Universitat de les Illes Balears

# MASTER'S THESIS

# TRADE-OFF BETWEEN PHOTOSYNTHESIS AND STRESS TOLERANCE: THE ROLE OF FOLIAR ANATOMY AND THE ANTIOXIDANT SYSTEM


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**Master's Degree in Applied Biotechnology**

**(Specialisation/Pathway *Environment*)**

**Centre for Postgraduate Studies**

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Key words:

Stress tolerance, trade-off, dehydration tolerance, freezing tolerance, maxima photosynthetic capacity.



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## ABSTRACT

Humanity has prioritized the selection of characters that allowed greater productivity since the domestication of crops approximately 12,000 years ago. However, considering the threat of climate change for agriculture, genetic improvement no longer only considers the improvement of productive traits but also of those that determine tolerance to stress. There is a generalized hypothesis that argues that productivity and stress tolerance follow an inverse relationship, that is, as productivity increases, stress tolerance decreases and vice versa. Considering the increase in the world population, it is predicted that being able to feed all the people of the planet is the greatest biotechnological and socioeconomic challenge of recent times. Since the resources available for plants are finite, the carbon assimilated from the environment through photosynthesis can be invested in plant growth, reproduction or defense in a highly balanced manner to maximize plant fitness. Additionally, maximum photosynthetical capacity ($A_{max}$) is driven by investments in leaf anatomy and the photosynthetic metabolism, these investments can also compete with resources needed for plant stress tolerance as well at anatomical level the antioxidant metabolism. Both levels define stress tolerance, as well as the photosynthetic process itself. In this way, in this TFM we proceeded with the characterization of 16 species of plants from different environments of the world, with some from the most extreme environments on the planet, such as the Arctic or Antarctica. A series of growth or photosynthetic measurements were carried out under optimal conditions for each of the species: photosynthetic measurements, freezing test, desiccation test, anatomical characterization, and different biochemical parameters. We observed that polar species usually tend to obtain higher stress tolerance values than "Mediterranean" and "Model & Crops" species, but however their photosynthetical capacity was lower than the others. Our results showed a new trade-off between $A_{max}$ and dehydration tolerance, which are related with anatomical parameters as $S_c/S$ and $T_{chl}$. Interestingly, we did not find a trade-off relationship with biochemical parameters as chlorophyll, flavanols and anthocyanin contents. Altogether, these results open the opportunity to establish the relationship between productivity and stress tolerance, which continues to be a global biotechnological challenge today.

## INTRODUCTION

### "Growth-defense trade-off"

In nature, plants live in complex and diverse environments with a wide variety of pathogens and insect herbivores. Considering that plants are sessile organisms, they have to deal with a whole set of abiotic and biotic factors, such as dry spells or different infection strategies, respectively (Huot et al., 2014). In that way, they need to keep growing with a constant adaptation to their external environment in order to survive and reproduce (Coley et al., 1985; Herms & Mattson, 1992). Plants have a limited pool of resources that can be invested in growth, defense and reproduction. Restrictive resources could lead to the inability to meet growth, defense and reproduction demands, simultaneously (Tuller et al., 2018). According to the "growth-defense trade-off" (figure 1) plants could face resource restriction by making 'choices': 'to grow or defend' (Hanley et al., 2007); understanding as "trade-off" '*a restriction imposed by the aptitude in two competing functions, which results in negative association patterns between the functions*' (Züst & Agrawal, 2017)**.** Additionally, it is important to keep in mind that these physiological trade-offs could not just reflect an inverse relationship between two processes that compete for the same resource, their consequences could operate at the whole individual, affecting plant fitness (the number of successful offspring in future generations) (figure 1). These adaptive trade-offs are considered major drivers in response to environmental heterogeneity leading divergent evolution (Haak et al., 2011). The "growth-defense trade-off" was first observed in field studies of plant-insect interactions (Huot et al., 2014)**.** Plants must grow and defend themselves to survive and reproduce, so the trade-offs between growth and defense have important agricultural, ecological and economic consequences (Huot et al., 2014)**.** Nevertheless, crops have been improved over time focusing on growth-related and carbon partition traits in order to maximize productivity, without considering the consequent loss of genetic diversity that could compromise defense (Strange & Scott, 2005)**.** In this way, understanding the molecular mechanisms of plants involved in the balance between growth and defense can improve their reproduction strategies for the selection of genetic traits that maximize their fitness (Huot et al., 2014).

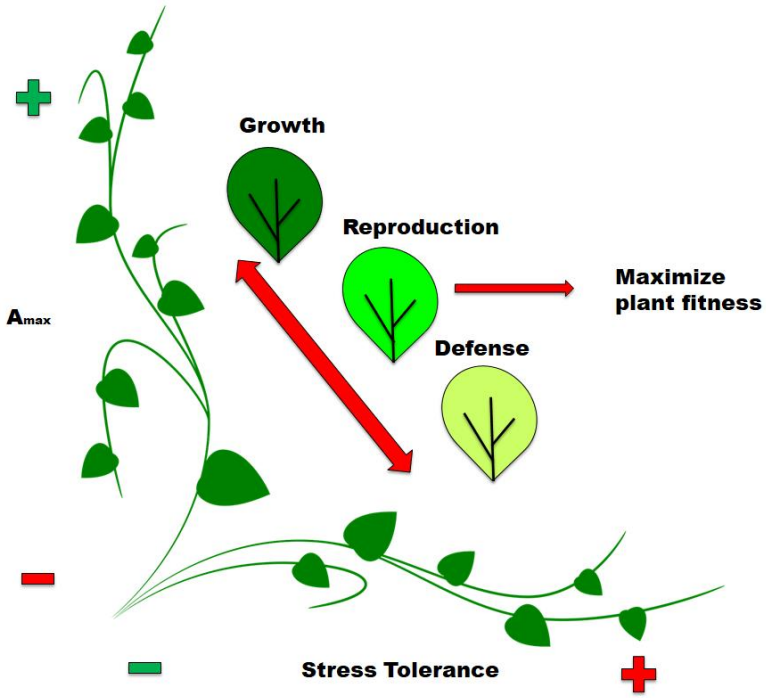


***Figure 1. Theoretical hypothesis of the trade-off between the maximal photosynthetic capacity ($A_{max}$) and stress tolerance.*** *Stress tolerance is plotted on the X axis and $A_{max}$ on the Y axis. The investment balance between growth, reproduction and defense, allows survival and tends to maximize the plan fitness. $A_{max}$: maximal photosynthetic capacity.*

Growth of vegetative tissue is a key physiological process for the overall plant's development. Plant growth can be determined, among others, by traits related to photosynthetic function and nutrient acquisition, such as leaf mass per unit area (*LMA*), leaf lifespan (*LL*), $CO_2$ assimilation rate ($A_N$) and N content, among others (Nadal et al., 2018). They are tightly intercorrelated across growth forms (Wright et al., 2004; 2005). These leaf traits depend on the conversion capacity of primary metabolites in the different cellular components. This conversion depends on metabolomic rates, which is limited by source-sink interactions. Source activity refers to the speed at which the plant makes essential external resources available to it. On the contrary, the sink activity consists in the storage capacity of resources in specific organs for a future use. Source-sink activity is highly regulated by a whole set of mechanisms (White et al., 2016; Züst & Agrawal, 2017). High-resource environments tend to favour fast-growing plants, but poorly defended; while low-resource environments tend to favour slow-growing plants, but well defended (Heckman et al., 2019). So, undesirable external factors for the plant, such as drought conditions, an excessively high temperature or herbivores attacks, set in motion a whole cascade of mechanisms that promote the redirection of energy and components of the primary

metabolism to the defense against the threat, which could compromise growth and reproduction; and therefore, compromise plant fitness (Züst & Agrawal, 2017). In this way, defense level depends on the degree of damage that the plant receives. Accordingly, it is known that divergent mechanisms lead to negative correlations between growth and defense (Karasov et al., 2017). This can be determined by studying **resistance** and **tolerance** mechanisms of the plant. Resistance refers to the ability to decrease damage caused by pathogens or herbivores, while tolerance refers to the ability to reduce the impact of damage on plant performance (Cronin et al., 2015).

A trade-off between two functions (growth and defense traits) can be studied at least at three different levels, non-mutually exclusive: (1) allocation costs, (2) genetic costs, and (3) ecological costs or energy limitation (Züst & Agrawal, 2017). Allocation costs refers to trade-offs based on reductions in growth or reproduction because of the redirection of limiting resources to defense processes. Direct competitive resource limitation between growth and defense may be the most commonly considered causal factor of trade-offs (Carmona & Fornoni, 2013; Pilson, 2000; Wise & Evolution, 2016), but it can also be a simplistic form to reflect the complex plant allocation processes (Züst & Agrawal, 2017). Moreover, genetic costs refer to trade-offs between growth and defense based on the study of the underlying genetic variation of traits. Differential expression of a single gene can lead to the modulation of different traits due to pleiotropy. There are a large number of studies of specific defensive traits of interest (Agren & Schemske, 1993; Kakes, 1989; Paul-Victor et al., 2010). Finally, ecological costs refer to the study of defense effects of a plant after being exposed to ecological interactions, such as negative impacts on mutualists or greater susceptibility to certain herbivores. For example, the interaction with a generalist herbivore, can activate a whole series of defense mechanisms that prevent its interaction with mutualists, such as pollinators, which could have its effect in a reduction of seed production (Strauss et al., 1999). In addition, defense against a generalist herbivore could lead to susceptibility to other specialist herbivores (Agren & Schemske, 1993; Giamoustaris & Mithen, 1995; Halkier & Gershenzon, 2006).

**Trade-off between maximal photochemical capacity and stress tolerance**

This work aims to study a specific trade-off: the maximal photosynthetic capacity ($A_{max}$) - Stress tolerance trade-off (figure 1). Photosynthesis ($A_N$) is a complex biological process in which organic matter is obtained from inorganic matter (Azcón Bieto & Talón, 2008). Before starting this process, atmospheric $CO_2$ has to enter into leaves by simple diffusion through the stomata (figure 2). This process is regulated by stomatal conductance ($g_s$), which is a function of foliar stomatal density, the opening level of stomata and its size. In this way, a certain $CO_2$ concentration ($C_i$) is achieved in the substomatal cavity inside the leaf. The $CO_2$ follows its way by apoplastic and simplistic pathways through mesophyll cells to the carboxylation sites of the Ribulose-1,5-bisphosphate carboxylase oxygenase (RUBISCO), in the stroma of the chloroplast, where the dark phase of photosynthesis takes place and, therefore, the carbon assimilation that has entered. In this path, the mesophyll conductance ($g_m$) is established, which determines the $CO_2$ concentration inside the chloroplast ($C_c$). $g_m$ is limited by a whole series of resistances of air, water and biophysics (cell walls and membranes) that compromise $C_c$. In addition, there is also an active regulation of $g_m$ by different enzymes, such as carbonic anhydrase and aquaporins (transmembrane proteins) (Flexas et al., 2016; Flexas et al., 2012; Gago et al., 2014). In addition, stomatal limitation ($l_s$) and mesophyll limitation ($l_m$), $C_c$ is also determined by biochemical limitation ($l_b$). It refers to the $CO_2$ rate consumption in the photosynthesis process, both by thylakoid electron transport rate (ETR), the maximum carboxylation rate of RUBICO ($V_{c,max}$) and the ribulose 1,5-bisphosphate (RuBP) regeneration in the Calvin cycle (Flexas et al., 2016)**.**

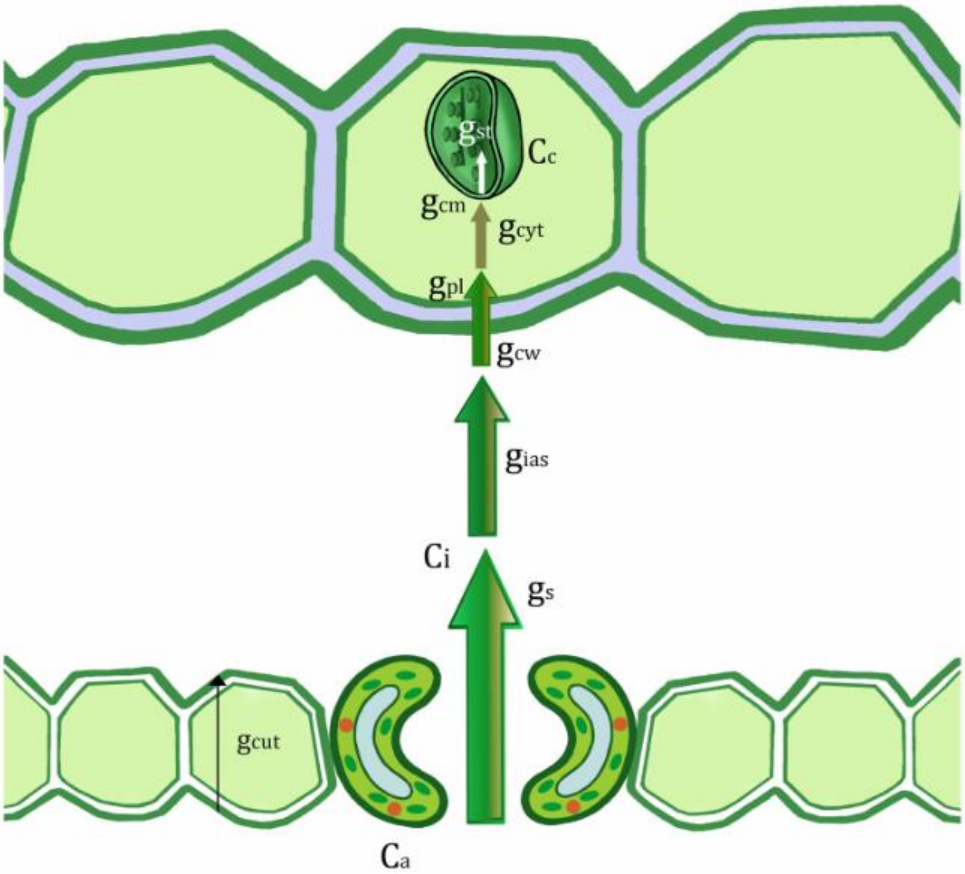


***Figure 2. Path of $CO_2$ to the carboxylation site.*** *From the atmosphere, $CO_2$ ($C_a$) diffuses through the guard cells of the stomata ($g_s$) into the sub-stomatal cavities at a certain concentration ($C_i$). Then, $CO_2$ crosses a series of biophysical barriers in the gas-phase ($g_{ias}$) to the outer surface of the mesophyll cell walls and diffuses in the liquid phase through the cell wall ($g_{cw}$), the plasma membrane ($g_{pl}$), the cytoplasm ($g_{cyt}$), the chloroplast membrane ($g_{cm}$) and the chloroplast stroma ($g_{st}$) to the carboxylation sites ($C_c$) of the Rubisco to perform photosynthetic process (taken from Gago et al. (2020)).*

### Has $A_N$ been studied from a trade-off point of view?

$A_N$ has been studied previously under different perspectives to assess putative trade-offs. It has been shown in the negative relationship between *LMA* and the light-saturated photosynthetic rate per unit leaf dry mass ($A_{mass}$), which is attributed to thicker mesophyll cell walls (Yusuke Onoda et al., 2017). Otherwise, a novel inverse relationship between $A_N$ and the bulk modulus of elasticity (ε) has been reported recently for vascular plants (Nadal et al., 2018). ε is a structural feature that represents the elasticity of leaf tissues (Bartlett et al., 2012) and is related to the ability of leaf tissue to resist drought in sclerophylls (Nadal et al., 2018). The results showed the incompatibility of a high $A_N$ (influenced by $g_m$) with rigid leaves. Furthermore, He et al.,

(2019) have shown a possible leaf mechanical strength-photosynthetic capacity trade-off, as traits related to tolerance and growth, respectively, across 57 subtropical forest species. Leave mechanical strength was determined as leaf force to tear. Nonetheless, they found only weak support for the proposed trade-off. Therefore, they concluded that observed relationships could be more the result of different traits coordination than any physiologically or physically enforced trade-off. $A_N$ has also been involved in the N allocation to cell walls-photosynthetic capacity inverse relationship (Onoda et al., 2004) in *Polygonum cuspidatum* Sieb. *et* Zucc. Given that N is a limiting resource, the more N allocated in cell walls, the less N available for photosynthetic enzymes.

**What do we understand by Freezing and Drought Stress Tolerance?**

Stress is understood as an external factor to the plant, whether biotic or abiotic, that exerts a negative influence on its optimal development. Faced with a certain stress, plants have different protection mechanisms: avoiding stress or increasing tolerance to such stress. Both are defense mechanisms of plants that allow their survival against adverse environmental conditions. The first one refers to the set of mechanisms that prevent the plant from being subjected to stress, such as the prevention of extrinsic or intrinsic ice formation in the case of freezing stress. Whereas stress tolerance refers to the set of coordinated physiological and biochemical tools that plants have as a defense mechanism to reduce the impact of damage on plant performance, for example the increase in the metabolites concentration in the case of freezing stress (Román-Figueroa et al., 2021). In this sense, tolerance to stress makes it possible to overcome unfavourable environmental conditions (Azcón Bieto & Talón, 2008).

One of the most important stressors affecting crop production is low temperature (Matzneller et al., 2016). Despite the great advances in research to reduce the impact of damage caused by low temperature, this continues to be one of the main causes of loss of production, in volume and quality of the fruit, which could mean devastating economic losses in the agricultural sector (Beyá-Marshall et al., 2019). It is for example the drop in temperature to below zero values in April 2021 in Manacor (Mallorca), which caused the loss of more than 800 tons of apricot and plum fruit in the company Terracor, one of the main producers of vegetables and fruits in the zone. The losses could exceed eight hundred thousand euros, according to one of those responsible,

Maties Adrover. These losses may have an even greater impact on other regions, such as the reduction of winter wheat production by 19%, peaches by 75%, apples by 67% and walnuts by 66% due to a frost in the spring in 2007 in the central and eastern United States, which meant an economic loss of more than two billion dollars (Liu et al., 2018).

In a climate change scenario, it is estimated that the frequency and duration of frosts may be modified, but their behaviour is still uncertain. It seems that the pattern is different depending on the bioclimatic zone. In the northern hemisphere a decrease in the frequency of frost was observed between 1980 and 2000, especially in northern Siberia, North America and the Tibetan Plateau. Nevertheless, in Europe and Central North America it increased, especially in spring (Román-Figueroa et al., 2021).

The drop in temperature is associated with the freeze point of the water (0ºC, 32ºF). Low temperatures can induce chilling as they drop to 0 °C. While temperatures below zero, can produce frost and freeze, which are often used interchangeably, but they are two different facts. Both concepts refer to physical phenomena that involve a change from liquid to solid phase of water. However, frost refers to the formation of ice crystals on the surface of the soil or foliage, which leaves a white layer of icy water. So, it doesn't have to involve cells freezing within the plant. On the contrary, freezing refers to the freezing of water inside the leaf, so it can occur without the formation of ice crystals (frost) (Smith, 2019).

Freezing temperatures can damage plants due to different mechanisms. First of all, extracellular ice formation leads to cell dehydration to maintain the water pressure balance between the inside and outside of the cell, which induces the contraction of cells, decreasing distances between membranes. At the same time, the growth of extracellular crystals causes a cellular mechanical deformation that can drive in cell lysis. All together results in increased membrane damage and cell death (Fujikawa et al., 1999; Nagao et al., 2007). On the other hand, freezing temperatures can influence photosynthetic efficiency, promoting a situation of photoinhibition and, therefore, a lower capacity for assimilating sunlight, which results in a situation of oxidative stress and direct damage to photosynthetic components (Román-Figueroa et al., 2021).

Water stress is another abiotic environmental stress that affects plant growth most seriously (Shinozaki & Yamaguchi-Shinozaki, 2000) and leads to substantial loss in

food production. According to the agricultural organization UPA, farmers suffered losses of 1.6 billion euros in the first five months of 2017 due to drought. Only at the Balearic level, in 2000, the president of the Balearic Agricultural and Livestock Federation (FAGB) exposed the loss of 1.67 billion euros in herbaceous crops, 995 in almonds, 164 in carob, 800 in forages and 50 in legumes; a total of 3.68 billion euros in the Balearic agricultural sector due to the drought.

"Drought, dehydration or desiccation" are often used to describe the level of water stress in plants. But a clear distinction must be made between these three concepts in order to make a comparative analysis in terms of morphological, physiological and molecular responses. Drought is often used to describe water deficit. It is a slow process during which water absorption cannot compensate for excessive water loss through transpiration. Dehydration is the process by which whole plants or detached organs suffer a constant loss of water. Finally, desiccation occurs when the state of the water is balanced with air, therefore is the final step of dehydration. In this manner, desiccation is the same as extremely dehydrated (Zhang & Bartels, 2018)**.**

Dehydration stress is induced by high salt, drought and freezing conditions (Bajaj, Targolli et al., 1999)**.** Desiccation tolerance (DHT) refers to the set of defense mechanisms of plants that allow balancing their internal water potential with that of the environment, followed by recovery after rehydration, in order to reduce the impact of desiccation damage on plant performance (Verhoeven et al., 2021a).

