (Leroux and Loreau, 2015). Achieving high sample sizes is limited by both internal (e.g. intraspecies variability) and external factors (e.g. time, human capital, and funding). Here, we discuss the importance of key internal factors constraining sample size, individual heterogeneity, sample size representativity, and context-dependent variability, to provide suggestions to ascertain the minimal optimal sample size that is compatible with hypothesis testing in plant ecophysiological studies. To illustrate our narrative, we employ the widely used functional trait relative water content (RWC).

The precision of any estimate is affected by its sampling variability, as well as by process variability, including environmental, inter- and intraindividual, and methodological variability (White, 2000). These sources of variability tend to be closely linked (Messier et al., 2010) and can influence the trueness of the measurement, thus constraining the statistical power necessary to detect potential intergroup significant differences (Bean et al., 2012). Although maximizing intraspecific sample size generally improves statistical power, the improvement is typically species- (Fiske et al., 2008) and trait-specific (Harmon and Losos, 2005), and may be limited by external factors such as time and funding.

When considering process variability (i.e. the combined effect of demographic, spatial, temporal, and individual variability), not only intraspecies but also inter- and intraindividual variability must be considered. Indeed, each species is characterized by a distinct array of functional trait values (Violle et al., 2007), which may vary at spatial and temporal scales (Messier et al., 2010). Individual differences may be

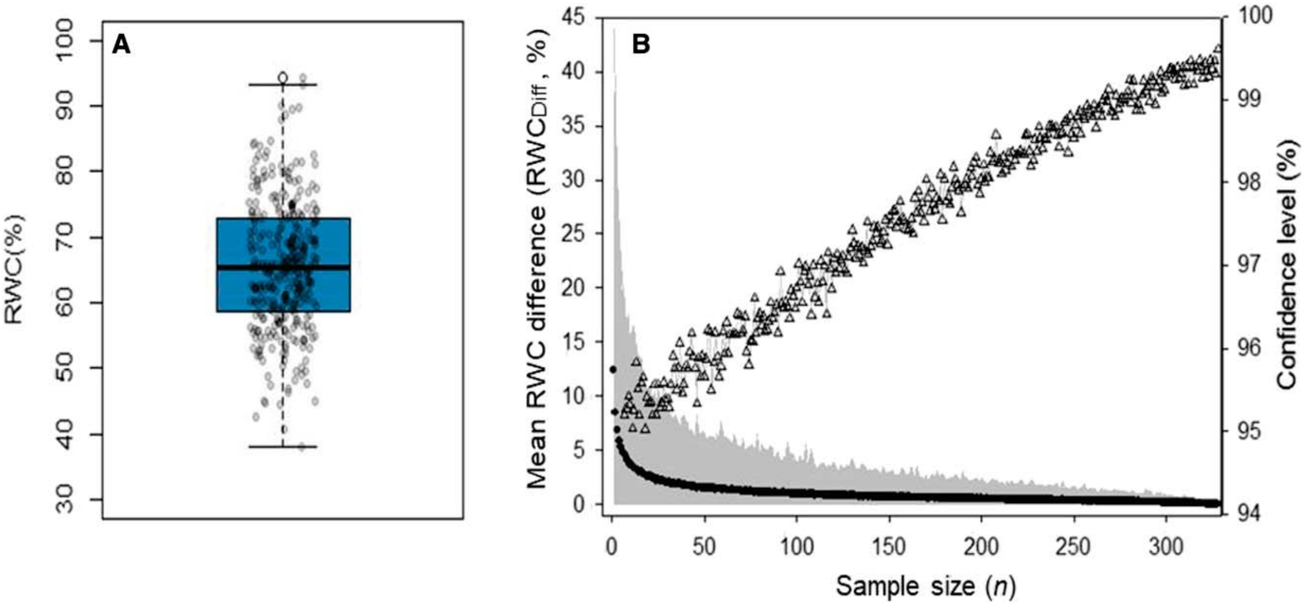

Figure 1. Sample size representativeness of RWC in a population of 328 individuals of *C. albidus*. A, Boxplot and scatter plot of the RWC data. The boxplot shows the median of the data between the first and the third quartiles; whiskers indicate the 10th and 90th percentiles. B, Percent mean difference between our population (*n* = 328) and a population of variable sample size (*n* = 1…328) after 10,000 iterations at each sample size (black dots). A sample size of *n* = 14 corresponds to the inflection point (estimated via the segmented function from the SEGMENTED R package). The gray area represents the minimum and maximum mean differences between the populations, and white triangles represent the confidence level, that is, the percentage of iterations in which the compared samples were found nonsignificantly different among 10,000 iterations.