**Foliar traits driving the trade-off**

As the resources available to plants are finite, the carbon assimilated from the environment through photosynthesis can be invested in characters that favour photosynthesis or stress tolerance; both processes directly depend on the leaf anatomy and its biochemistry/antioxidant metabolism. Understanding the mechanisms involved in this relationship is essential to get a better understanding of this theoretical trade-off. Anatomical and biochemical determinants present a wide range of variation between species or even genotypes, which partially explains the differences in their photosynthetic capacity (Carriquí et al., 2020, 2019; Peguero-Pina et al., 2017)**.**

The general relationship between leaf anatomy and $A_N$ is well established (I. J. Wright et al., 2004). Subsequent studies focused on the mechanisms that relate anatomical

features to $A_N$ through $g_m$. $g_m$ refers to $CO_2$ conductance/diffusion from substomatal cavities to the carboxylation sites of RUBISCO in the chloroplast stroma (Clemente-Moreno et al., 2019). On certain occasions, mesophyll conductance could be the most significant photosynthetic limitation, depending on the plants and environmental conditions (Flexas et al., 2012; Gago et al., 2020). $CO_2$ needs to cross different barriers along the mesophyll to reach the carboxylation site: apoplast, plasma membrane, cytoplasm, chloroplast membrane and stroma. Therefore, the $g_m$ depends on total diffusion efficiency through each component in the liquid and gas phase between the intercellular air spaces and the carboxylation sites (Gago et al., 2020). Unfortunately, methods are lacking to quantitatively verify the conductance associated with each of these barriers (Evans, 2021) and the biophysical diffusion properties of the diffusion pathway components that influence $g_m$ have not yet been fully elucidated (Marc Carriquí et al., 2020; Flexas et al., 2012). It is known that at least three properties determine the conductance to $CO_2$ diffusion of each of the different components (Gago et al., 2020)**.** First of all, $CO_2$ diffusivity in the liquid phase is at least $10^4$ times lower than the intercellular gas phase (Carriquí et al., 2019; Flexas et al., 2021; Gago et al., 2020). Additionally, the length of the diffusion path must be considered, the longer it is, the more it will cost the $CO_2$ to reach the stoma. The last property that determines the $CO_2$ conductance through the mesophyll and, therefore, related to $A_N$, is the cell wall resistance. The last one, in turn, can depend on at least two physical wall properties: thickness and effective diffusivity (porosity and tortuosity) (Carriquí et al., 2020; Evans et al., 2009; Gago et al., 2020); as well as the presence of mediators such as aquaporins (AQP) and carbonic anhydrases (CAs). However, the biophysical diffusion properties of the diffusion pathway components that influence $g_m$ remains elusive (Carriquí et al., 2020; Flexas et al., 2012).

In recent years there has been a great effort to characterize $g_m$ in different plant species (Flexas, 2018) and the importance of differences in the anatomical properties of leaves over differences in maximum $g_m$ between species has been established ( Flexas & Carriquí, 2020). Plant anatomical studies have suggested that anatomical features, such as chloroplast surface exposed to mesophilic air spaces ($S_c/S$) and cell wall thickness (*CWT*), are among the strongest determinants of mesophilic conductance (and also from $A_N$) (Carriquí et al., 2019; Terashima et al., 2011; Tosens et al., 2016)**.** Increasing $S_c/S$ and decreasing *CWT* result in increasing $g_m$ (Carriquí et

al., 2020, 2019; Tosens et al., 2016). Whereas the cytosol between plasma and chloroplast membranes plays only a minor role in $g_m$ limitation, because chloroplasts tend to align closely to cell walls (*CW*) in bright light conditions to reduce the $CO_2$ effective pathway (Evans et al., 2009; Gago et al., 2020). On certain occasions, rapid variations in response to environmental changes could be regulated by other factors such as the conductance of aquaporins and carbonic anhydrases (Flexas et al., 2016). However, cases have been reported in which chloroplasts shed the plasma membrane, which causes a decrease in $g_m$ (Gago et al., 2020).

The mesophyll cell assemblage is loose enough to optimize the available surface area for the exposure of chloroplasts to intermembrane space. In this way, the role of $S_c/S$ is understood as a way to shorten the $CO_2$ pathway to the carboxylation sites of the chloroplast stroma (Evans, 2021). On the other hand, cell walls can impose up to 90 % of the $g_m$ limitations (Carriquí et al., 2019; Gago et al., 2020). As mentioned above, the characteristics of cell walls are marked by the thickness and effective diffusivity (porosity and tortuosity). Recent data compilation of *CWT* has been measured in dozens of species (Gago et al., 2019, 2020). The results suggested that the thicker the cell wall the higher the $g_m$ limitation. Despite this, species with thicker *CW* could compensate for this disadvantage by modifying other traits, such as the effective diffusivity of the cell wall (porosity and tortuosity), thus achieving $g_m$ values similar to those of other species regardless *CWT* (Carriquí et al., 2020). However, methodological limitations prevent the study of effective cell wall diffusivity in terrestrial plants, despite this, several authors have tried to estimate it (Evans et al., 2009; Nobel, 1999; Terashima et al., 2006).

Recently, it has been observed that *CWT* plays an important role in relation to the stress tolerance capacities of different plant species (Houston et al., 2016; Tenhaken, 2015). Plant species adapted to live in dry environments or cold deserts such as Antarctica showed thicker *CWs* than the angiosperms average (Sáez et al., 2018). However, studies within the same species of the negative relationship between $g_m$ and *CWT* were not so easily observed under different stress treatments and conditions (M. Carriquí et al., 2015; Tomás et al., 2014).

Leaf anatomical traits are not the only elements that can explain stress tolerance, but also antioxidant metabolism. Both growth and defense involve the expression of

thousands of genes and the biosynthesis of innumerable compounds (Monson, Weraduwage, Rosenkranz et al., 2021). Resource limitation at the molecular level implies that the allocation of a nutrient to defense may lead to a reduction in the allocation to growth. In this way, growth-defense trade-off involves a whole series of cellular signalling networks that involve changes at gene expression level (Karasov et al., 2017).

Abiotic factors such as extreme temperatures, drought, salinity, among others, lead to an oxidative stress situation. Stomatal closure is induced, which results in a reduction in $CO_2$ input. In addition, the two photosynthesis phases (light and dark) are uncoupled and a saturation level is reached in which the excess energy cannot be completely dissipated in the heat or fluorescence form. In this way, the excess of photons converted to electrons with high energy are not directed to photosynthesis, but can react with molecular oxygen, thus forming reactive oxygen species (ROS). The main sites of generation of cellular ROS are chloroplasts, mitochondria, peroxisomes, the apoplast, and plasma membranes (Banerjee et al., 2019), but they can also form as a result of normal cellular metabolism. There are different form of ROS, including free radicals (superoxide anion, $O_2^{\bullet-}$; hydroxyl radical, $^{\bullet}OH$; alkoxy radical, $RO^{\bullet}$; and hydroperoxyl radical, $HO_2^{\bullet}$) and non-radical molecules (hydrogen peroxide, $H_2O_2$, and singlet oxygen, $^1O_2$) (Hasanuzzaman et al., 2020; Waszczak et al., 2018)**.** When ROS are generated transiently and in low concentrations, they play an important role as signalling compounds. For example, during growth, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are activated to transiently increase ROS concentration in the apoplast, because ROS gradients play a key role in the leaves and roots growth and differentiation (Foreman et al., 2003; Morales & Munné-Bosch, 2016). The problem is when ROS formation is high and sustained over time and occurs simultaneously with the failure of the antioxidant system, as can occur in the responses of plants to various biotic or abiotic stresses. Due to the highly reactive nature of ROS, they can trigger the oxidation of biomolecules such as carbohydrates, the breakdown of membranes by interacting with membrane lipids and proteins and the inactivation of enzymes directly or by altering the cofactors necessary for enzyme activity and causing nucleic acids damage (Mittler, 2002; Nath et al., 2016; Wyrwicka & Skłodowska, 2006). Furthermore, ROS can directly damage photosynthetic complexes (mainly photosystem II) by reacting with chlorophyll during photosynthesis,

rapidly inducing the triplet state of chlorophyll ($^1O_2$) and disrupting the photosynthetic pathway (Buchert & Forreiter, 2010; Sharma et al., 2019)**.**

Mitochondrial respiration plays a key protective role in the release of excess ROS produced in the chloroplast during photosynthesis and in the same mitochondria due to the loss of cellular homeostasis in an environmental stress situation (Dahal et al., 2014; Del-Saz et al., 2018; Van Aken, 2021)**.** In this way, mitochondrial respiration exerts a protective role, dissipating reductants from both mitochondria and chloroplast as well as providing energy and carbon skeletons (Igamberdiev, 2020).

In addition, the loss of cellular homeostasis due to the increase in the concentration of ROS in a environmental stress situation activates the signal transduction pathways, transporting the stress signal to the nuclear interior through redox reactions and the involvement of the mitogen-activated protein kinase (MAPK) pathway. All this leads to a modification of gene expression patterns (Singh et al., 2019)**,** which could mean an increase in the defense-related genes expression and the inhibition of other growth-related genes expression. In this way, in a stress situation, there is an inhibition of photosynthesis and an increase in oxidative damage in the plant, which sets in motion a whole series of stress tolerance mechanisms and inhibits others related with plant growth (Isah, 2019; Luciano et al., 2017). But what are these stress tolerance mechanisms of plants that compromise growth in a stress situation?

The signalling pathways in response to stress, promote the osmoprotective phenolic compounds biosynthesis, the secondary metabolites, which help to minimize the effects of different stresses by its antioxidant role (Hodaei et al., 2018; Jogawat, 2019; Knight, 1999). Phenolic compounds are natural products that were not originally considered essential for plant growth, reproduction and survival (Wink, 1988). They are derived from the primary metabolites produced by plants to combat adverse physiological changes under different environmental stresses (Khare et al., 2020).

The main reason for the accumulation of these phenolic compounds in the cytoplasm is the activation of the phenylpropanoid pathway (Sharma et al., 2019). Studies carried out on transgenic *Arabidopsis thaliana* showed how the overexpression of genes related to flavonoid biosynthesis such as MYB75, MYB12, FLAVANOL PRODUCTION GLYCOSIDES1, and PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1) promote anthocyanin-mediated antioxidant activity, which resulted in improved

drought tolerance (Nakabayashi et al., 2013). Interestingly, a recent study showed that microRNAs and their target genes can play a very important role in the biosynthesis of secondary metabolites (especially anthocyanins) in response to heat and cold stress (Yu et al., 2020).

A recent study on *Chrysanthemum sp*. showed an increase in antioxidant capacity promoted by the increase in total phenolic compounds, total flavonoids, anthocyanins and polyphenolic compounds in drought conditions (Hodaei et al., 2018). Flavonoids are phenolic compounds that lower ROS levels by catalysing oxygenation reactions, thus contributing to stress tolerance (Nakabayashi et al., 2013)**.** Flavonoids are classified into different subgroups such as flavanols, (iso) flavones or anthocyanins (Nakabayashi et al., 2013)**.** There are several studies that have shown modifications in the flavonoids composition, for example, wheat, peanuts and sesame showed a differential accumulation of polyphenols and flavonoids under drought conditions (Ghotbzadeh et al., 2019; Juliano et al., 2020). While other studies related the flavonoid content under a drought situation at the tissue level, showing a higher flavonoids concentration and, therefore, a greater drought tolerance in spikes than in flag leaf (Li et al., 2020). Studies carried out on the species *Crataegus laevigata*, *Crataegus monogyna* and *Trifolium repens* under drought stress conditions also showed an increase in biosynthesis and accumulation of flavanols. In addition to greater resistance to drought stress (Ara Kirakosyan et al., 2003; Ballizany et al., 2012). Sharma et al. (2019) stated that plants increase phenolic compounds concentration such as anthocyanins, flavonoids, flavanols and phenolic acids in response to low temperature, as observed in Chan et al. (2010), but it also happens at high temperatures, marking a common protection mechanism against to temperature stress, either hot or cold. In this way, it has been seen that the biosynthesis of phenolic compounds is related to an increase in tolerance to different stress situations (drought, low and high temperature), due to improved antioxidant capacity. Hence, it highlights the important protective role of secondary metabolism as an essential defense mechanism to protect the plant in a stress situation, but with an important cost in resources and energy that could limit primary metabolism and investments in photosynthetic capacities.

**How can the trade-off be studied?**

In order to study the hypothesis of the existence of a compensation at the foliar level between the maximum photosynthetic capacity and the tolerance to different stresses (dehydration and freezing) mediated by anatomical and biochemical features, a wide ecological range of herbaceous angiosperms has been used in this work. Most of the studies carried out on plant physiology and ecophysiology have been realized using only crops and/or model species and, therefore, much less is known about, for example, plant species adapted to environments under extreme conditions. Model species have been used to obtain relatively rapid knowledge on plant physiology and biochemistry, many of them have emphasized the biotechnological development of crop stress tolerance optimization (Provart et al., 2016). However, the use of these model species presents a series of limitations. Previous results have shown that the photosynthetic capacity of crops has already been optimized, as they have been shown to have a high maximum photosynthetic capacity. This fact has been attributed as an indirect consequence of breeding (Nadal & Flexas, 2019). Despite this, they have also shown a low capacity to tolerate stress, such as desiccation (Turcotte et al., 2014). In addition, this study expands the ecological range by using herbaceous angiosperm species from extreme environments. These species inhabit environments that combine dry and cold conditions, including Antarctica and the Svalbard Islands in the Arctic. Due to adverse weather conditions, they should develop much more stress tolerance mechanisms than models or crops; so, they could help us to understand the mechanisms underlying stress tolerance.

## HYPOTHESIS AND OBJECTIVE

In this project, I hypothesize that

1) A trade-off at leaf level exists between the maximal photosynthetic capacity ($A_{max}$) and stress tolerance.
2) This trade-off at leaf level between the maximal photosynthetic capacity ($A_{max}$) and stress tolerance can be explained by leaf anatomy and/or biochemistry/antioxidant metabolism traits.

Hence, the objective of the present research project is to determine if a trade-off relationship exists between $A_{max}$ and stress tolerance at leaf level that can be explained by anatomical and/ or antioxidant biochemical traits.

## MATERIALS AND METHODS

### Collection sites and parameters used between species: literature search

In this work a total of 16 non-woody angiosperm species from different bioclimatic zones have been used, eight of them coming from polar regions. These species differ between those native to Antarctica and those from Svalbard, a Norwegian archipelago that is part of the Arctic region. The native species of Antarctica are *Colobanthus quitensis* described by Humboldt, Bonplant & Kunth, 1831; and *Deschampsia antarctica*, described by Étienne-Émile Desvaux, 1854. On the other hand, the native species from the Arctic are: *Cerastium articum x C. regelii; Draba rupestris,* described by Carl Axel Magnus Lindman, 1926; *Oxyria digyna,* described by John Hill, 1768; *Saxifraga cespitosa, Saxifraga oppositifolia* and *Poa pratensis,* described by Carlos Linneo, 1753. In addition, species from more temperate bioclimatic zones are included: those of Mediterranean origin and those that make up the crops and model plants group. Mediterranean species are: *Alisma lanceolatum*, described by William Withering, 1796; and *Phlomis italica*, described by Philip Miller, 1759. Finally, the species that make up the group: Model & Crops are *Arabidopsis thaliana,* described by Johannes Thal, 1588; *Solanum lycopersicum*, described by Joseph Pitton de Tournefort, 1694; *Helianthus annuus, Nicotiana tabacum, Oryza sativa, Spinacia oleracea* and *Triticum aestivum*, described by Carlos Linneo, 1753.

Antarctic plants were collected from King George Island in the South Shetlands, near to the Henryk Arctowski Polish Antarctic Station (62°09′S, 58°28′W, December 2015). Arctic species were collected in the surroundings of Longyearbyen from the Svalbard Archipelago (79°49′N, 47.4” N 11°39'10.6”, 2019) and Mediterranean species were evaluated in a common garden at the Sóller Botanic Garden (Mallorca, 39° 45′ 51.58″ N, 2° 42′ 33.75″ E, May 2012) under optimal growing conditions (at 17.3 ºC and 1500 µmol $m^{-2}$ $s^{-1}$ PAR).

This thesis combines data acquired during my own experimental work, with data kindly shared by other authors previously published or obtained by personal communication.

Gas-exchange and anatomical data were obtained from eleven different species published in 8 articles of different researchers from the same research group. In addition, 5 of the species data comes from a Svalbard campaign in 2019, in which different measurements were carried out despite not being published yet. Table 1 summarizes the origin of the data used according to the species.

**Table 1.** *Origin, location and authorship of the data employed in this TFM*

| Group | specie | Dehydration test | Freezing test | Gas exchange | Leaf anatomy | DUALEX |
|---|---|---|---|---|---|---|
| Antarctic | Colobanthus quitensis | Svalbard campaign | Own data | (Clemente-Moreno, MJ. et al. 2020ab) | Sáez, P.L. et al. (2017) | Own data |
| Antarctic | Deschampsia antarctica | Svalbard campaign | Own data | (Clemente-Moreno, MJ et al. 2020ab) | Sáez, P.L. et al. (2017) | Own data |
| Arctic | Cerastium articum x C. regelii | Own data | Own data | Own data | No data | Own data |
| Arctic | Draba rupestris | Own data | Own data | Own data | No data | Own data |
| Arctic | Oxyria digyna | Svalbard campaign | Own data | Svalbard campaign | No data | Own data |
| Arctic | Saxifraga cespitosa | Own data | Own data | No data | No data | Own data |
| Arctic | Saxifraga oppositifolia | Svalbard campaign | Own data | Svalbard campaign | No data | Own data |
| Arctic | Poa pratensis | Svalbard campaign | Own data | Own data | No data | Own data |
| Mediterranean | Alisma lanceolatum | Gago et al. 2021 (in preparation) | Own data | Carriquí, M. et al. (2015) | Carriquí, M. et al. (2015) | Own data |
| Mediterranean | Phlomis italica | Gago et al. 2021 (in preparation) | Own data | Carriquí, M. et al. (2015) | Carriquí, M. et al. (2015) | Own data |
| Model & Crop | Arabidopsis thaliana | Gago et al. 2021 (in preparation) | Own data | Xiong, D. et al. (2017) | Tholen, D. et al. (2008) | Own data |
| Model & Crop | Helianthus annuus | Gago et al. 2021 (in preparation) | Own data | Nadal, M. et al. (2018) | Nadal, M. et al. (2018) | Own data |
| Model & Crop | Nicotiana tabacum | Gago et al. 2021 (in preparation) | Own data | Flexas, J. et al. (2006) | Flexas, J. et al. (2006) | Own data |
| Model & Crop | Oryza sativa | Gago et al. 2021 (in preparation) | Own data | Xiong, D. et al. (2016) | No data | Own data |
| Model & Crop | Solanum lycopersicum | Gago et al. 2021 (in preparation) | Own data | Nadal, M. et al. (2018) | Nadal, M. et al. (2018) | Own data |
| Model & Crop | Spinacia oleracea | Gago et al. 2021 (in preparation) | Own data | Nadal, M. et al. (2018) | Nadal, M. et al. (2018) | Own data |
| Model & Crop | Triticum aestivum | Gago et al. 2021 (in preparation) | Own data | Nadal, M. et al. (2018) | Nadal, M. et al. (2018) | Own data |

**Growing conditions**

Growing conditions of the species and measurements done by other authors are provided in the publications referred in Table 1. All the experiments were carried out in a greenhouse and phytotron provided by the plant physiology research group at the University of the Balearic Islands Spain (UIB).

The polar plants, both Arctic and Antarctic; and *Spinacia oleracea* were grown in 0.45 L plastic pots, using a peat-perlite mixture (2:1 v/v) and maintained inside a growth chamber with a daytime temperature of 15 ºC and 12 ºC at night, with an irradiance of 300 µmol $m^{-2}$ $s^{-1}$ photosynthetically active radiation (PAR) and a 12:12 h photoperiod. Plants were watered every 2-3 days depending on the pot weight and were fertilized with 50 % Hoagland's solution once a week (Hoagland & Arnon, 1938).

*Arabidopsis thaliana, Nicotiana tabacum*, *Oryza sativa* and *Triticum aestivum* were grown in plastic pots filled with a substrate containing peat-perlite mixture (2:1 v/v). The size of the pots was different between them, being 0.45 L, 3 L, 2.5 L and 2.5 L, respectively. In the case of *Oryza sativa* and *Triticum aestivum*, three plants were grown in each pot. All of them were maintained in a growth chamber with a daytime temperature of 25ºC and 22ºC during the night, with an irradiance of 300 µmol $m^{-2}$ $s^{-1}$ PAR (except for *Arabidopsis thaliana*, which was 100 µmol $m^{-2}$ $s^{-1}$ PAR) and 12:12 h photoperiod. Plants were watered every day at field capacity and fertilized with 50% Hoagland's solution twice a week (Hoagland & Arnon, 1938).

Finally, *Solanum lycopersicum* and *Helianthus annuus* were grown outdoors in the experimental field of the UIB in pots (5-10 L) using a peat-perlite mixture (2:1 v/v) and were grown at full sunlight with a maximum daytime temperature of 26 ºC and 18 ºC at night and an average photoperiod of 15: 9 (June 2021).

**Fluorescence and gas exchange measurements**

In the case of the gas exchange and fluorescence data obtained from other databases, the measurements were made on completely expanded sheets without malformations and under non-stressed conditions. All compiled gas exchange measurements were performed between 15-25 ºC, at a saturation light intensity of (1500-2.000 µmol photon $m^{-2}$ $s^{-1}$), atmospheric $CO_2$ concentrations (400 ppm). The mesophyll conductance

calculation, the methods of variable J and isotopic discrimination were followed (Harley et al., 1992; Warren, 2011)

Due to the small leaf size of the different arctic species (the only gas-exchange data taken during this thesis), a set of fully expanded leaves were selected to occupy the maximum area within the cuvette (2 $cm^2$). If leaves do not completely cover the cuvette, an orthogonal photo was taken and area was measured using the ImageJ image analysis software (Schneider et al., 2012). In the selected leaves, simultaneous measurements of gas exchange and fluorescence of chlorophyll *a* using an infrared gas analyser (Li-6400-40, Li-Cor Inc., Lincoln, NE, USA) coupled with a Chl fluorimeter (Li-6400-40, 2 $cm^2$ chamber; Li-Cor). Measurements were made in the same growth chamber where the plants were grown (15 ºC) and the flow rate within the chamber was fixed at 300 µmol air $s^{-1}$, 400 ppm $CO_2$ and light saturation (1500 PAR µmol $m^{-2}$ $s^{-1}$) Between four and seven individuals were carried out to obtain the data of the different species.

In the case of the species of *Colobanthus quitensis* and *Deschampsia antarctica* there are two sets of gas-exchange measurements under laboratory conditions (Clemente-Moreno et al., 2020a; 2020b) and at field conditions (Sáez et al., 2018). Photosynthesis gas-exchange data were used for comparison with the freezing test and biochemical data, because they were carried out under the same conditions. However, to compare with the dehydration test and the anatomical data, two values published in (Sáez et al., 2018) obtained under field conditions were used: 9.88 ± 1.71 and 15.11 ± 0.51, for *Colobanthus quitensis* and *Deschampsia antarctica*, respectively.

**Freezing tolerance measurements**

Freezing tolerance assessment was based on the Freezing test. Freezing test consisted in placing leaf segments (ca. 1 $cm^2$ leaf disc) into an isolated cryothermocycler machine, which allowed to impose different temperature cycles of freezing (-6/-9/-12/-18 ºC target temperature, ramp 8 ºC/h) and subsequent recovery, including an additional step at 0 ºC for 30 min, and subsequently reaching 15ºC at the previous described rate. Foliar freezing tolerance was quantified as a function of the recovery percentage of the maximum photochemical efficiency of photosystem II ($F_v/F_m$), measured with a BLUE junior-PAM portable fluorometer (Heinz Walz GmbH,

Effeltrich, Germany) before and after the freezing-recovery process. Relative $F_v/F_m$ was normalized by the calculation of the final $F_v/F_m$ after one freezing/recovery cycle per the initial $F_v/F_m$ values. The $F_v/F_m$ data prior to the freezing/recovery cycle was taken after 30 minutes of leaf submission to dark conditions, considered the necessary adaptation time of photosystems II to dark conditions (Clemente-Moreno et al., 2020; Maxwell & Johnson, 2000). The temperature during this adaptation period was 15ºC.

**Dehydration tolerance measurements**

Tolerance to desiccation were estimated according to the previous published protocol (López-Pozo et al., 2019). The procedure was based on the introduction of foliar segments of approximately 100 mg of fresh weight previously hydrated for 24 h at dark conditions and 25ºC at room temperature. Subsequently, foliar pieces were placed in 50 mL closed Falcon tubes with three different desiccant concentrations: NaCl, $MgCl_2$ and silica, which allowed a relative humidity of 80 %, 50 % and 10 %, respectively. The excess of interstitial water was removed using absorbent paper before placing them inside the tubes. Leaves were maintained within the tubes for 48 h at dark conditions and 25ºC temperature. Finally, the leaf tissue was rehydrated in water for 24 h, 25ºC and dark conditions. After each step leaf tissues were weighted and $F_v/F_m$ measured with a BLUE junior-PAM portable fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Relative $F_v/F_m$ was normalized by the calculation of the final $F_v/F_m$ after one desiccation/rehydration cycle per the initial $F_v/F_m$ values. Leaves samples that were not dehydrated below 30 % RWC were discarded.

**Structural and anatomical measurements**

All anatomical data were obtained from external databases, except for leaf mass per unit area (*LMA*). *LMA* was determined using Image-J software (Schneider et al., 2012), and the leaf mass per area (*LMA*) was calculated as the ratio of leaf dry mass to leaf area, as described in Tosens et al. (2016). Leaf mass measurements were performed using a precision analytical balance (RADWAG AS 600 / 220.R2, Poland) and the samples were dried at 70 ºC in a TCF 400 oven (ArgoLab, California, USA).

Ultra-anatomical measurements as the chloroplast surface exposed to mesophilic air spaces ($S_c/S$), the cell surface exposed to mesophilic air spaces ($S_m/S$), the chloroplast thickness ($T_{chl}$), the cell wall thickness and the fraction of air space within

the leaf ($f_{ias}$) were obtained following the protocol described in (Tomás et al., 2014). Briefly, the protocol employs 1 x 1 mm segments cut between the main veins of the leaves. The samples were fixed with 4% glutaraldehyde and 2% paraformaldehyde in a 0.1 m phosphate buffer (pH 7.4); and then in 2% osmium tetroxide for 2 h. They were then dehydrated in a gradual series of ethanol and embedded and solidified in LR-White resin and an oven at 60°C for 48 h, respectively. Ultrathin sections were made, stained with 1% toluidine blue and observed under the light microscope at 200x magnification and under the electron microscope at 2000x magnification. All images were analyzed with ImageJ (Wayne Rasband/NIH) and AutoCAD (AutoCAD 2011 Mac; Autodesk, San Rafael, CA, USA).

**Foliar chlorophyll and polyphenols content measurements**

The balanced nitrogen index, the content of chlorophyll (µg/cm²), flavanols (relative absorbance units) and anthocyanins (relative absorbance units) were quantified with the optical leaf sensor Dualex (Force A, France). Fully expanded, mature leaves were selected, without previous damage or signs of stress between the third and fifth leaves from the apical meristem whenever possible were clamped to carry out. In case of some herbaceous or polar species, the last criterion was not applied due to their structural characteristics. The measurement area is 5 mm in diameter and the quantification range of chlorophyll is 5-80 µg/cm², while that of flavanols and anthocyanins is 0 to 3 and 0 to 1.5 relative absorbance units, respectively.

**Statistical analysis**

Statistical analysis was performed using the free software "R Core Team (2020)". The freezing and dehydration test graphics; in addition to the graph of the freezing cycle and recovery of the freezing test, were carried out by using the Excel 2016 software (v.16.0).

The study of significant differences between the different response variables (freezing and dehydration tests) was carried out in different steps. In a first step, it was assessed whether the variables followed a normal distribution using the Shapiro-Wilks test. They were considered to follow a normal distribution if the resulting p-value was greater than 0.05. The analysis of the homogeneity of variances was carried out by using the Levene's test. For those response variables that showed a normal distribution and

homogeneity of variances, according to the applied tests, a one-way analysis of variance (ANOVA) was carried out to analyse if there were significant changes between the different treatments. For those response variables that did not follow a normal distribution, the non-parametric Kruskal-Wallis test was applied in order to know the existence of significant changes between the different species or groups; considering the p-value significant if was less than 0.1. After performing the Kruskal-Wallis test, significant changes between groups were studied using the Bonferroni test; considering significant if the p-value was less than 0.1. In the case of the comparison between “species” factor with the Bonferroni test, no significant results were obtained due to the lack of degrees of freedom.

Regression analyses were carried out with the "stats" function. The differences of the response variables depending on the species and group were carried out by means of a one-way ANOVA. It was considered significant if the resulting p-value was less than 0.1, with a confidence interval of 90 %. On the other hand, the Pearson correlation values were obtained with the "rcorr" function of "R", while the heat map was performed with the "pheatmap" function.

## RESULTS

### Photosynthetic, biochemical and anatomical characterization of the studied species

In order to investigate the relationship between the maximum photosynthetic capacity and stress tolerance, a compilation of the photosynthetic data characterization of the different species was carried out under optimal conditions. Table 2 shows the results obtained from the gas exchange measurements for each species of the databases indicated in Table 1. The parameters characterized for each species were: $A_{max}$, $g_s$, $g_m$, $ETR$, $C_i$ and $C_c$.

One of the hypotheses of this work is that the trade-off at the leaf level between maximum photosynthetic capacity ($A_{max}$) and stress tolerance can be explained by leaf anatomy and biochemical/antioxidant metabolism traits. In order to investigate the role of leaf anatomy and antioxidant metabolism traits. A data collection was carried out for the characterization of both parameters, which are shown in Tables 3 and 4, respectively.

In the case of the study of the foliar anatomy of each species, the *LMA, $S_c$/S, $S_m$/S, $T_{chl}$, CWT* and $f_{ias}$ parameters were characterized (table 3). Unfortunately, only *LMA* was obtained for all species. No ultra-anatomical data is shown in this study or all the species that form the Arctic group (*C. arcticum x C. regelii, D. rupestris, O. digyna, S. cespitosa, S. oppositifolia* and *Poa pratensis*); these data are in process. Unfortunately, this lack of information would constraint the conclusions of this work.

Additionally, in the biochemical characterization (table 4), two parameters involved in the primary metabolism (NBI and Chlorophyll *a*) and two parameters involved in the protective secondary antioxidant metabolism (flavanols and anthocyanins) were studied for each species.

***Table 2.*** *Gas-Exchange mesurements under optimal conditions*

| | | Maximal photosynthetic capacity | Stomatal conductance to $CO_2$ | Mesophyll conductance to $CO_2$ | Electron Transport Rate | $[CO_2]$ substomatic | $[CO_2]$ chloroplastic |
|---|---|---|---|---|---|---|---|
| **Group** | **Species** | $A_{max}$ | $g_s$ | $g_m$ | **ETR** | $C_i$ | $C_c$ |
| | | µmol $CO_2$ $m^{-2}$ $s^{-1}$ | mol $CO_2$ $m^{-2}$ $s^{-1}$ | mol $CO_2$ $m^{-2}$ $s^{-1}$ | µmol photons $m^{-2}$ $s^{-1}$ | mol $CO_2$ $mol^{-1}$ air | mol $CO_2$ $mol^{-1}$ air |
| Antarctic | *Colobanthus quitensis* | 18.2 ± 1.1 | 0.310 ± 0.030 | 0.09 ± 0.01 | 150.20 ± 3.70 | 326.6 ± 5.0 | 103.70 ± 8.90 |
| Antarctic | *Deschampsia antarctica* | 14.3 ± 0.7 | 0.220 ± 0.020 | 0.10 ± 0.01 | 95.10 ± 3.00 | 318.0 ± 2.0 | 172.60 ± 6.70 |
| Arctic | *Cerastium articum x C. regelii* | 7.2 ± 1.8 | 0.125 ± 0.030 | *NA* | 79.20 ± 8.08 | 281.6 ± 18.2 | *NA* |
| Arctic | *Draba rupestris* | 10.0 ± 0.9 | 0.367 ± 0.070 | *NA* | 86.99 ± 8.26 | 341.5 ± 5.2 | *NA* |
| Arctic | *Oxyria digyna* | 15.93 ± 2.31 | *NA* | *NA* | *NA* ± | 98.26 ± 50.32 | *NA* |
| Arctic | *Saxifraga oppositifolia* | 6.4 ± 0.6 | *NA* | *NA* | 67.53 ± 2.23 | 201.2 ± 23.1 | *NA* |
| Arctic | *Poa pratensis* | 10.0 ± 1.2 | 0.130 ± 0.020 | 0.13 ± 0.05 | 73.82 ± 2.24 | 305.5 ± 6.8 | 178.31 ± 31.86 |
| Mediterranean | *Alisma lanceolatum* | 17.0 ± 0.6 | 0.170 ± 0.024 | 0.14 ± 0.02 | 150.40 ± 5.80 | 278.0 ± 11.0 | 159.00 ± 17.00 |
| Mediterranean | *Phlomis italica* | 24.0 ± 1.1 | 0.240 ± 0.019 | 0.17 ± 0.01 | 218.90 ± 2.30 | 277.0 ± 3.0 | 133.00 ± 7.00 |
| Model & Crop | *Arabidopsis thaliana* | 7.7 ± 0.5 | 0.085 ± 0.003 | 0.15 ± 0.02 | *NA* | 292.0 ± 21.0 | 239.00 ± 27.00 |
| Model & Crop | *Helianthus annuus* | 32.8 ± 0.6 | 0.360 ± 0.015 | 0.54 ± 0.02 | *NA* | *NA* | *NA* |
| Model & Crop | *Nicotiana tabacum* | 19.7 ± 0.7 | 0.240 ± 0.013 | 0.21 ± 0.01 | *NA* | 240.6 ± 5.0 | 151.80 ± 6.40 |
| Model & Crop | *Oryza sativa* | 31.8 ± 1.9 | 0.330 ± 0.030 | 0.25 ± 0.03 | *NA* | *NA* | *NA* |
| Model & Crop | *Solanum lycopersicum* | 25.0 ± 25.0 | 0.250 ± 0.035 | 0.36 ± 0.03 | *NA* | *NA* | *NA* |
| Model & Crop | *Spinacia oleracea* | 22.5 ± 0.9 | 0.210 ± 0.018 | 0.29 ± 0.03 | *NA* | *NA* | *NA* |
| Model & Crop | *Triticum aestivum* | 18.8 ± 1.5 | 0.190 ± 0.016 | 0.15 ± 0.02 | *NA* | *NA* | *NA* |

***Table 3.*** *Leaf anatomy data*

| Group | Species | Leaf Mass per unit Area ***LMA*** (g $m^{-2}$) | Chloroplast surface exposed to mesophilic air spaces ***$S_c/S$*** ($m^2$ $m^{-2}$) | Cellular surface exposed to mesophilic air spaces ***$S_m/S$*** ($m^2$ $m^{-2}$) | Chloroplast thickness ***$T_{chl}$*** (µm) | Cell wall thickness ***CWT*** *(µm)* | Air spaces fraction inside the leaf ***$f_{ias}$*** *(%)* |
|---|---|---|---|---|---|---|---|
| Antarctic | *Colobanthus quitensis* | 63.07 ± 4.73 | 2.06 ± 0.32 | 4.33 ± 0.48 | 5.12 ± 0.66 | 0.35 ± 0.060 | 13 ± 1 |
| Antarctic | *Deschampsia antarctica* | 84.87 ± 8.43 | 2.24 ± 0.48 | 6.4 ± 0.75 | 2.96 ± 0.24 | 0.26 ± 0.010 | 46 ± 6 |
| Arctic | *Cerastium articum x C.regelii* | 48.31 ± 1.15 | *NA* | *NA* | *NA* | *NA* | *NA* |
| Arctic | *Draba rupestris* | 59.81 ± 0.93 | *NA* | *NA* | *NA* | *NA* | *NA* |
| Arctic | *Oxyria digyna* | 20.74 ± 0.75 | *NA* | *NA* | *NA* | *NA* | *NA* |
| Arctic | *Saxifraga cespitosa* | 28.89 7.02 | *NA* | *NA* | *NA* | *NA* | *NA* |
| Arctic | *Saxifraga oppositifolia* | 44.98 ± 7.22 | *NA* | *NA* | *NA* | *NA* | *NA* |
| Arctic | *Poa pratensis* | 45.4 ± 2.33 | *NA* | *NA* | *NA* | *NA* | *NA* |
| Mediterranean | *Alisma lanceolatum* | 53.6 ± 2.7 | 7.4 ± 0.4 | 15.3 ± 1.4 | 3.05 ± 0.09 | 0.28 ± 0.027 | 44.1 ± 4.6 |
| Mediterranean | *Phlomis italica* | 67.5 ± 6.7 | 9.0 ± 0.2 | 12.3 ± 0.6 | 2.26 ± 0.280 | 0.149 ± 0.008 | 49.6 ± 3.1 |
| Model & Crop | *Arabidopsis thaliana* | 11.38 ± 0.73 | 8.17 ± 0.54 | 9.02 ± 0.77 | *NA* | 0.174 ± 0.006 | 23.4 ± 3.4 |
| Model & Crop | *Helianthus annuus* | 57.5 ± 0.7 | 8.89 ± 0.39 | 9.89 ± 0.19 | 1.41 ± 0.03 | 0.14 ± 0.018 | 35.5 ± 1.6 |
| Model & Crop | *Nicotiana tabacum* | 25.7 ± 2.3 | 9.1 ± 0.6 | 12.3 ± 1.1 | *NA* | *NA* | *NA* |
| Model & Crop | *Oryza sativa* | 41.65 ± 2.12 | 19.6 ± 0.9 | 20.8 ± 2 | *NA* | 0.164 ± 0.014 | 21.7 ± 2.9 |
| Model & Crop | *Solanum lycopersicum* | 51.6 ± 0.9 | 9.77 ± 1.29 | 12.91 ± 3.1 | 2.31 ± 0.22 | 0.11 ± 0.005 | 22.1 ± 2.2 |
| Model & Crop | *Spinacia oleracea* | 66.4 ± 3.7 | 10.04 ± 2.25 | 14.02 ± 2.27 | 2.32 ± 0.23 | 0.38 ± 0.023 | 33.2 ± 3.85 |
| Model & Crop | *Triticum aestivum* | 74.9 ± 2.4 | 12.91 ± 1.95 | 18.31 ± 4.37 | 2.13 ± 0.2 | 0.23 ± 0.032 | 11.4 ± 1.73 |

***Table 4.*** *DUALEX fluorescence data. "NBI: Nitrogen Balance Index"*

| **Group** | **Species** | Nitrogen Balanced Index ***NBI*** Relative units | Chlorophyll a content ***Chlorophyll a*** µg/cm² | Flavanols content ***Flavanols*** Relative units | Anthocianin content ***Anthocianin*** Relative units |
|---|---|---|---|---|---|
| Antarctic | *Colobanthus quitensis* | 38.70 ± 4.20 | 22.12 ± 2.94 | 0.58 ± 0.05 | 0.16 ± 0.01 |
| Antarctic | *Deschampsia antarctica* | 28.57 ± 3.47 | 22.22 ± 2.97 | 0.77 ± 0.02 | 0.10 ± 0.01 |
| Arctic | *Cerastium articum x C. regelii* | 142.38 ± 34.39 | 48.37 ± 6.14 | 0.40 ± 0.07 | 0.08 ± 0.03 |
| Arctic | *Draba rupestris* | 75.77 ± 12.43 | 33.32 ± 6.55 | 0.45 ± 0.05 | 0.17 ± 0.02 |
| Arctic | *Oxyria digyna* | 141.71 ± 6.66 | 35.10 ± 2.17 | 0.28 ± 0.02 | 0.13 ± 0.01 |
| Arctic | *Saxifraga oppositifolia* | 73.85 ± 10.43 | 29.13 ± 2.84 | 0.41 ± 0.04 | 0.13 ± 0.02 |
| Arctic | *Poa pratensis* | 54.88 ± 6.54 | 31.03 ± 4.02 | 0.57 ± 0.03 | 0.12 ± 0.03 |
| Mediterranean | *Alisma lanceolatum* | 39.87 ± 4.25 | 34.02 ± 5.01 | 0.85 ± 0.05 | 0.09 ± 0.00 |
| Mediterranean | *Phlomis italica* | 37.45 ± 2.53 | 28.53 ± 1.68 | 0.82 ± 0.05 | 0.13 ± 0.01 |
| Model & Crop | *Arabidopsis thaliana* | 39.77 ± 1.44 | 36.19 ± 0.78 | 0.97 ± 0.04 | 0.13 ± 0.01 |
| Model & Crop | *Helianthus annuus* | 104.24 ± 7.41 | 21.28 ± 1.57 | 0.24 ± 0.02 | 0.19 ± 0.01 |
| Model & Crop | *Nicotiana tabacum* | 60.63 ± 5.75 | 31.43 ± 2.78 | 0.71 ± 0.06 | 0.14 ± 0.01 |
| Model & Crop | *Oryza sativa* | 54.18 ± 3.07 | 31.08 ± 1.44 | 0.58 ± 0.03 | 0.12 ± 0.00 |
| Model & Crop | *Solanum lycopersicum* | 70.47 ± 5.09 | 35.10 ± 3.36 | 0.50 ± 0.02 | 0.12 ± 0.01 |
| Model & Crop | *Spinacia oleracea* | 95.53 ± 5.09 | 32.70 ± 0.94 | 0.35 ± 0.01 | 0.10 ± 0.00 |
| Model & Crop | *Triticum aestivum* | 63.38 ± 7.17 | 28.38 ± 1.41 | 0.52 ± 0.05 | 0.13 ± 0.01 |

**Freezing test results**

In order to investigate the freezing tolerance at the species level and at the group level, a whole series of graphs were made for the different target temperatures -6, -9, -12 and -18 ºC, as well as the mean of all the target temperatures. The results of the percentage of the $F_v/F_m$ recovery of the freezing test as a function of the target temperature for each species are shown below: Figures 3, 4, 5 and 6, for the -6, -9, -12 and -18 ºC target temperatures, respectively. In addition, a graph is added with the mean of the percentage of the $F_v/F_m$ recovery of the four target temperatures of the freezing test for each of the species in figure 7.

Figure 3 shows the results of the percentage of the % $F_v/F_m$ recovery of the freezing test with a target temperature of -6 ºC for the study species. Shapiro's test showed that the percentage of the $F_v/F_m$ recovery of the freezing test with a target temperature of -6 ºC did not follow a normal distribution, with a value of $p < 0.001$. In addition, the Levene test showed that the study parameter did not have homogeneity of variances as a function of the species, but rather as a function of the group, with a value of $p = 0.01$ and $p = 0.15$, respectively. As it did not follow a normal distribution, the non-parametric Kruskal-Wallis test was applied. The analysis showed significant changes between species and groups, with a value of $p = 0.01$ and $p = 0.045$, respectively. The Bonferroni test could not be performed to compare the different species because they did not have enough degrees of freedom. Considering “Groups” as a factor the same test showed significant differences between the Arctic and Model & Crops groups ($p = 0.085$). Interestingly at this temperature that is the highest tested with this methodology, all groups achieved a $F_v/F_m$ recovery above 90%, so all of them achieved an almost complete recovery. The group of the “Models & Crops” species showed higher heterogeneity than the others, with some species showing the lowest recoveries around 80%; however, any other species from the other groups at -6ºC test showed values below 90% (Figure 3).