BOX 1. Recommendations to accurately determine the minimal optimal sample size of ecophysiological measurements in natural populations and to improve the quality of in situ estimates:

- Run a pilot experiment on a reduced sample size and carry out power analysis (McDonald, 2008). The results of the power analysis will report how large the sample size should be.
- Implement jack-knife techniques on your data to estimate the inflection point of variance, which indicates the sample size beyond which increases in sampling effort do not significantly improve accuracy (See Figure 2).
- Whenever possible, quantify and report the variance components associated with sampling (i.e. spatial, temporal, and inter- and intraspecific variability), as governed by the experimental design.
- Endeavour to reduce methodological variability. This can be achieved by having only one highly trained person collecting the data, or training and centralising a team of fieldworkers, such that data are collected from individual plants within the same environmental and temporal conditions, and in the protocol and instruments.
- Take advantage of background noise; do not ignore it! Environmental conditions can vary from one day to the next, and harnessing and quantifying that variation can actually help contextualize the measured values (e.g. when all data are collected under a clear, sunny day after a month of drought, vs. a clear, sunny day but after a day of rains).

In conclusion, although the choice of sample size is usually constrained by external factors (e.g. funding, time, and portable, fast technology), researchers must carefully keep in mind the magnitude of the effects of internal factors (e.g. environmental variability, inter-individual variability, and method accuracy) on the power of analysis conferred by those sample sizes. This is particularly important when researchers account for the total variability that may constrain sample representativity and statistical power to optimize experimental designs and report results that are in fact most representative of natural settings. For instance, in terms of the representativity of an estimate of RWC in our natural population of *C. albidus* (a situation that also may be relevant to other traits and species), a minimal optimal sample size of four may suffice to obtain a good proxy for the actual value in natural settings, but differences between populations are not detected unless more than 20 individuals are sampled in a single time point: $n = 26$ in our case study. Therefore, we recommend carefully considering the effects of sampling size in ecophysiological studies to best depict phenomena in nature. We urge the scientific community to go well beyond the standard of $n = 3$ individuals still found in several recent examples in the literature and to carefully consider the sampling size adequate for each functional trait and species. To that end, we provide a few recommendations in Box 1.

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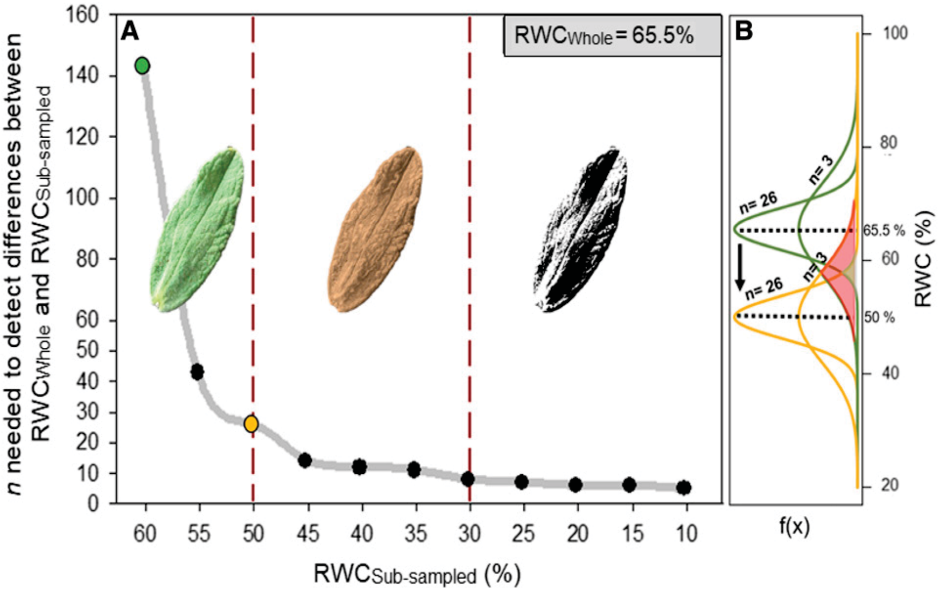