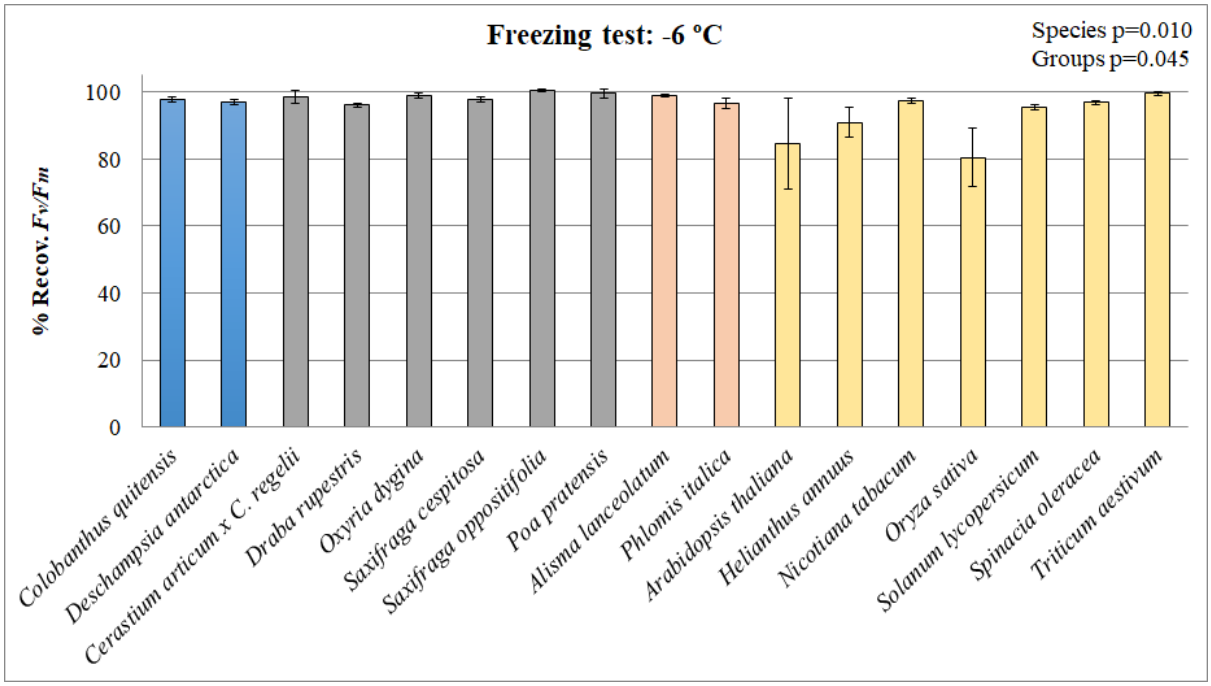


***Figure 3. Results of the freezing test for -6 ºC target temperature for the studied species.*** *The recovery percentage of the $F_v/F_m$ after the freezing-recovery cycle (Y axis) as a function of the species (X axis) is shown. Colours distinguish the groups that form different species: Antarctic (blue), Arctic (grey), Mediterranean (orange) and Models & Crops (yellow). Data are average values ± ET of 6 biological replicates per species.*

Figure 4 shows the results of the freezing test at -9 ºC for all the studied species, except for *Oxyria digyna*, from the Arctic group, due to a technical problem. The Shapiro test showed that the percentage of the $F_v/F_m$ recovery of the freezing test with a target temperature of -9 ºC did not follow a normal distribution, with a value of $p < 0.001$. As it did not follow a normal distribution, the Levene test was applied to study the homogeneity of variances. The Levene test showed that the study parameter did not have homogeneity of variances as a function of the species, but it had depending on the group, with a value of $p = 0.01$ and $p = 0.20$, respectively. As it did not follow a normal distribution, the non-parametric Kruskal-Wallis test was applied. The analysis showed significant changes between species and groups, with a value of $p < 0.001$ for both cases. The Bonferroni test could not be performed to compare between the different species because they did not have enough degrees of freedom. However, when "Groups" was analysed, significant differences of about 25 % in % $F_v/F_m$ recovery were found between the Model & Crops and Antarctic groups ($p = 0.03$), followed by significant differences of about 10% between the Model & Crops and Arctic groups ($p = 0.04$). In this case, with the drop of only 3 ºC with respect to the target temperature analysed previously (-6 ºC), the greatest drop in the % of the $F_v/F_m$

recovery was observed in the Model & Crops group, of approximately 45 %, obtaining a recovery value of approximately 45 %; the drop and value was similar in the case of the Mediterranean group; followed by a drop of approximately 40 % in the case of the Arctic group, obtaining a value of approximately 60 %; while, the Antarctic group, only showed a drop of approximately the 20 % obtaining a value of approximately 80 %.

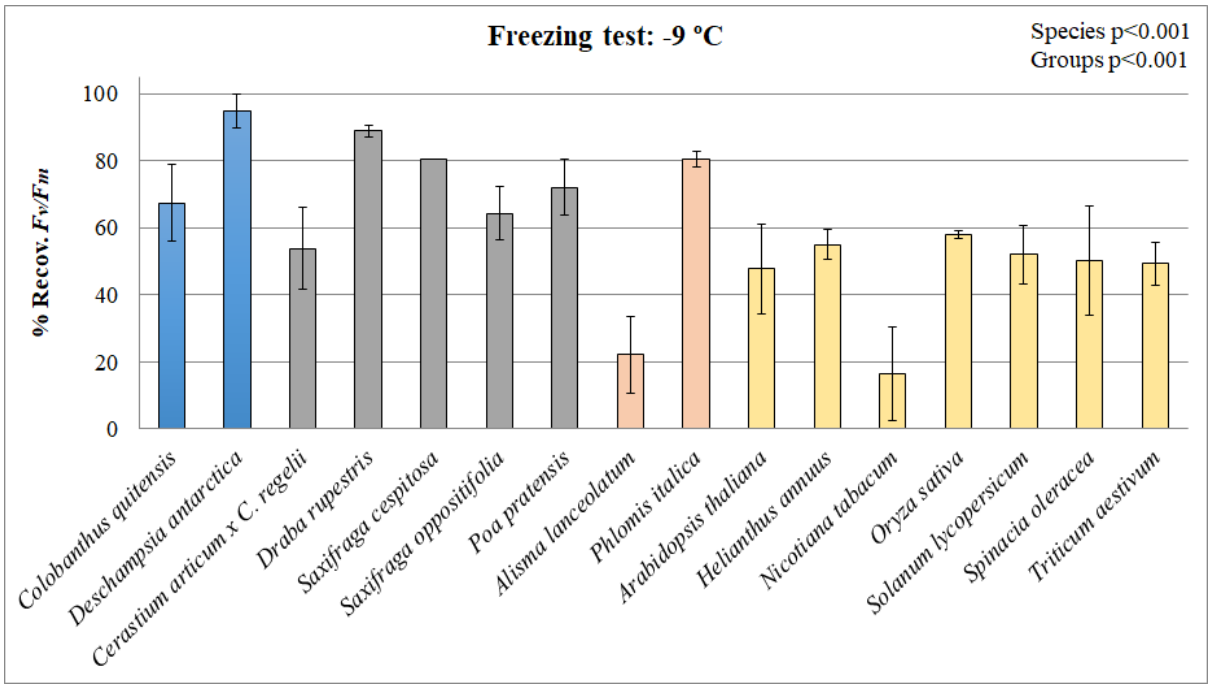


***Figure 4. Results of the freezing test for -9 ºC target temperature for the studied species.*** *The percentage of the $F_v/F_m$ recovery after the freezing-recovery cycle (Y axis) as a function of the species (X axis) is shown. Colours distinguish the groups that form different species: Antarctic (blue), Arctic (grey), Mediterranean (orange) and Models & Crops (yellow). Data are average values ± ET of 6 biological replicates per species.*

Figure 5 shows the results of the percentage of the $F_v/F_m$ recovery of the freezing test with a target temperature of -12 ºC for the species under study. The Shapiro test showed that the percentage of the $F_v/F_m$ recovery of the freezing test with a target temperature of -12 ºC did not follow a normal distribution, with a value of $p < 0.001$. As it did not follow a normal distribution, Levene's test was applied to study the homogeneity of the variances. Levene's test showed that the study parameter had homogeneity of variances as a function of the species, but not as a function of the group, with a value of $p = 0.19$ and $p < 0.01$, respectively. As it did not follow a normal distribution, the non-parametric Kruskal-Wallis test was applied. The analysis showed significant changes between species and groups, with a value of $p < 0.001$ and $p < 0.01$, respectively. The Bonferroni test could not be performed to compare between the

different species because they did not have enough degrees of freedom. When "Groups" were analysed, significant differences of about 25 % in % $F_v/F_m$ were found between the Arctic group with respect to the Mediterranean ($p < 0.01$) and Model & Crops groups ($p < 0.001$). Any of the groups reached a recovery higher than 60% at this temperature. The greatest drop with respect to the previous target temperature (-9ºC) was seen in the Antarctic group, with a decrease of almost 30 %, obtaining a value of approximately 45% of the % $F_v/F_m$ recovery, while the Arctic group maintained with respect to the previous target temperature was around 60 %. In the case of the Model & Crops and Mediterranean groups, a % $F_v/F_m$ recovery below 40 % was obtained.

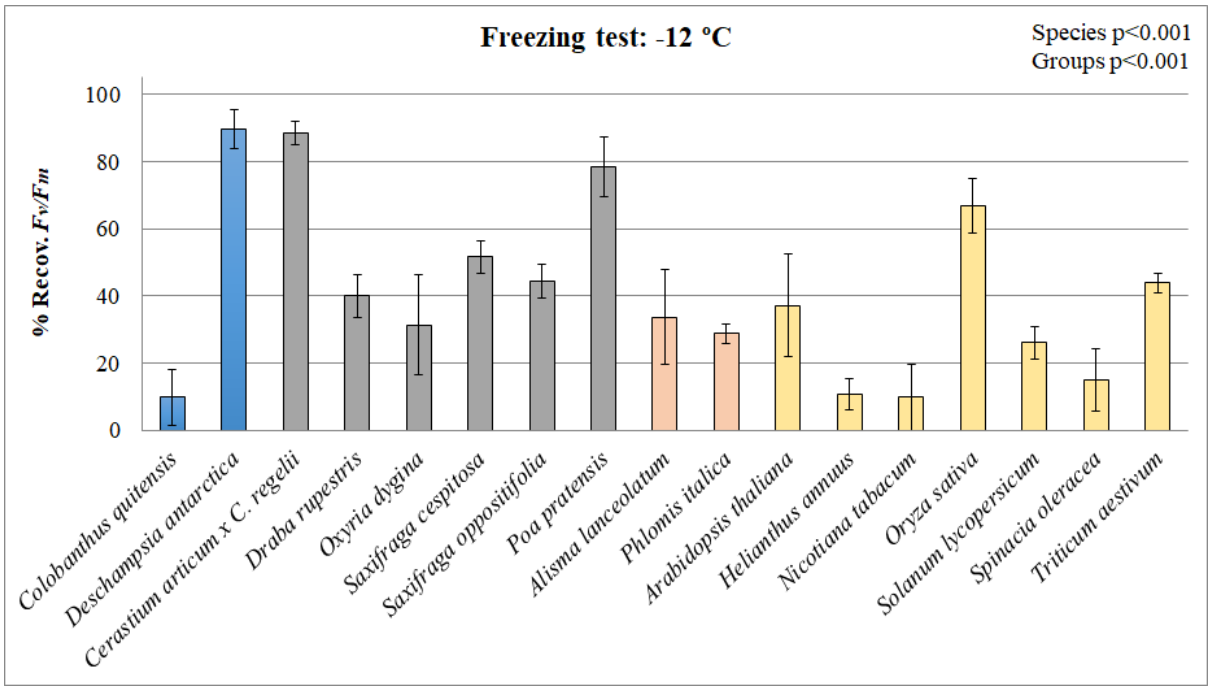


***Figure 5. Results of the freezing test for -12 ºC target temperature for the studied species.*** *The percentage of the $F_v/F_m$ recovery after the freezing-recovery cycle (Y axis) as a function of the species (X axis) is shown. Colours distinguish the groups that form different species: Antarctic (blue), Arctic (grey), Mediterranean (orange) and Models & Crops (yellow). Data are average values ± ET of 6 biological replicates per species.*

Figure 6 shows the results of the percentage of the $F_v/F_m$ recovery of the freezing test with a target temperature of -18 ºC for the species under study. The Shapiro test showed that the percentage of recovery of the $F_v/F_m$ of the freezing test with a target temperature of -18 ºC did not follow a normal distribution, with a value of $p < 0.001$. As it did not follow a normal distribution, Levene's test was applied to study the homogeneity of the variances. Levene's test showed that the study parameter had

homogeneity of variances both as a function of the species and the group, with a value of p = 0.18 and p <0.08, respectively. Kruskal-Wallis test showed significant changes between species and groups, with a value of p <0.001 and p = 0.08, respectively. The Bonferroni test could not be performed to compare between the different species because they did not have enough degrees of freedom. When “groups” were compared with the same test, significant differences between the Model & Crops group with respect to the Antarctic (p = 0.07) were found. However, all groups showed an approximate % $F_v/F_m$ between 20 and 40 %, which shows that at -18ºC, the lowest temperature tested, most of the species do not recover. Interestingly, the species *Poa pratensis*, a highly cosmopolite invasive grass showed the highest recovery values.

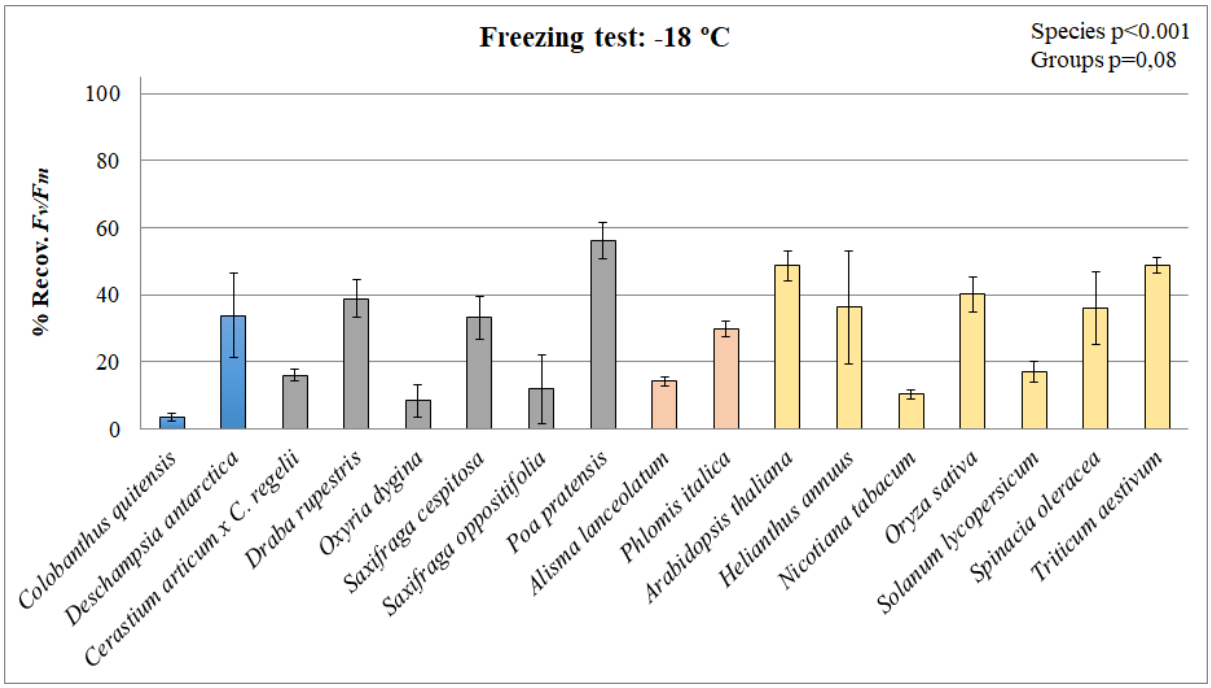


***Figure 6. Results of the freezing test for -18 ºC target temperature for the studied species.*** *The percentage of the $F_v/F_m$ recovery after the freezing-recovery cycle (Y axis) as a function of the species (X axis) is shown. Colours distinguish the groups that form different species: Antarctic (blue), Arctic (grey), Mediterranean (orange) and Models & Crops (yellow). Data are average values ± ET of 6 biological replicates per species.*

Finally, an analysis was also carried out of the results of the percentage of the $F_v/F_m$ recovery of the freezing test with the average of all the target temperatures (-6, -9, -12, -18 ºC) for the species under study, shown in figure 7. Shapiro's test showed that the recovery percentage of $F_v/F_m$ from the freezing test did not follow a normal distribution, with a value of p = 0.01. As it did not follow a normal distribution, Levene's test was applied to study the homogeneity of the variances. Levene's test showed that

the study parameter did not have homogeneity of variances both as a function of the species and the group, with a value of p = 0.04 and p = 0.001, respectively. Kruskal-Wallis test showed significant changes between species and groups, with a value of p <0.001 in both cases. The Bonferroni test could not be performed to compare between the different species because they did not have enough degrees of freedom. When "groups" were compared with the same test, significant differences of approximately 10 % between the Arctic group with respect to the Mediterranean (p <0.001) and Model & Crops (p = 0.04) groups were found. In the case of polar groups (Arctic and Antarctic), they showed an average % $F_v/F_m$ of approximately 60%, while the groups from more temperate regions (Model & Crops and Mediterranean) obtained average values below, of around 50 %.

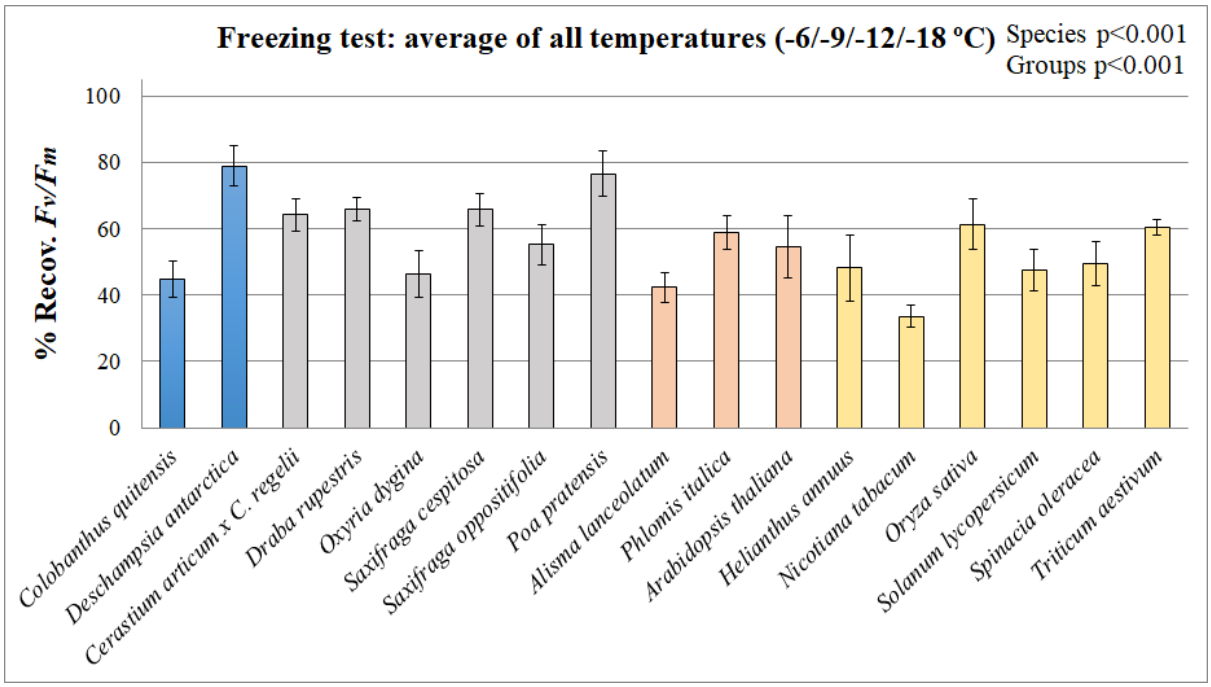


***Figure 7. Results of the freezing test for the average of all the target temperatures (-6, -9, -12, -18 ºC) for the studied species.*** *The recovery percentage of the $F_v/F_m$ after the freezing-recovery cycle (Y axis) as a function of the species (X axis) is shown. Colours distinguish the groups that form different species: Antarctic (blue), Arctic (grey), Mediterranean (orange) and Models & Crops (yellow). Data are average values ± ET of 24 biological replicates per species.*

### Dehydration test results

The results of the recovery percentage of the $F_v/F_m$ of the dehydration test as a function of the treatment for each species are shown below: Figures 8, 9 and 10, for the NaCl, $MgCl_2$ and silica gel treatments, respectively. In addition, a graph is added

with the mean of the recovery percentage of the $F_v/F_m$ of the three treatments of the dehydration test for each of the species in figure 11.

Figure 8 shows the results of the recovery percentage of the $F_v/F_m$ of the dehydration test with the NaCl treatment for the species under study. The Shapiro test showed that the percentage of recovery of the $F_v/F_m$ of the dehydration test with the NaCl treatment did not follow a normal distribution, with a value of $p < 0.001$. As it did not follow a normal distribution, Levene's test was applied to study the homogeneity of the variances. Levene's test showed that the study parameter did not have homogeneity of variances as a function of the species, with a value of $p = 0.04$, but it did as a function of the group, with a value of $p = 0.1$. The Kruskal-Wallis analysis showed significant changes between species and groups, with a value of $p < 0.01$ and $p < 0.001$, respectively. The Bonferroni test could not be performed to compare between the different species because they did not have enough degrees of freedom.

However, the same test showed significant differences between the Mediterranean-Antarctic groups ($p = 0.08$), Model & Crops-Antarctic groups ($p = 0.03$) and Model & Crops-Arctic groups ($p = 0.02$) with a confidence interval of 90%. No significant changes were shown between the rest of the combinations.

However, when “Groups” was analysed, % $F_v/F_m$ recovery values greater than 50 % were observed in the case of the polar groups, with a maximum value greater than 80 % of the Antarctic group, while in the case of the Mediterranean and Model & Crops groups, they were approximately 20 %. Significant differences of about 60 % in % $F_v/F_m$ recovery were found between the Antarctica group with respect to the Mediterranean ($p = 0.08$) and Model & Crops groups ($p = 0.03$), followed by significant differences of about 40% between the Arctic and Model & Crops groups ($p = 0.04$).

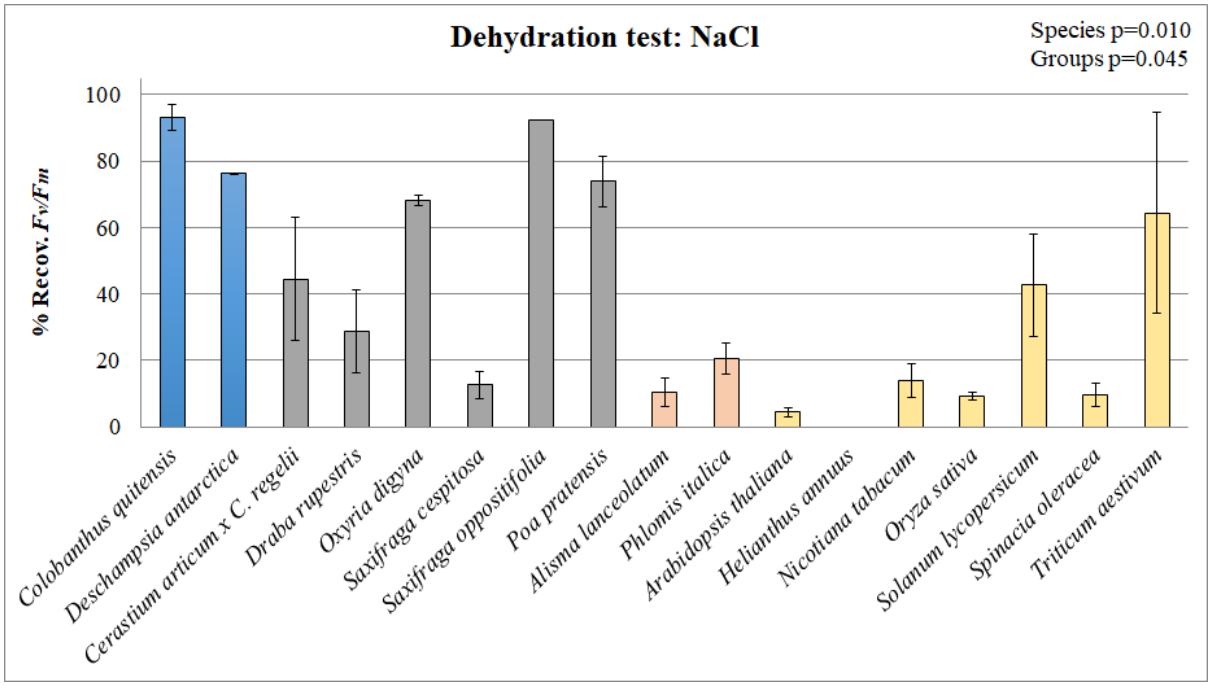


***Figure 8. Results of the dehydration test for NaCl treatment for the study species.*** *The recovery percentage of the $F_v/F_m$ after the dehydration-rehydration cycle (Y axis) as a function of the species (X axis) for NaCl treatment is shown. Colours distinguish the groups that form different species: Antarctic (blue), Arctic (grey), Mediterranean (orange) and Models & Crops (yellow). Data are average values ± ET of 3 biological replicates per species.*

Figure 9 shows the results of the recovery percentage of the $F_v/F_m$ of the dehydration test with the $MgCl_2$ treatment for the species under study. The Shapiro test showed the percentage of recovery of the $F_v/F_m$ of the dehydration test with the $MgCl_2$ treatment, with a value of $p < 0.001$. As it did not follow a normal distribution, Levene's test was applied to study the homogeneity of the variances. Levene's test showed that the study parameter had homogeneity of variances depending on the species and the group, with a value of $p = 0.79$ and $p = 0.65$. The non-parametric Kruskal-Wallis test showed significant changes between species and groups, with a value of $p = 0.01$ and $p = 0.05$, respectively. The Bonferroni test could not be performed to compare between the different species because they did not have enough degrees of freedom. When "Groups" were analysed, % $F_v/F_m$ fell below 80 % for all groups, with the Antarctic group remaining at approximately 70 %, the highest of all; while the rest of the groups remained below 40 %. The Bonferroni test showed significant differences of approximately 40 % between the Antarctic and Model & Crops groups ($p = 0.05$).

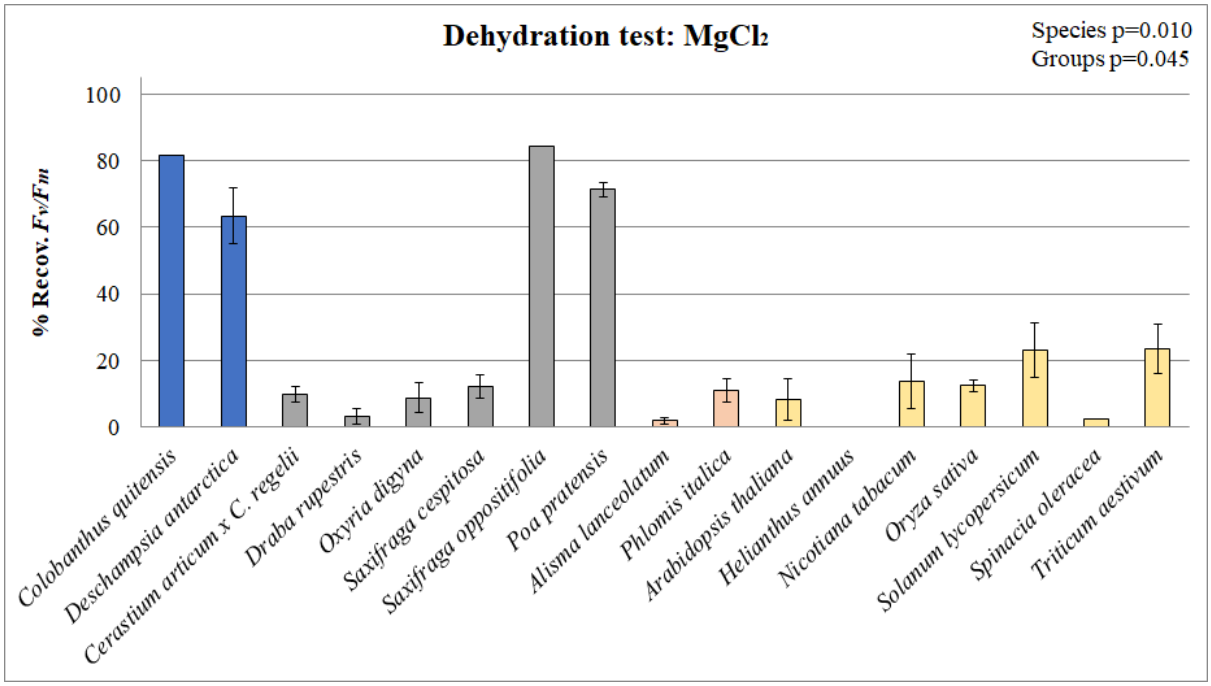


***Figure 9. Results of the dehydration test for $MgCl_2$ treatment for the study species.*** *The recovery percentage of the $F_v/F_m$ after the dehydration-rehydration cycle (Y axis) as a function of the species (X axis) for $MgCl_2$ treatment is shown. Colours distinguish the groups that form different species: Antarctic (blue), Arctic (grey), Mediterranean (orange) and Models & Crops (yellow). Data are average values ± ET of 3 biological replicates per species.*

Figure 10 shows the results of the recovery percentage of the $F_v/F_m$ of the dehydration test with the silica gel treatment for the species under study. The Shapiro test showed that the percentage of recovery of the $F_v/F_m$ of the dehydration test with the silica gel treatment, did not follow a normal distribution, with a value of $p < 0.001$. As it did not follow a normal distribution, Levene's test was applied to study the homogeneity of the variances. Levene's test showed that the study parameter had homogeneity of variances as a function of the species ($p = 0.74$), but not as a function of the group ($p = 0.03$). The non-parametric Kruskal-Wallis test showed significant changes between species and groups, with a value of $p = 0.01$ and $p > 0.01$, respectively. The Bonferroni test could not be performed to compare between the different species because they did not have enough degrees of freedom.

When “Groups” were analysed, % $F_v/F_m$ fell below 45 % for all groups, with the Antarctic group remaining at approximately 40 %, the highest of all; while the rest of the groups remained below 20 %. The Bonferroni test showed significant differences of approximately 20 % between the Antarctic and Arctic groups ($p = 0.09$), followed by

the significant differences of approximately 30 % between the Antarctic with respect to the Mediterranean (p = 0.03) and to the Model & Crops (p <0.01) groups.

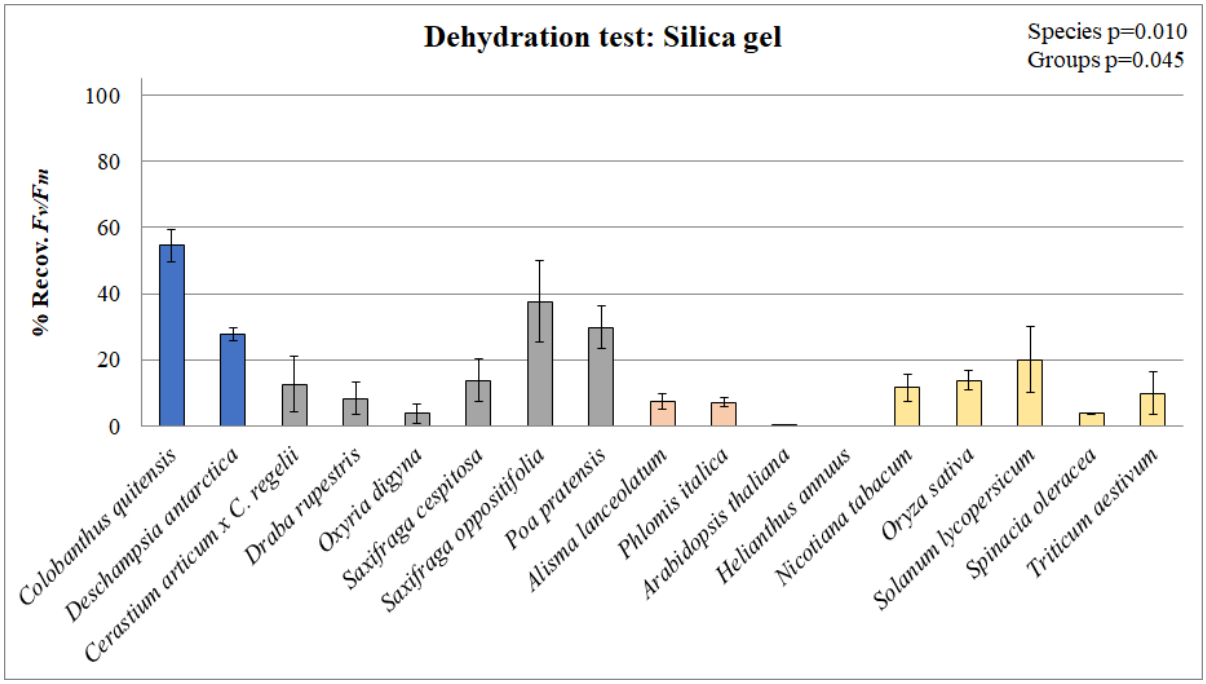


***Figure 10. Results of the dehydration test for silica gel treatment for the studied species.*** *The recovery percentage of the $F_v/F_m$ after the dehydration-rehydration cycle (Y axis) as a function of the species (X axis) for silica gel treatment is shown. Colours distinguish the groups that form different species: Antarctic (blue), Arctic (grey), Mediterranean (orange) and models & crops (yellow). Data are average values ± ET of 3 biological replicates per species.*

Finally, an analysis was carried out of the results of the recovery percentage of the $F_v/F_m$ of the dehydration test with the average of all treatments (NaCl, $MgCl_2$ and silica gel) for the species under study, shown in figure 11. Shapiro's test showed that the recovery percentage of $F_v/F_m$ from the dehydration test did not follow a normal distribution, with a value of p = 0.001. As it did not follow a normal distribution, Levene's test was applied to study the homogeneity of the variances. Levene's test showed that the study parameter did not have homogeneity of variances both as a function of the species and the group, with a value of p <0.01 in both cases. As it did not follow a normal distribution, the non-parametric Kruskal-Wallis test was applied. The analysis showed significant changes between species and groups, with a value of p <0.001 in both cases. The Bonferroni test could not be performed to compare between the different species because they did not have enough degrees of freedom.

When the "Groups" were analysed, the % $F_v/F_m$ remained below 40 % for all groups, except for the Antarctic group, which remained approximately above 60 %. The

Bonferroni test showed significant differences of approximately 50 % between the Antarctic with respect to the Mediterranean ($p < 0.001$) and Model & Crops groups ($p < 0.001$). Furthermore, significant differences of approximately 25 % were observed between the Arctic group with respect to the Model & Crops ($p = 0.04$) group. Considering all the treatments together (Figure 11), significant changes were shown between the Model & Crops group with respect to the Antarctic, in contrast to the freezing test, where greater significant changes were shown between the Model & Crops groups with respect to both polar groups: Arctic and Antarctic (Figure 7).

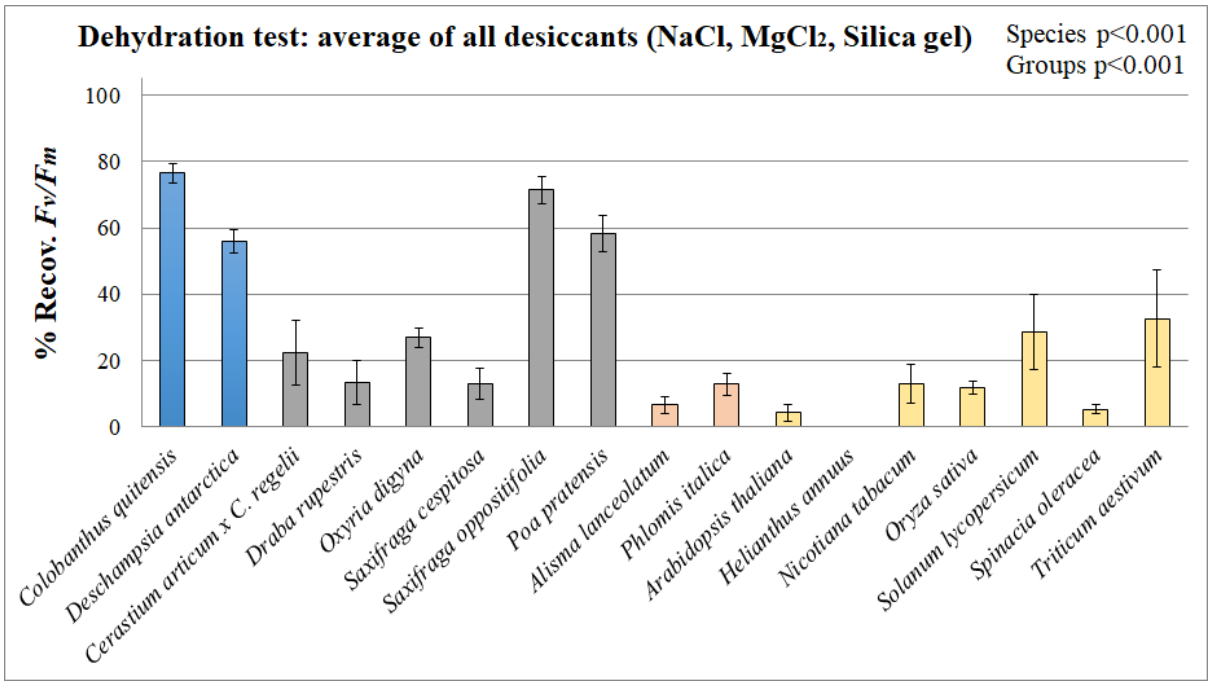


***Figure 11. Results of the dehydration test for the average of all treatments (NaCl, $MgCl_2$ and silica gel) for the studied species.*** *The recovery percentage of the $F_v/F_m$ after the dehydration-rehydration cycle (Y axis) as a function of the species (X axis) is shown. Colours distinguish the groups that form different species: Antarctic (blue), Arctic (grey), Mediterranean (orange) and Models & Crops (yellow). Data are average values ± ET of 9 biological replicates per species.*

### Freezing and dehydration tolerance: multiple stress cross-tolerance mechanisms?

In order to study if there are some cross-tolerance mechanisms to tolerate freezing and dehydration stress along our species experimental set-up, a regression analysis was carried out. The results of the regression analysis are shown in figure 12. A positive relationship was shown between both parameters; considering "species" as a factor, any significant trend was signalled by ANOVA ($p = 0.17$ and a $R^2 = 0.09$) (Figure

12a). However, when “group” was analysed a positive trend emerged (p = 0.045 and an $R^2$ = 0.636) (Figure 12b), leaded by the Antarctic group, with high recovery values for both freezing and dehydration tests, followed by the Arctic group, with high values of recovery for the freezing test, but lower than the previous group in the dehydration test; meanwhile the other groups from a template region (Mediterranean and Model & Crops) obtained lower recovery for both parameters, freezing and dehydration tests.

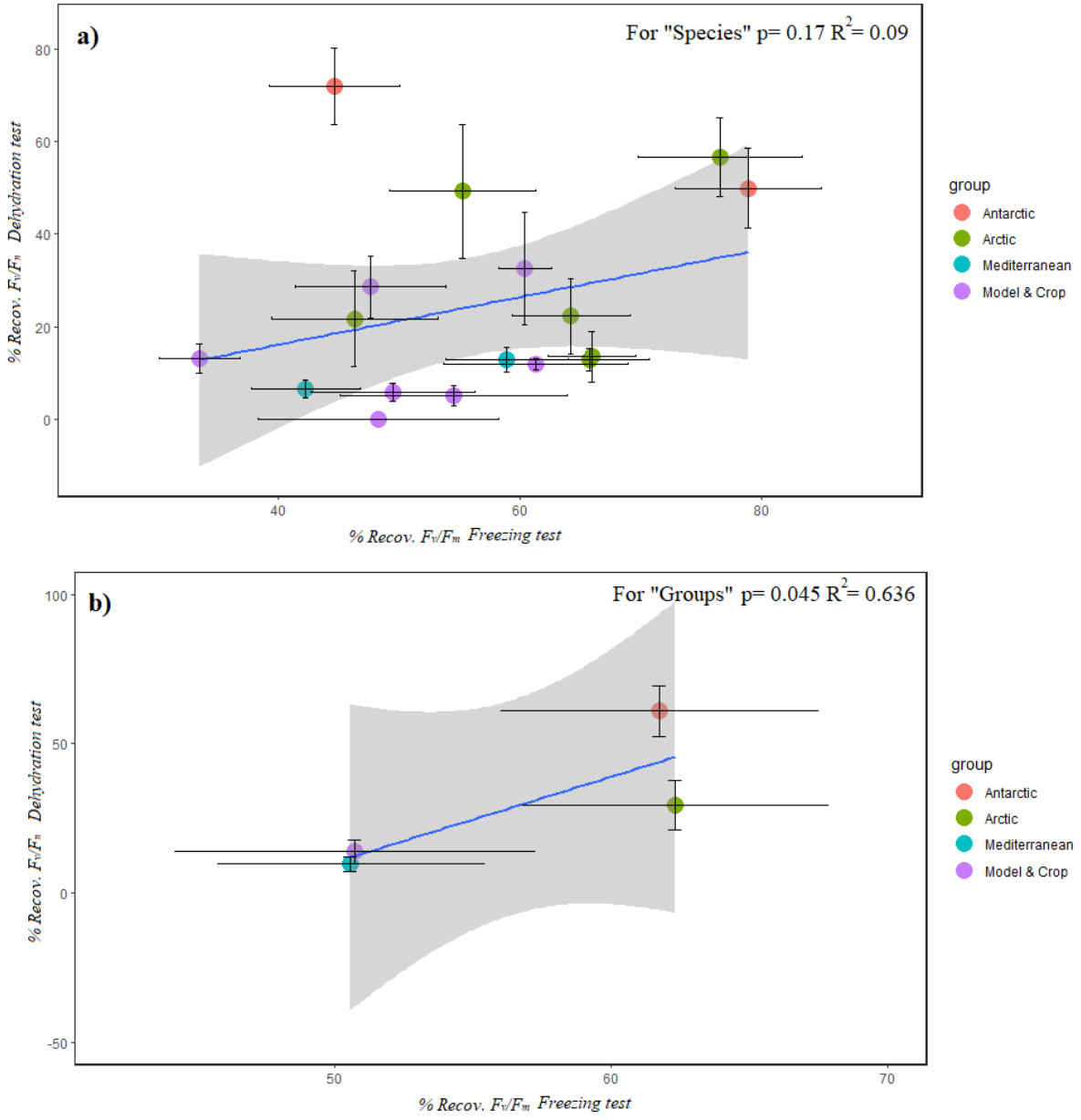


***Figure 12. Relationship between freezing and dehydration test.*** *Factor: a)”Species”; b)“Groups”. The average of $F_v/F_m$ recovery percentage after freezing cycles (target temperatures: -6, -9, -12 and -18ºC)/dehydration (treatments: NaCl, $MgCl_2$ and silica gel)-recovery is plotted for freezing (X-axis) and dehydration (Y-axis) tests, respectively. The grey shading indicates the confidence interval of the regression function (blue line).*

**Is there a trade-off between freezing and dehydration test with respect to the maximal photosynthetic capacity?**

The main hypothesis of this work is that there is a trade-off at the leaf level between maximum photosynthetic capacity ($A_{max}$) and stress tolerance. A regression analysis was carried out between the two tolerance tests studied in this work: freezing and dehydration tests with respect to the maximal photosynthetic capacity, shown in figures 13 and 14, respectively.

Figure 13 shows the regression analysis between recovery percentage of the $F_v/F_m$ of the freezing test for the average of all the target temperatures (-6, -9, -12, -18 ºC) with respect to $A_{max}$. No relationship between both parameters, neither considering "species" as a factor, nor "groups", was signalled by ANOVA, with a value of p = 0.11 and an $R^2$ = 0.145 and p = 0.57 and an $R^2$ = 0.579, respectively.