Figure 2. Sample size required to detect differences in RWC between subsampled populations of *C. albidus* (see Fig. 1) compared to the whole population containing 328 individuals. A, Dots represent the sample size necessary to detect significant differences at a $P < 0.05$ in RWC between a whole population ($n = 328$, RWC_{Whole}: 65.55% ± SE = 0.54) and subsampled populations with different mean values, as represented by the *x* axis, while keeping the SD constant. Dashed vertical lines at RWC 50% and 30% represent severe water stress and the point of irreversible water loss, respectively. Sampled subpopulations were performed using 10,000 iterations. RWC_{Whole} is the mean RWC of the whole population, and $RWC_{Subsampled}$ is the mean RWC of the subsampled populations. B, Comparison of the whole population to a subsampled, simulated population ($RWC_{Subsampled}$: 50%) with two different sample sizes representing these populations (n = 26 and 3). The red-shaded area represents $P > 0.05$, whereas the green-shaded area represents $P < 0.05$).

detected by sampling among different individual plants, whereas intraindividual differences may emerge when sampling within the same individual at different spatiotemporal scales. For instance, Valladares et al. (2000) found significant differences for structural and physiological leaf traits (including leaf mass per unit area, photosynthetic capacity, and root:shoot ratio, among others) across a light gradient in growth chambers. Most recently, Aguilar-García et al. (2018) found a great deal of variation in floral traits as a function of their position around individuals of *Myrtillocactus geometrizans.* These findings emphasize the fact that sample size may affect the accuracy of estimates of ecophysiological traits (White, 2000; Bean et al., 2012). Furthermore, increased variability in the trait of interest (e.g. due to high phenotypic plasticity; West-Eberhard, 2003) may compromise the sample representativeness in a complex way.

To illustrate how the sources of variability influence the sample size in an ecophysiological study, we quantified RWC across 328 individuals of *Cistus albidus* (Cistaceae) in four sites, located more than 300 m apart from each other, within a natural population in Spain (41.589N, 1.835E, 987 m a.s.l., Spain) from July 4 to 14, 2014. When defining our population as all 328 individuals, the mean RWC_{Whole} was μ = 65.55% ± 0.54 SE (Fig. 1A). Indeed, although we found a great deal of individual variance, no significant differences in RWC were found as a function of sampled day and location (generalized linear model; site: $F_{df = 3} = 1.01$, $P > 0.05$; day: $F_{df = 4} = 0.61$, $P > 0.05$). To understand the relationship between intraspecific variability, sample size, and representativeness, we subsampled these data 10,000 times for increasing sample sizes ($n = 1, 2, 3\ldots328$), and then we contrasted the obtained mean value ($RWC_{Subsampled}$) with the sampled whole-population mean (RWC_{Whole}). The mean RWC difference ($RWC_{Diff} = RWC_{Whole} - RWC_{Subsampled}$) between each of these simulated subsampled populations and the whole population for each sample size was calculated to determine the asymptote in this relationship, whereby no increase in the marginal benefit was achieved by increasing sample size (Fig. 1B). In spite of the fact that the inflection point in Figure 1B corresponded to $n = 14$, a sample size of four individuals was enough to guarantee sample representativeness at an accuracy of 95%. Strikingly, however, to achieve an accuracy of 99%, a sample size of 279 individuals was needed (Fig. 1B). Furthermore, in studies where multiple comparisons exist, larger sample sizes are required. As an example, we show that a sample size of 26 individuals would be required to detect significant differences between two populations with means of 65.55% of RWC and 50% at an accuracy of 95% (Fig. 2).

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