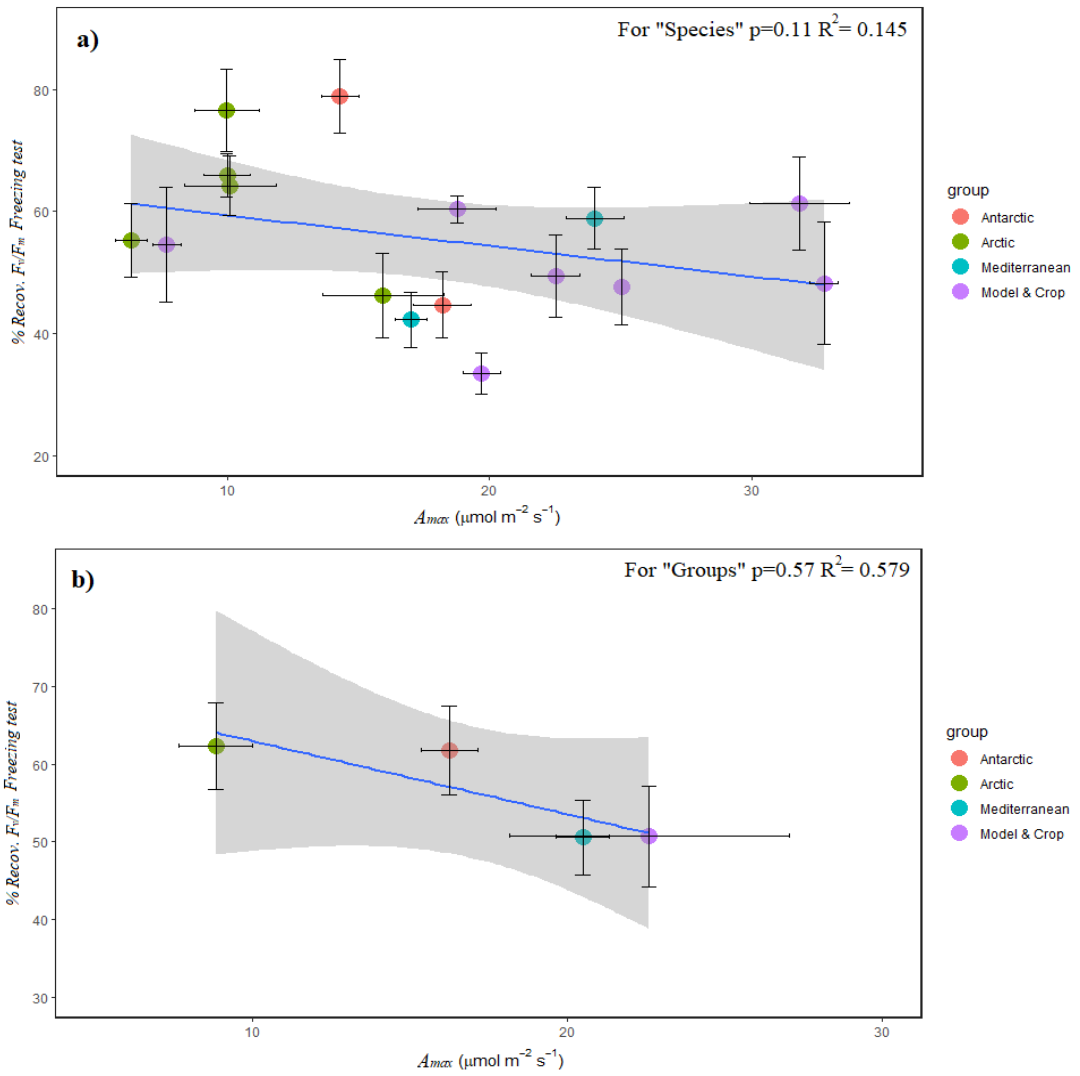

***Figure 13. Regression between the percentage of $F_v/F_m$ recovery from the freezing test for the average of all the target temperatures (-6, -9, -12, -18 ºC) with respect to the maximum photosynthetic capacity ($A_{max}$, µmol $m^{-2}$ $s^{-1}$).*** *Factor: a)"Species"; b)"Groups". The grey shading indicates the confidence interval of the regression function (blue line).*

Figure 14 shows the regression analysis between recovery percentage of the $F_v/F_m$ of the dehydration test for the average of all treatments (NaCl, $MgCl_2$ and silica gel) with respect to $A_{max}$. A negative relationship was shown between both parameters, considering "species" and "Groups" as a factor, with a value of p = 0.09 and an $R^2$ = 0.148 and p = 0.07 and an $R^2$ = 0.618, respectively. Relationship was leaded by the Model & Crops group, with the highest $A_{max}$ and lower recovery for the dehydration test values, followed by the Mediterranean group, with similar recovery for the dehydration test values, but lower $A_{max}$ than the previous group; meanwhile the other groups from a polar region (Antarctic and Arctic) obtained lower $A_{max}$, but higher recovery of the dehydration test.

The negative relationship between both study parameters may be due to the limited number of resources available to plants. So, they could not satisfy demands of growth, defense and reproduction simultaneously (Tuller et al., 2018)**.** In this way, through different mechanisms, they can promote the redirection of energy and components of the primary metabolism (involved in growth) towards defense processes against the threat, which could compromise growth (Züst & Agrawal, 2017).

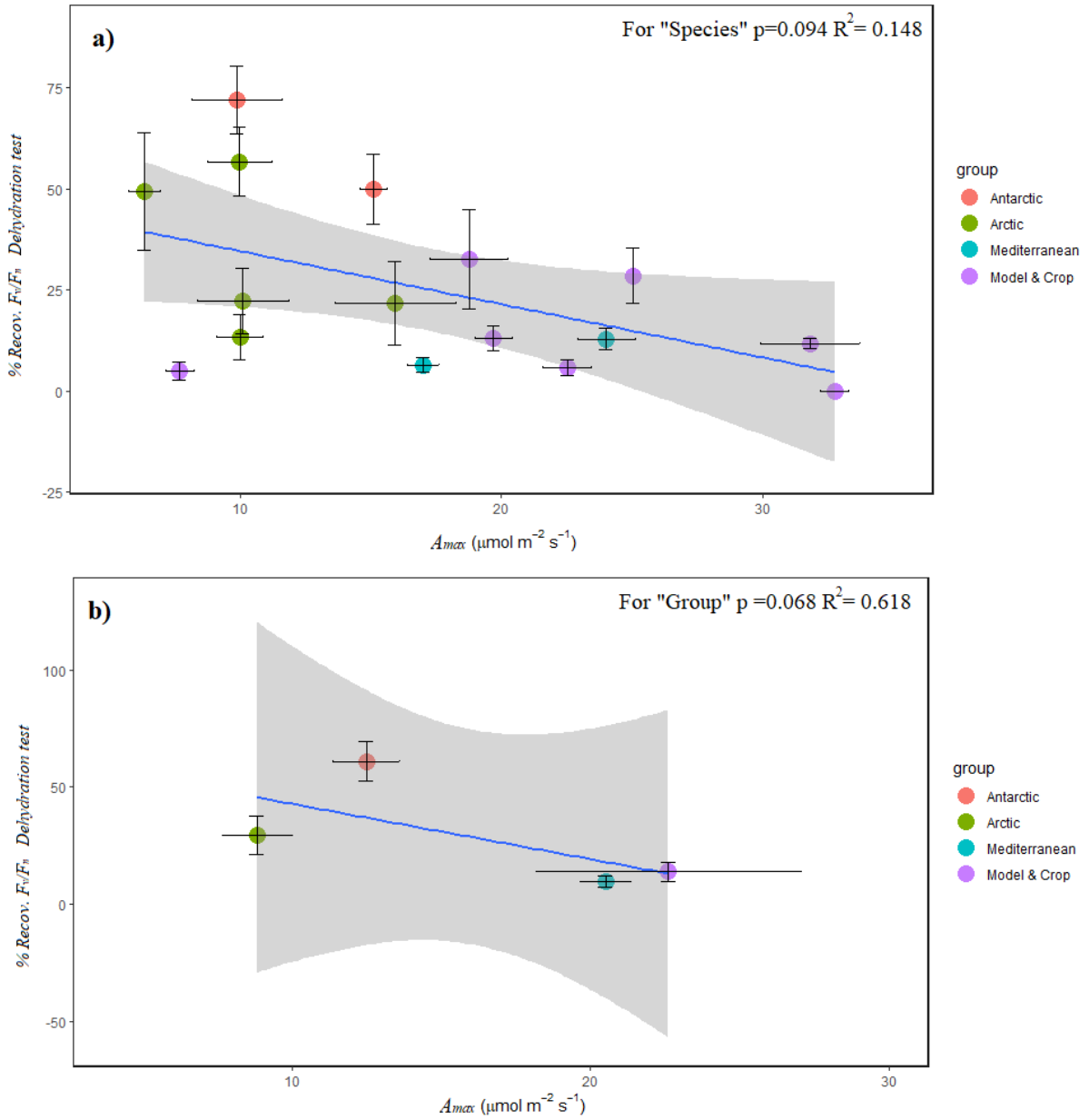


***Figure 14. Regression between the percentage of $F_v/F_m$ recovery from the dehydration test for the average of all treatments (NaCl, $MgCl_2$ and silica gel) with respect to the maximum photosynthetic capacity ($A_{max}$, µmol $m^{-2}$ $s^{-1}$).*** *Factor: a)"Species"; b)"Groups". The grey shading indicates the confidence interval of the regression function (blue line).*

### General screening of the relationships between $A_{max}$, stress tolerance, leaf anatomy and biochemistry

One of the hypotheses of this work is that the trade-off at leaf level between the maximal photosynthetic capacity ($A_{max}$) and stress tolerance can be explained by leaf anatomy and biochemistry/antioxidant metabolism traits. A Pearson correlation heatmap was performed (figure 15) to explore the relationship between anatomical and biochemical parameters with respect to stress tolerance (freezing and dehydration

tests) and maximum photosynthetic capacity in field conditions ($A_{max}$ dehydration) and in laboratory conditions ($A_{max}$ freezing), as described in the Material & Methods section. The results showed a significant negative relationship between $A_{max}$ and $T_{chl}$ ($A_{max}$ dehydration data, p <0.01). In addition, a significant positive relationship was observed between $A_{max}$ and $S_c/S$ ($A_{max}$ dehydration data, p = 0.03), that becomes more significant when also consider the cell thickness in a ratio between both $(S_c/S)/CWT$ (p = 0.02), and also positive for $(S_m/S)/CWT$ (p = 0.03). $A_{max}$ (freezing data) showed a very similar trend with significant positive relationship for $S_c/S$ and $(S_c/S)/CWT$ (p = 0.05). For recovery from the dehydration test it was observed significant negative and positive relationships for $S_c/S$ and $T_{chl}$, respectively. The results show significant relationships between the anatomical parameters with respect to maximum photosynthesis and the dehydration test. However, no significant relationship was observed between freezing and the antioxidant biochemistry data with respect to the rest of the parameters.

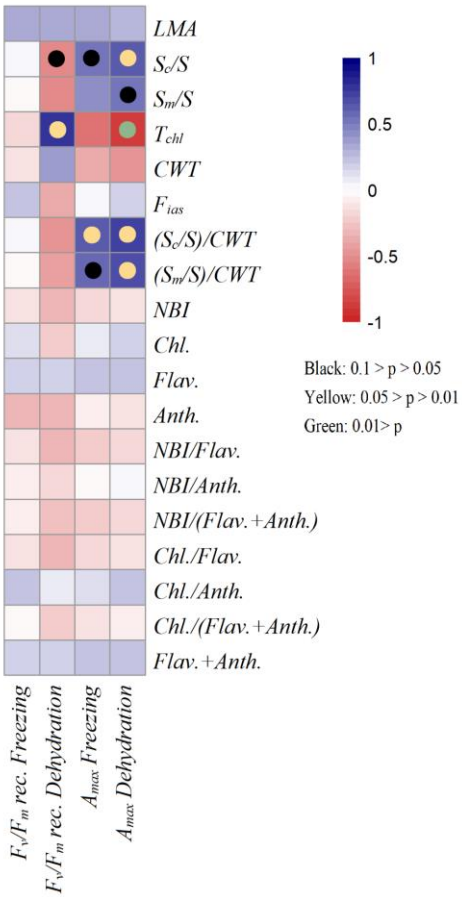


***Figure 15. Pearson's correlation heat map*** *of the anatomical (LMA, $S_c/S$, $S_m/S$, $T_{chl}$, CWT, $f_{ias}$,$(S_c/S)/CWT$ and $(S_m/S)/CWT$) and biochemical (NBI, chlorophyll a, flavanols,, anthocyanins, NBI/flavanols, NBI/anthocyanins, NBI/(flavanols+anthocyanins), Chl./flavanols,*

*Chl./anthocyanins, Chl./(flavanols+anthocyanins) and flavanols+anthocyanins) parameters with respect to the % $F_v/F_m$ recovery from the freezing and dehydration test; and the maximum photosynthetic capacity ($A_{max}$) data used for the freezing and dehydration test, respectively. Blue and red signify positive and negative relationships, respectively. Significant correlations with a confidence interval of 90, 95 and 99 %, are underlined in black, yellow and green, respectively. LMA, leaf mass per unit area (g $m^{-2}$); $S_c/S$, chloroplast surface exposed to mesophilic air spaces ($m^2$ $m^{-2}$); $S_m/S$, cellular surface exposed to mesophilic air spaces ($m^2$ $m^{-2}$); $T_{chl}$, chloroplast thickness (µm); CWT, cell wall thickness (µm); $f_{ias}$, air spaces fraction inside the leaf (%); NBI, nitrogen balance index; Chl., Chlorophyll a (µg/$cm^2$); Flav.; flavanols (relative units); Anth., anthocyanins (relative units).*

### Is the trade-off between $A_{max}$ and Stress Tolerance driven by anatomical and/or biochemical traits?

With the aim of deepening the relationship between foliar anatomical features with respect to maximal photosynthetic capacity and stress tolerance, ANOVA analysis at species level and group level (origin biome) were carried out after the exploration by Pearson's correlation analysis of the heatmap. In the figures 16, 17 and 18 is shown the previous significant relationships between $S_c/S$ and $T_{chl}$ versus $A_{max}$; and $T_{chl}$ versus the recovery from the dehydration test.

Figure 16 shows the regression analysis between $S_c/S$ with respect to $A_{max}$. A positive relationship was shown between both parameters; considering “group” as a factor, any significant trend was signalled by ANOVA ($p = 0.24$ and an $R^2 = 0.668$). However, when “species” was analysed a positive trend emerged ($p = 0.05$ and an $R^2 = 0.412$), leaded by the species of the Model & Crops group, showing the highest values for both parameters, $A_{max}$ and $S_c/S$; meanwhile species from the Antarctic group obtained the lowest $A_{max}$ and $S_c/S$ values.

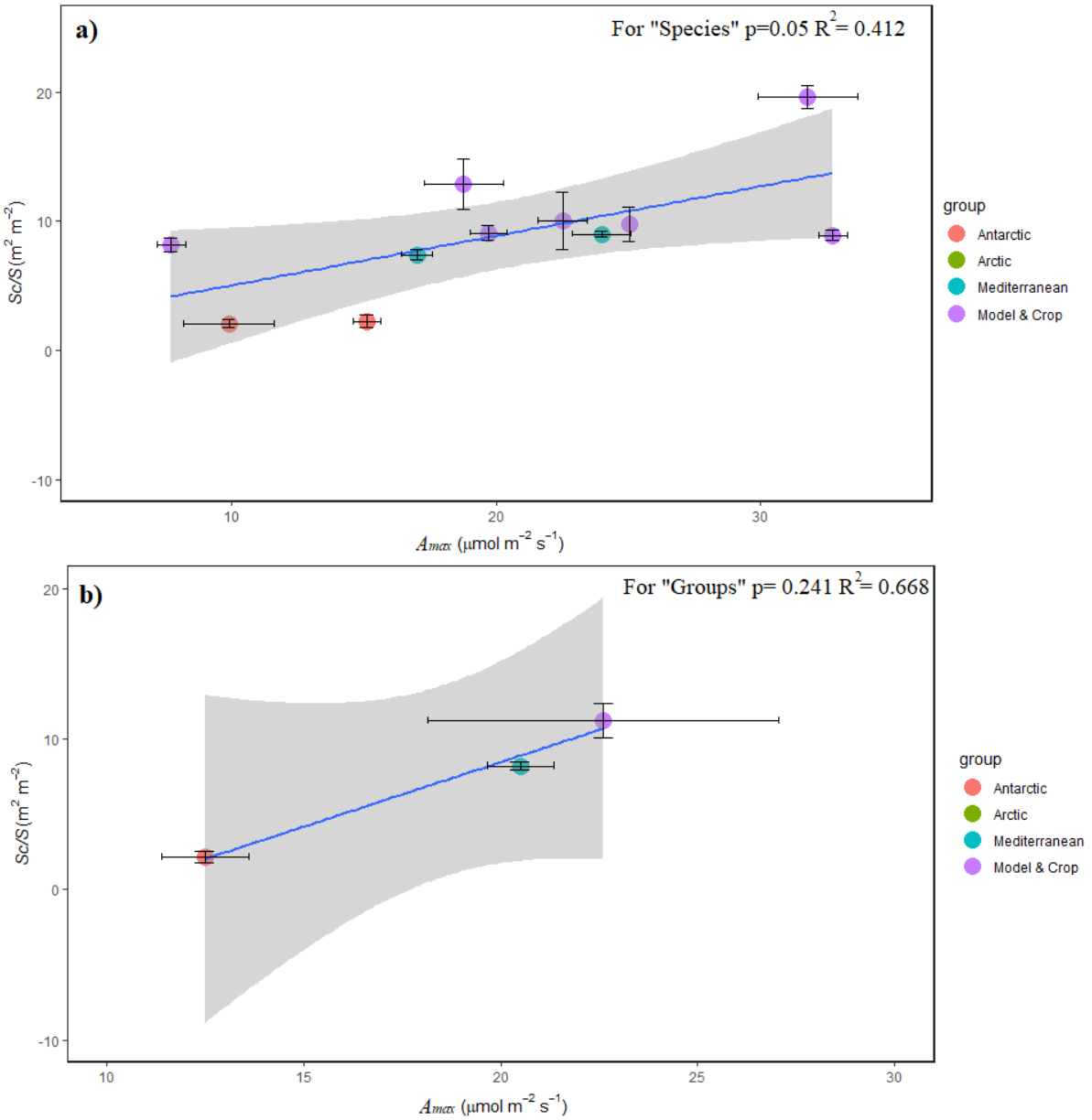


***Figure 16. Regression between chloroplast surface exposed to mesophilic air spaces ($S_c/S$, $m^2$ $m^{-2}$) and maximal photosynthetic capacity ($A_{max}$, µmol $CO_2$ $m^{-2}$ $s^{-1}$).*** *Factor: a)"Species"; b)"Groups". The grey shading indicates the confidence interval of the regression function (blue line).*

Figure 17 shows the regression analysis between $T_{chl}$ with respect to $A_{max}$. A negative relationship was shown between both parameters; considering "group" as a factor, any significant trend was signalled by ANOVA (p = 0.38 and an $R^2$ = 0.977). However, when "species" was analysed a positive trend emerged (p = 0.01 and an $R^2$ = 0.720), leaded by the species of the Model & Crops group, showing the lowest values of $T_{chl}$ content and the highest of $A_{max}$; meanwhile species from the Antarctic group obtained the lowest $A_{max}$ values, but the highest $T_{chl}$ values.

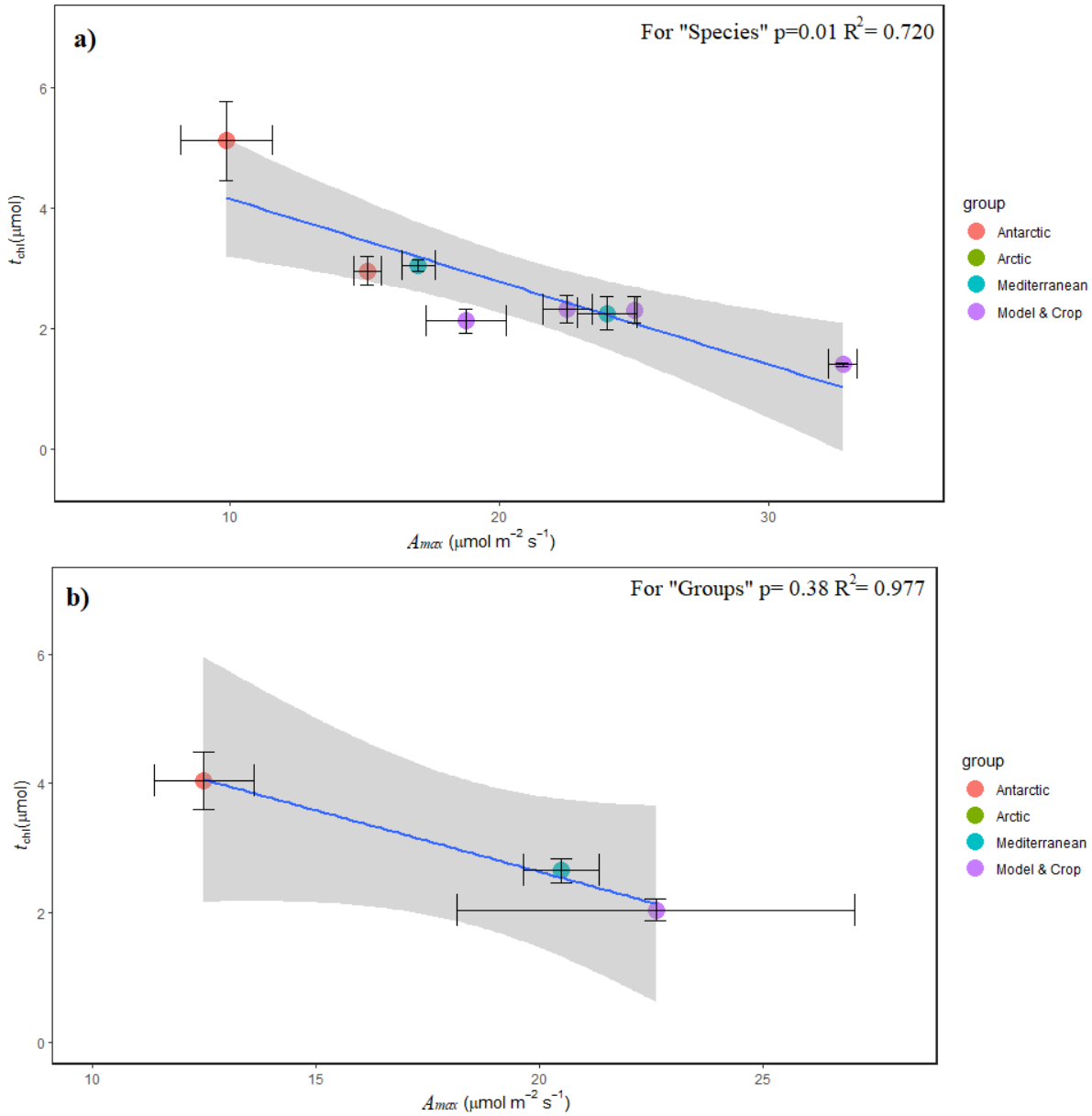


***Figure 17. Regression analysis between chloroplast thickness ($T_{chl}$, µm) and maximal photosynthetic capacity ($A_{max}$, µmol $CO_2$ $m^{-2}$ $s^{-1}$).*** *Factor: a)"Species"; b)"Groups". The grey shading indicates the confidence interval of the regression function (blue line).*

Finally, Figure 18 shows the only regression analysis with significant results between an anatomical parameter with respect to a stress tolerance test: $T_{chl}$ with respect to the recovery from the dehydration test. A positive relationship was shown between both parameters; considering "group" as a factor, no significant trend was signalled by ANOVA (p = 0.26 and an $R^2$ = 0.956). However, when "species" was analyzed a positive trend emerged (p = 0.03 and an $R^2$ = 0.628), leaded by the species of the Antarctic group, showing the highest values of both parameters, $T_{chl}$ content and $A_{max}$;

meanwhile species from the Mediterranean and Model & Crops groups obtained lower $T_{chl}$ content and % recovery values.

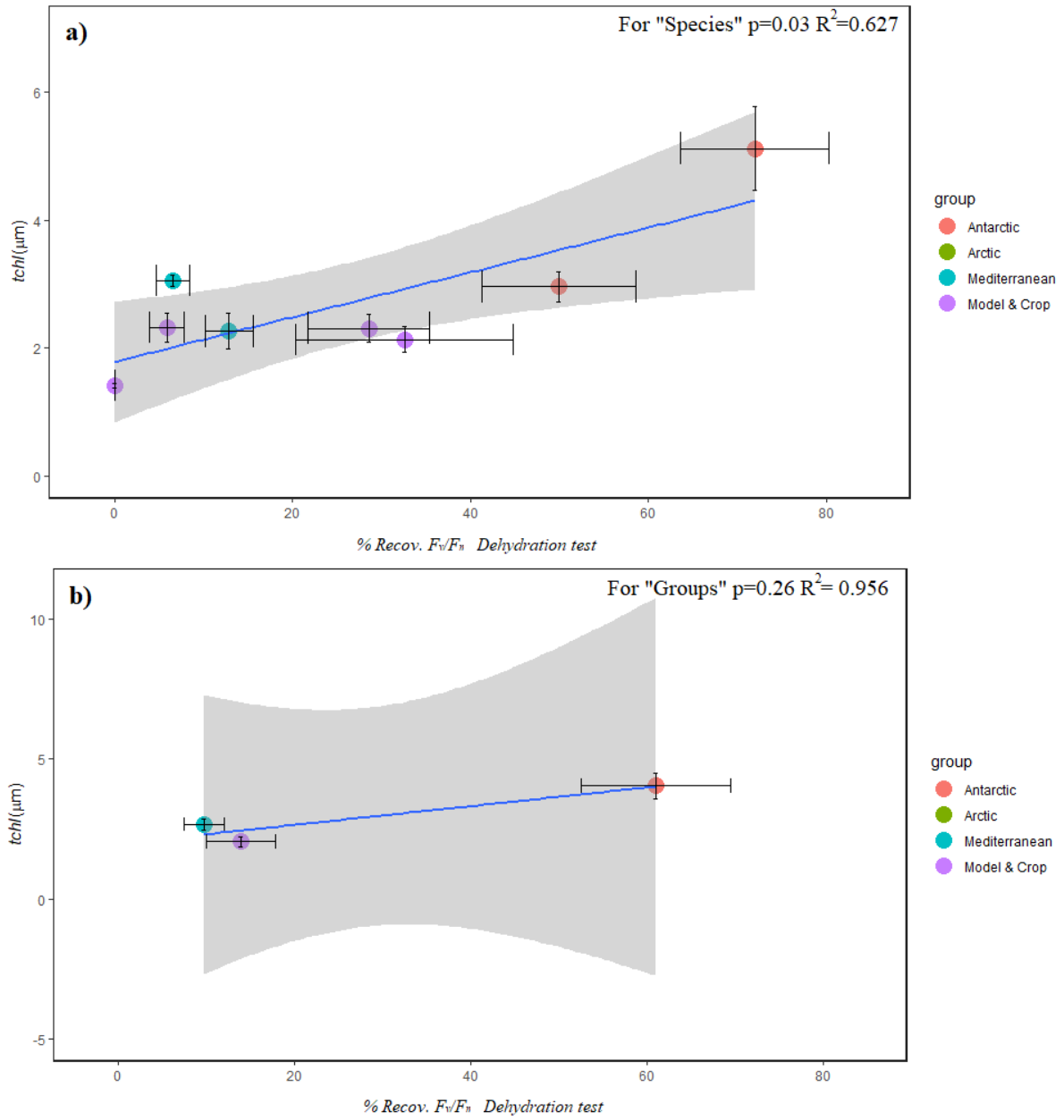


***Figure 18. Regression between chloroplast thickness ($T_{chl}$, µm) and the percentage of $F_v/F_m$ recovery from the dehydration test.*** *Factor: a)"Species"; b)"Groups". The grey shading indicates the confidence interval of the regression function (blue line).*

For this case, it was observed that all the results of significant relationships of the regression analyses between the foliar anatomical characteristics with respect to the maximum photosynthesis and the dehydration test were observed between species, but, in no case, the regressions were significant between the different study groups.

All biochemical parameters (*NBI, chlorophyll a, flavanols, anthocyanins, NBI/flavanols, NBI/anthocyanins, NBI/(flavanols+anthocyanins), Chl./flavanols, Chl./anthocyanins, Chl./(flavanols+anthocyanins)* and *flavanols+anthocyanins*) were also analyzed by one-way ANOVA. No relationship between both parameters, neither considering "species" as a factor, nor "groups", was signalled by ANOVA with respect to $A_{max}$, and the recovery from both, the freezing and dehydration tests.

## DISCUSSION

### Freezing tolerance

In case of freezing tolerance, our results showed a decrease in the percentage of the $F_v/F_m$ recovery as a function of the decreasing freezing temperatures applied in the different treatments (-6, -9, -12 and -18 ºC). With the highest target temperature with this methodology (-6 ºC), all species showed the ability to tolerate this freezing stress, with a recovery above 90 %, which suggests that there were no high disturbances of the status of the species at this temperature. However, a decrease of only 3 ºC, (plus the increase in the exposure time to this target temperature), with a target temperature of -9 ºC, resulted in the reduction of the recovery below 60 % for all the species of the Mediterranean and Model & Crops groups, which showed significant differences in freezing tolerance between Model & Crops group with respect to the species from the Arctic and Antarctic groups.

Polar plants could develop further resistance to freezing because of an environmental signal. Some authors have found that signal could be in autumn = 4 ºC and it could be kay in the ability to survive the next colder conditions (sub-zero temperatures) which triggers a freeze tolerance mechanism (Bravo et al., 2001; Gianoli et al., 2004); in this sense, it was found that *D. antarctica* and *C. quitensis* species from the Antarctic group have different strategies to cope with freezing temperatures. The first one was classified as a freezing-tolerant species, whereas *C. quitensis* could resist freezing by avoidance (Bravo et al., 2001).

Polar grass species could be more resistant to freezing due to the accumulation of soluble sugars (Alberdi & Corcuera, 1991). As may be a common feature with the dehydration tolerance, its mechanism is discussed in section 3 of the discussion. Furthermore, a constitutive high activity of antifreeze proteins in the apoplast (Bravo

& Griffith, 2005) could be involved in the cryoprotection against freezing stress in polar species (Doucet et al., 2000).

At the target temperature of -12 ºC, our results showed that any of the groups reached a recovery higher than 60%, highlighting the decrease of almost 30% in the case of the Antarctic group. When the target temperature was -18 ºC, the most extreme studied in this work, a reduction below 40 % recovery for all the studied species, regardless of the group, was shown. In this sense, not only were the species from more temperate climates unable to tolerate freezing stress, but it also happened in the case of polar species, especially Antarctic species, which showed greater recovery at lower temperatures. Similar values were found in the bibliography, i. e. in the case of *N. tabacum*, a survival % around 35 % was registered (Takumi et al., 2008). Moreover, the species of the Antarctic group non-acclimated to cold temperatures (kept in a phytotron at 15ºC, as was done in this experiment) also showed low survival at such extreme temperatures (Bravo et al., 2001)**.**

**Dehydration tolerance**

Dehydration tolerance refers to the set of defense mechanisms of plants that allow balancing their internal water potential with that of the environment, followed by recovery after rehydration, in order to reduce the impact of dehydration damage on plant performance (Verhoeven et al., 2021b). Desiccation is the final step of dehydration, once the state of the water is balanced with air, considered when RWC< 30 % recovery. A distinction is made between plant species tolerant and sensitive to desiccation. Desiccation tolerant plants are those that can resist complete cellular dehydration, in which the water content (RWC) of photosynthetic tissues is below 30%. In contrast, plants sensitive to desiccation are those that cannot survive a water loss > 70% (López-Pozo et al., 2019). In this sense, dehydration tolerance is a continuum from DS plants with low tolerance, through plants with intermediate tolerances, to plants with very high DT. Furthermore, it is infrequent for angiosperms to be desiccation tolerant (Nadal et al., 2021).

Our results showed a decrease in the percentage of the $F_v/F_m$ recovery as the desiccant was stronger. With the lower desiccant used in this TFM (NaCl), Mediterranean and Model & Crops showed a lower capacity to tolerate dehydration stress, with a recovery value of approximately 20 %, thus showing the high sensitivity

to dehydration of these species (figure 8). Previous studies using species from our Model & Crops, such as *H, annuus* and *Triticum aestivum* showed similar recovery values and classified them as DS (López-Pozo et al., 2019). While polar groups showed a higher capacity, lidered by the Antarctic group, with a recovery value greater than 80 % and followed by the Arctic group, with a recovery value of approximately 60 % (figure 8). The results showed proportional decreases between the different groups as the desiccant was stronger ($MgCl_2$ and silica gel), always keeping the Antarctic group with the highest recovery and the Mediterranean and Model & Crops groups at the tail (figures 9 and 10). Greater significant differences were found between the Antarctic groups with respect to the Mediterranean and Model & Crops groups, in the three treatments, showing a high tolerance to desiccation in the case of the Antarctic group and a low in the case of the Mediterranean and Model & Crops groups (figure 11). The Arctic group could have an intermediate tolerance to desiccation.

Polar species can prevent desiccation due to the reduced height of the shoots and changes in the shape of the leaves, which is associated with the prevention of desiccation by convection caused by the wind in environments of high altitude or latitude (Korner, 2003)**.** Additionally, these species showed high efficiency in water use rates, and their anatomical characteristics are similar to those observed from species from xerophytic environments (Sáez et al., 2017), altogether, indicating significant adaptations to survive to dry environments, and so, explaining their high recovery values under this dehydration test.

**Are there common mechanisms for dehydration and freezing tolerances?**

It has been observed that the polar groups have a higher freezing and dehydration tolerance capacity than the Mediterranean and Model & Crops groups at moderate stress level. Moreover, our results of the regression analysis between both stress tolerances, considering all treatments for both tests and using “Groups” as a factor, demonstrated a positive significant relationship among them, shown in figure 12b. The polar species showed the greatest capacity for tolerance to both types of stress, while the species from more temperate zones showed the opposite behaviour. Hence, there could be common mechanisms for the tolerance of both stresses.

In the case of the Mediterranean and Model & Crops groups, the low capacity to tolerate different stresses could be due to different processes, some of them could be

common for dehydration and freezing stress. First, extracellular ice formation or drought leads to cell dehydration to maintain the balance of water pressure between the inside and outside of the cell, which induces the cell contraction, decreasing the distances between membranes (McDowell, 2011). At the same time, the growth of extracellular crystals in case of freezing stress, causes a cellular mechanical deformation that can lead to cell lysis. Together they result in increased membrane damage and cell death (Fujikawa' et al., 1999; Nagao et al., 2007). On the other hand, freezing temperatures or drought can affect photosynthetic efficiency, favouring a situation of photoinhibition and, therefore, a lower capacity for assimilating sunlight, which translates into a situation of oxidative stress and direct damage to the photosynthetic components (Román-Figueroa et al., 2021).

Polar grass species could have several anatomical xerophytic characteristics such as small leaf and epidermal cells and thick leaves, among others, which could be involved in tolerance to both stresses (Giełwanowska et al., 2005; Romero et al., 1999). However, the literature shows that, in the case of *C. quitensis*, individual morphology may not be as important as in other species that grow in cold environments, which is attributed to cushion-shaped growth, which moderates the effects of extreme environments (Armesto et al., 1980).

Polar species may also be more dehydration and freezing resistant due to the presence of turgid papillae, which could be a carbohydrate storage mechanism (Romero et al., 1999). The accumulation of soluble sugars is considered a functional response to cold stress in plants (Alberdi & Corcuera, 1991). *D. antarctica*, which showed one of the highest recoveries in this study at moderate freezing and dehydration stress, has been reported a high concentration of different non-structural carbohydrates (Chatterton et al., 1989; Piotrowicz-Cieslak et al., 2005; Zúñiga-Feest et al., 2003, 2009; Zuñiga et al., 1996). Sugar accumulation plays a role in the depression of the freezing point of cell sap and the avoidance of plasmolysis caused by cell dehydration induced by freezing (Livingston & Olien, 1989; Santarius, 1992). In this sense, species with a higher carbohydrate content should be more resistant to both stresses.

Furthermore, a constitutive high activity of stress-induced proteins such as dehydrins (Olave-Concha et al., 2004) could be involved in the cryoprotection against

dehydration and freezing stress in polar species (Doucet et al., 2000). Finally, a higher proportion of unsaturated fatty acids in the cell membranes of leaves could play an important role in freezing tolerance maintaining membrane fluidity (Zúñiga et al., 1994).

However, at more severe stress levels, all the mechanisms involved in the tolerance of polar species may not be sufficient to combat dehydration and freezing stress. This could induce a whole series of processes that lead to cell, tissue and / or organism death (explained earlier in this section) that could explain the decrease in recovery in polar species in the most extreme treatments: target temperature of -18 ºC for freezing test and silica gel desiccant in the dehydration test.

**Is there a trade-off between freezing and dehydration tests with respect to $A_{max}$?**

Taking into account that plants are sessile organisms, they have to deal with a whole set of abiotic and biotic factors (Huot et al., 2014). In that way, they need to keep growing with a constant adaptation to their external environment in order to survive and reproduce (Coley et al., 1985; Herms & Mattson, 1992). According to the “growth-defense trade-off” plants could face resource restriction by making ‘choices’: ‘to grow or defend’ (Hanley et al., 2007); understanding a trade-off as a negative association between two functions that have as the final consequence the limitation of their reproductive capacity ("fitness"). There are many ways to study the "growth-defense trade-off". The trade-off has been widely studied at least at three different levels, not mutually exclusive: (1) allocation costs, (2) genetic costs and (3) ecological costs (Züst & Agrawal, 2017). The first one may be the most commonly considered causal factor of trade-offs (Carmona & Fornoni, 2013; Pilson, 2000; Wise & Evolution, 2016). This work aims to study a specific trade-off at allocation costs level: the trade-off between the maximal photosynthetic capacity ($A_{max}$) and stress tolerance.

Growth of vegetative tissue is a key physiological process for the overall plant’s development. Crops have been improved over time focusing on growth-related and carbon partition traits to maximize productivity (Strange & Scott, 2005)**.** In this work, the growth rate of herbaceous angiosperms has been studied based on the maximum photosynthesis capacity ($A_{max}$). The maximum photosynthesis range, shown in table 2, is within that described for herbaceous-type angiosperms (Gago et al., 2019). The

species of the Mediterranean group, as well as the model plants & Crops, have $A_{max}$ values like those that are all described in the same article. In the case of Antarctic species, the range was like that described in (Edwards & Smith, 1988). The value of $A_{max}$ is within that described by other authors for *Saxifraga oppositifolia* (Sekikawa, Muraoka, & Uchida, n.d.) and *Poa pratensis* Arctic species (Selzer & Busso, 2016). The rest of the Arctic species have not been characterized, but it does drop within the range described for herbaceous angiosperms from extreme environments such as the Arctic, Antarctic and Mountain Tundra (Semikhatova & Gerasimenko, 1992).

The results of this TFM demonstrated a negative significant relationship between dehydration tolerance with respect to the maximum photosynthetic capacity, shown in figure 14. In this way, it has been shown that, as the features related to the dehydration tolerance stress capacity increase, the maximum photosynthetic capacity decreases.

However, no significant relationship was expressed between freezing tolerance versus $A_{max}$. This may be due to the limited fit of the regression line due to the use of a reduced number of individuals (n = 16). In this way, a greater number of species could be needed in order to see a freezing tolerance vs. $A_{max}$ trade-off.

**What are the elements that can direct this trade-off?**

One of the hypotheses of this work is that the trade-off at leaf level between the maximal photosynthetic capacity ($A_{max}$) and stress tolerance can be explained by leaf anatomy and biochemistry/antioxidant metabolism traits. As seen above, only a significant negative trade-off was demonstrated between $A_{max}$ and the dehydration test. Understanding the mechanisms involved in this relationship is essential to get a better understanding of this trade-off. Anatomical and biochemical determinants present a wide range of variation between species or even genotypes, which partially explains the differences in their photosynthetic capacity (Carriquí et al., 2020, 2019; Peguero-Pina et al., 2017)

The general relationship between leaf anatomy and $A_N$ is well established (Wright et al., 2004). Subsequent studies demonstrated that anatomical features are related to $A_N$ through $g_m$ (Flexas & Carriquí, 2020; Gago et al., 2020)**.** Plant anatomical studies have suggested that anatomical features, such as chloroplast surface exposed to mesophilic air spaces ($S_c/S$) is one of the strongest determinants of mesophilic

conductance (and also from $A_N$) (Carriquí et al., 2019; Terashima et al., 2011; Tosens et al., 2016). Increasing $S_c/S$ results in increasing $g_m$, and, therefore, an increase in photosynthetic capacity (Carriquí et al., 2019; Carriquí et al., 2020; Tosens et al., 2016). This was shown in our results, where a significant positive relationship between $S_c/S$ with respect to $A_{max}$ were found (figure 16a). The role of $S_c/S$ is understood as a way to shorten the $CO_2$ pathway to the carboxylation sites of the chloroplast stroma (Evans, 2021). In this regard, the closer the chloroplasts are to the cell walls (*CW*), the less route that $CO_2$ has to travel to reach the carboxylation site. Interestingly, a negative significant relationship was found between $S_c/S$ with respect to dehydration stress tolerance in the Pearson correlation analysis (Figure 15), with a confidence interval of 90 %. This same relationship was not significant using the one-way ANOVA, which could indicate the need for a greater number of individuals to be able to study the behaviour between both parameters.

The exposure of chloroplasts exposed to the surface of the mesophyll is not the only parameter of interest that could be involved in the trade-off between the maximum photosynthetic capacity and the dehydration tolerance, but also the number of chloroplasts exposed to the surface of the mesophyll, reflected by the chloroplast thickness ($T_{chl}$). In *Arabidopsis*, it has been recorded that mutants with lower number of chloroplasts, but high size reduces the $g_m$ by up to 50% compared to the wild type (Weise et al., 2015).

Interestingly, the only regression analysis with significant results between an anatomical parameter with respect to $A_{max}$ and a stress tolerance test in the Pearson correlation analysis and the one-way ANOVA was $T_{chl}$, using “Species” as factor, showing a significant negative and positive relationship between both parameters, respectively (figure 17a and 18a). Size of the chloroplast, shape and their distribution are essential traits defining photosynthetic capacity, including the ratio surface/volume that facilitates $CO_2$ diffusion inside them (Terashima et al., 2011; Tholen et al., 2008); clearly, through land plant phylogeny size of chloroplast was reducing from the basal groups (mosses, liverworts) to the most modern angiosperms, as well their exposure to the mesophyll airspaces (Gago et al., 2019). Interestingly, bigger chloroplasts are generally observed in basal groups, that are also known to have a higher frequency of "resurrection species", meanwhile angiosperms showed a much lower frequency of highly tolerant species to desiccation (Proctor & Tuba, 2002). It is possible to speculate

that thicker chloroplasts have reduced surface/volume ratio, difficulting $CO_2$ diffusion to the Rubisco carboxylation sites, and so, favouring photorespiration. Photorespiration is an old known actor dealing with oxidative stress, consuming energy and reducing equivalents that are going to be used through the N and S metabolism pathways, and so directly avoiding ROS formation when photosynthesis is blocked (Voss et al., 2013).

In the case of *D. antarctica* it has been found that some of the mesophyll chloroplasts are different, showing small vesicles or pockets. Both components increase the surface area of chloroplasts, which, as explained above, can favour the $CO_2$ diffusion to the carboxylation sites. In addition, this specie shows mitochondrias and peroxisomes very close to chloroplasts, which can facilitate the exchange of $CO_2$ between respiration and photosynthesis processes (Giełwanowska et al., 2005). Recent studies have shown that the transport of reductants from chloroplasts to the mitochondria could be through the “Malate Valve”. In this sense, the malate produced in the chloroplast could be transported and catalysed by malate dehydrogenases in the cytosol, mitochondria and peroxisomes, thus reducing the reductants concentration and increasing NADH concentration (Selinski & Scheibe, 2018). Increasing the concentration of mitochondrial NADH has been shown to drive citrate export to the chloroplast through the "Citrate Valve." The increase in the redox level induces the transformation of the Krebs cycle to a hemicycle-shaped structure, which allows the citrate exportation to the cytosol and regulates the $NADPH/NADP^+$ balance, contributing to the biosynthesis of amino acids and other compounds (Igamberdiev, 2020). In this way, mitochondrial respiration exerts a protective role, dissipating reductants from both mitochondria and chloroplast as well as providing energy and carbon skeletons (Igamberdiev, 2020).

However, faced with a situation in which the $A_N$ is blocked due to a stress situation, mitochondrial respiration tries to reverse the situation, but in turn it can generate an excess of energy (ATP), reducing power (NADH) and carbon skeletons. N and S metabolism could play a very important role in cellular homeostasis, eliminating the excess of energy, equivalents and carbon skeletons from mithochondrias (Long et al., 2015) and reducing equivalents from the photorespiration. All needed for several primary and secondary metabolic routes related to stress tolerance (Del-Saz et al., 2018; Igamberdiev, 2020).

Leaf anatomical traits are not the only elements that can explain stress tolerance, but also antioxidant metabolism. Abiotic factors such as lower temperatures or drought, lead to an oxidative stress situation, marked by an increase in the ROS concentration (Banerjee et al., 2019). ROS can trigger the oxidation of biomolecules such as carbohydrates, lipids and proteins (Mittler, 2002; Nath et al., 2016; Wyrwicka & Skłodowska, 2006). In response to stress, the secondary metabolites biosynthesis is activated, which helps to minimize the effects of different stresses by its antioxidant role (Hodaei et al., 2018; Jogawat, 2019; Knight, 1999). Sharma et al. (2019) stated that plants increase phenolic compounds concentration such as anthocyanins, flavonoids, flavanols and phenolic acids in response to low temperature, as observed in Chan et al. (2010). Nevertheless, no significant relationship between antioxidant metabolism with respect to $A_{max}$, and the recovery from both the freezing and dehydration tests was signalled by ANOVA, neither considering "species" as a factor, nor "groups". This may be due to the limited fit of the regression line due to the use of a reduced number of individuals (n = 16). In this way, a greater number of species could be needed in order to see the biochemistry role on $A_{max}$ and both dehydration and freezing tolerance.

**Perspectives and future lines**

For future perspectives, I think that the study of the trade-off between maximum photosynthetic capacity versus freezing and dehydration tolerance would be improved employing a greater number of species from the different environments. This could be a key-point to understand the mechanisms leading the new trade-off between photosynthetic capacity with respect to stress tolerance.

In addition, it would be of special interest to complete the data that have not been possible in the realization of this TFM, it is for example the anatomical data of the Arctic species. Its anatomical characterization could be key in finding a greater number of anatomical features that explain the novel trade-off found.

The expansion of the biochemical characterization, such as the study of a greater number of parameters involved in the primary and secondary metabolism, could expand the knowledge about the antioxidant mechanisms involved in the trade-off found.

All this, together, could increase knowledge about the strategies involved to improve crop tolerance without penalty in photosynthetic capacity, a key aspect in the global change scenario.

## CONCLUSIONS

1. Polar groups (Antarctic and Arctic) showed a greater tolerance capacity to freezing and dehydration stresses than the groups from more temperate regions (Mediterranean and Model & Crops).
2. Our results demonstrated a significant positive relationship between freezing and dehydration stresses, so there could be common mechanisms for the tolerance of both stresses.
3. The results of this work demonstrated a novel trade-off between dehydration tolerance with respect to the maximum photosynthetic capacity.
4. No significant relationship was found between freezing tolerance with respect to the maximum photosynthetic capacity.
5. The trade-off at leaf level between the maximal photosynthetic capacity ($A_{max}$) and dehydration tolerance can be explained by leaf anatomy traits, as $S_c/S$ and $T_{chl}$, but no by biochemistry/antioxidant metabolism traits studied.

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## SUPPORTING INFORMATION

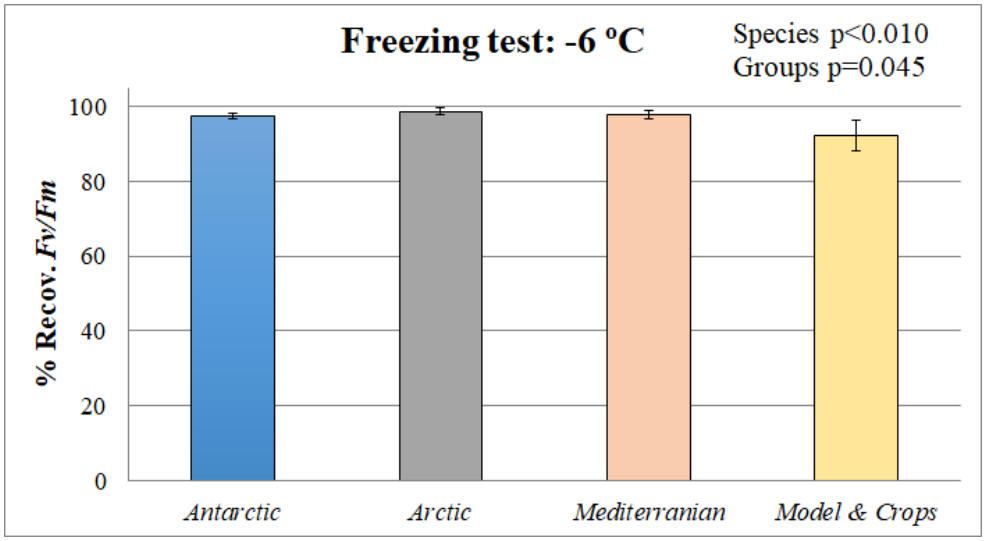


***Figure S1. Results of the freezing test for -6 ºC target temperature between groups.*** *The recovery percentage of the $F_v/F_m$ after the freezing-recovery cycle (Y axis) as a function of the group (X axis) is shown. Colours distinguish the groups that form different species: Antarctic (blue), Arctic (grey), Mediterranean (orange) and Models & Crops (yellow). Data are average values ± ET of 6 biological replicates per species.*

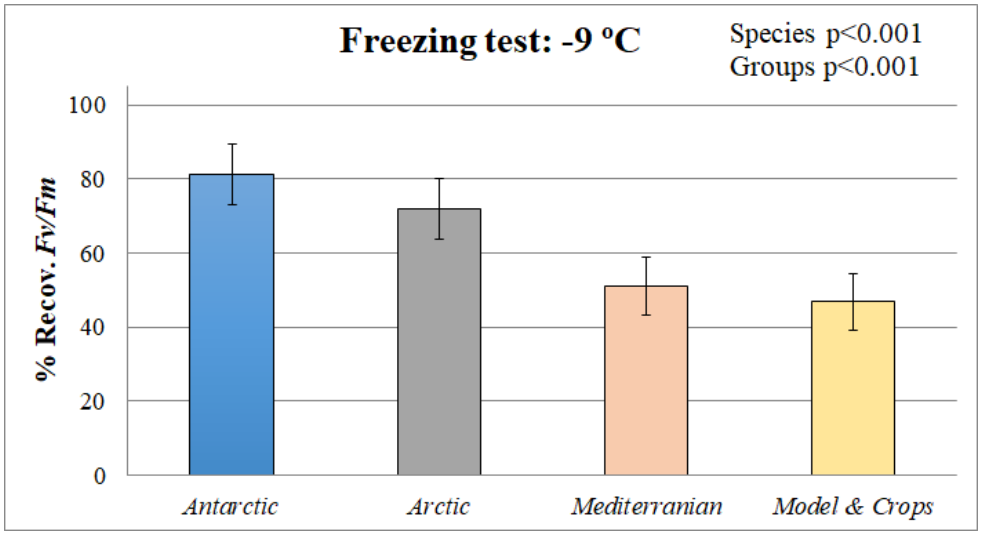


***Figure S2. Results of the freezing test for -9 ºC target temperature between species.*** *The percentage of the $F_v/F_m$ recovery after the freezing-recovery cycle (Y axis) as a function of the group (X axis) is shown. Colours distinguish the groups that form different species: Antarctic (blue), Arctic (grey), Mediterranean (orange) and Models & Crops (yellow). Data are average values ± ET of 6 biological replicates per species.*

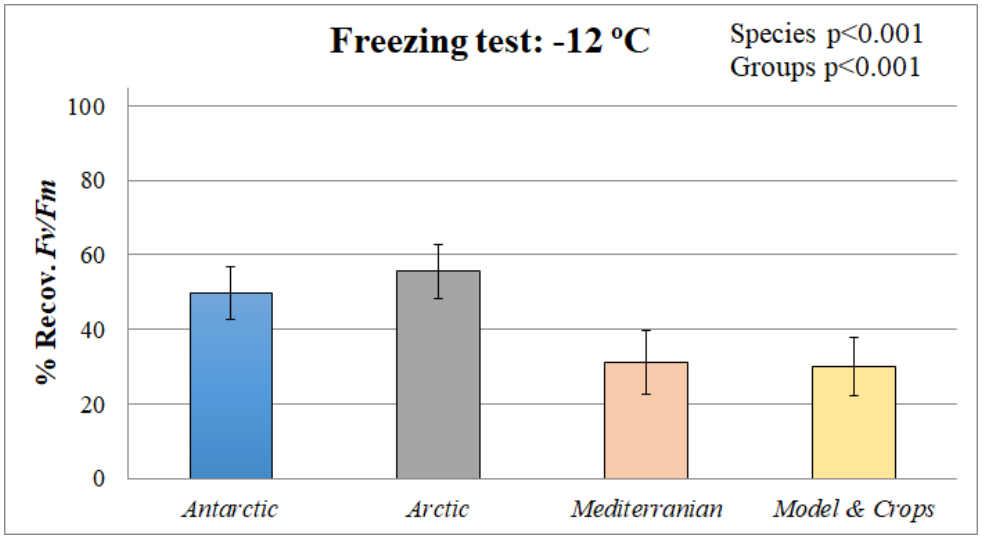


***Figure S3. Results of the freezing test for -12 ºC target temperature between species.*** *The percentage of the $F_v/F_m$ recovery after the freezing-recovery cycle (Y axis) as a function of the group (X axis) is shown. Colours distinguish the groups that form different species: Antarctic (blue), Arctic (grey), Mediterranean (orange) and Models & Crops (yellow). Data are average values ± ET of 6 biological replicates per species.*

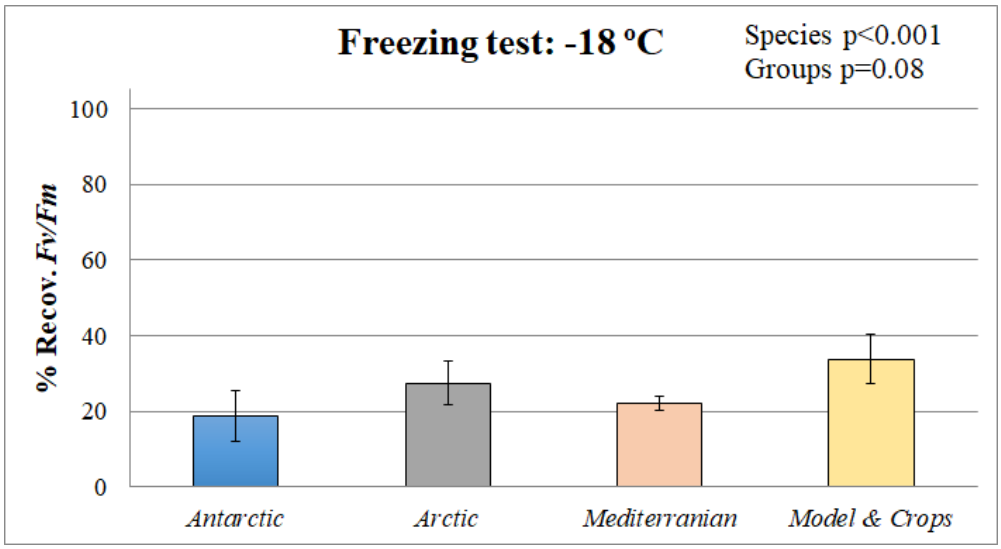


***Figure S4. Results of the freezing test for -18 ºC target temperature between groups.*** *The percentage of the $F_v/F_m$ recovery after the freezing-recovery cycle (Y axis) as a function of the group (X axis) is shown. Colours distinguish the groups that form different species: Antarctic (blue), Arctic (grey), Mediterranean (orange) and Models & Crops (yellow). Data are average values ± ET of 6 biological replicates per species.*

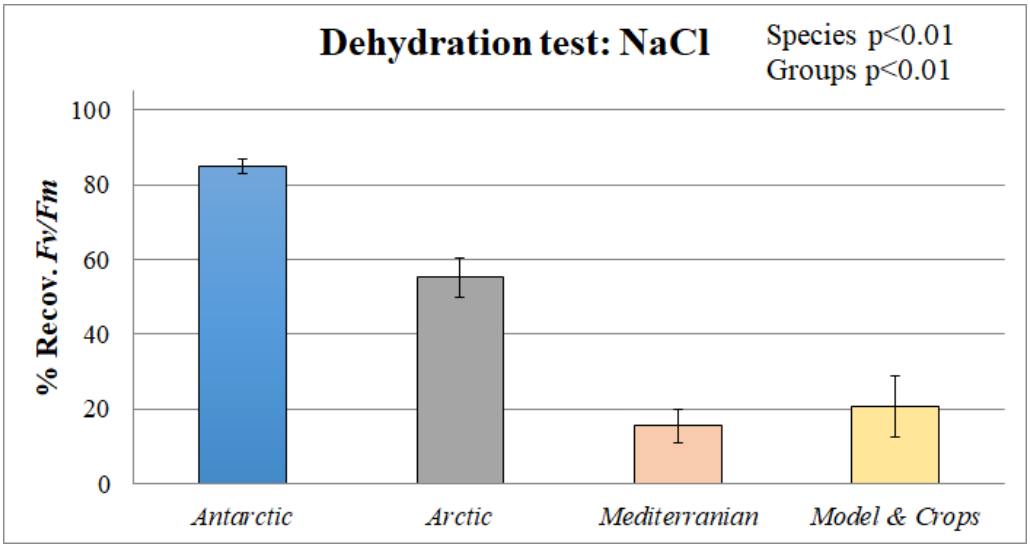


***Figure S5. Results of the dehydration test for NaCl treatment between groups.*** *The recovery percentage of the $F_v/F_m$ after the dehydration-rehydration cycle (Y axis) as a function of the group (X axis) for NaCl treatment is shown. Colours distinguish the groups that form different species: Antarctic (blue), Arctic (grey), Mediterranean (orange) and Models & Crops (yellow). Data are average values ± ET of 3 biological replicates per species.*

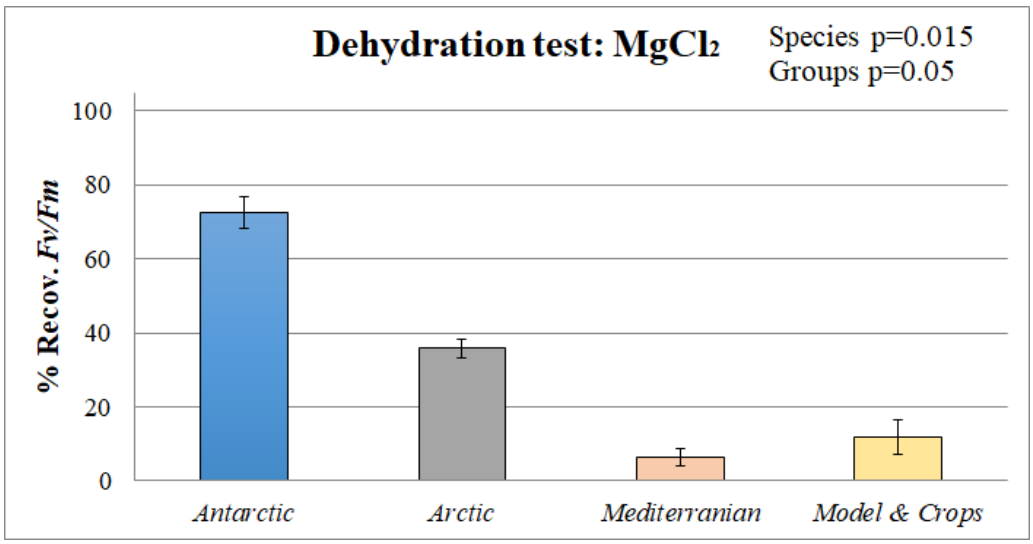


***Figure S6. Results of the dehydration test for $MgCl_2$ treatment between groups.*** *The recovery percentage of the $F_v/F_m$ after the dehydration-rehydration cycle (Y axis) as a function of the group (X axis) for $MgCl_2$ treatment is shown. Colours distinguish the groups that form different species: Antarctic (blue), Arctic (grey), Mediterranean (orange) and Models & Crops (yellow). Data are average values ± ET of 3 biological replicates per species.*

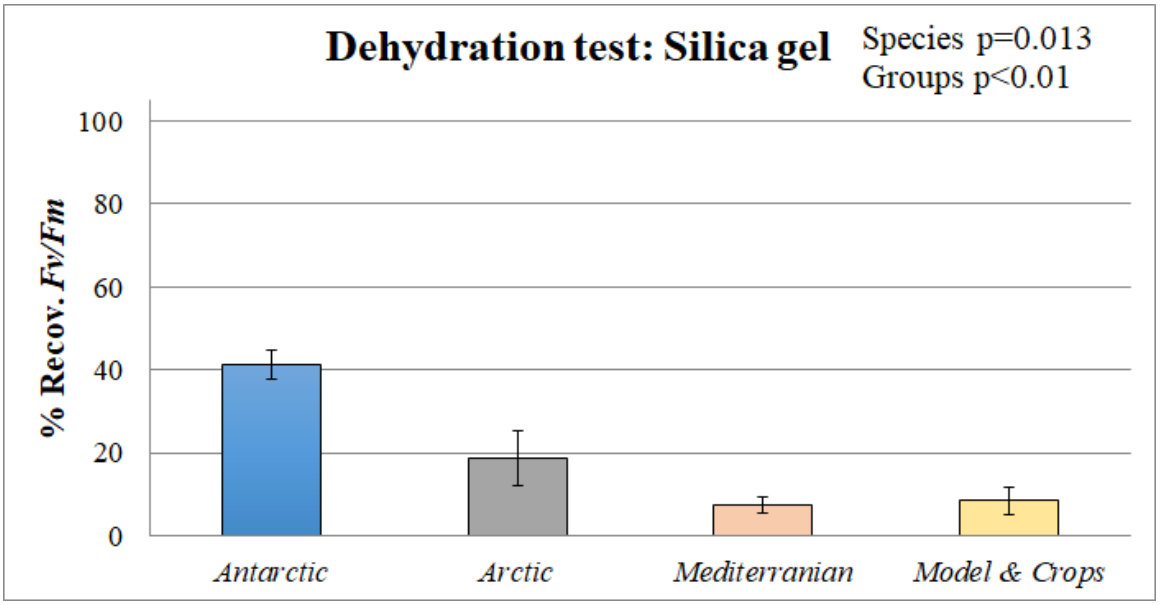


***Figure S7. Results of the dehydration test for silica gel treatment between groups.*** *The recovery percentage of the $F_v/F_m$ after the dehydration-rehydration cycle (Y axis) as a function of the species (X axis) for silica gel treatment is shown. Colours distinguish the groups that form different groups: Antarctic (blue), Arctic (grey), Mediterranean (orange) and models & crops (yellow). Data are average values ± ET of 3 biological replicates per species.*

As indicated in the materials & methods section, replicates dehydrated (RWCd) below 30% were employed for the final $F_v/F_m$ recovery assessment. To ensure this selection criteria, a frequency histogram of the percentage of RWCd was carried out, shown in figure S8, in which it is observed that all the data used were below 30% of the RWCd.

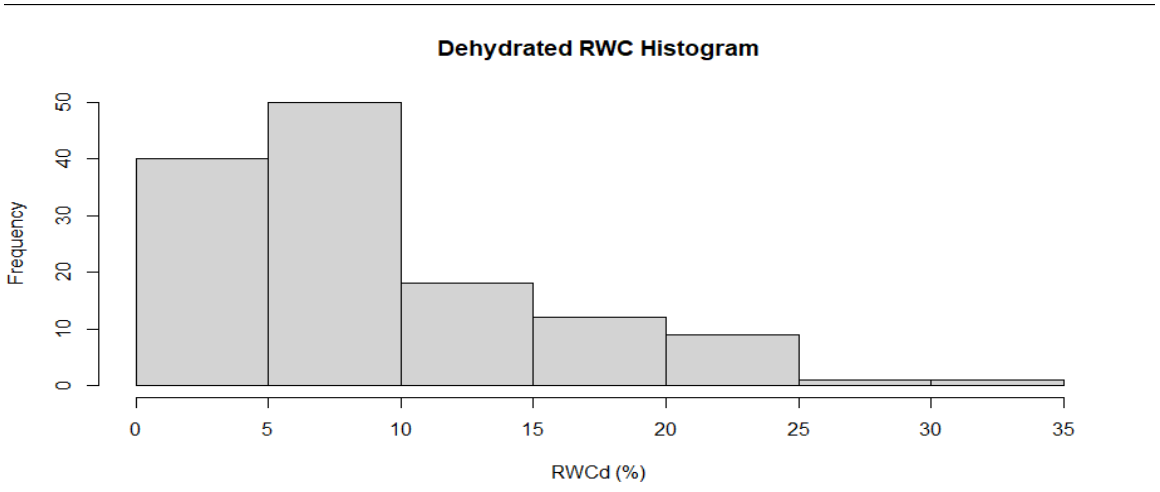


***Figure S8. Results of frequency histogram of the percentage of RWCd.*** *The frequency (Y axis) as a function of the relative water content under dehydration conditions (RWCd) after 24 h of leaf submission in each treatment (X axis) is shown.*