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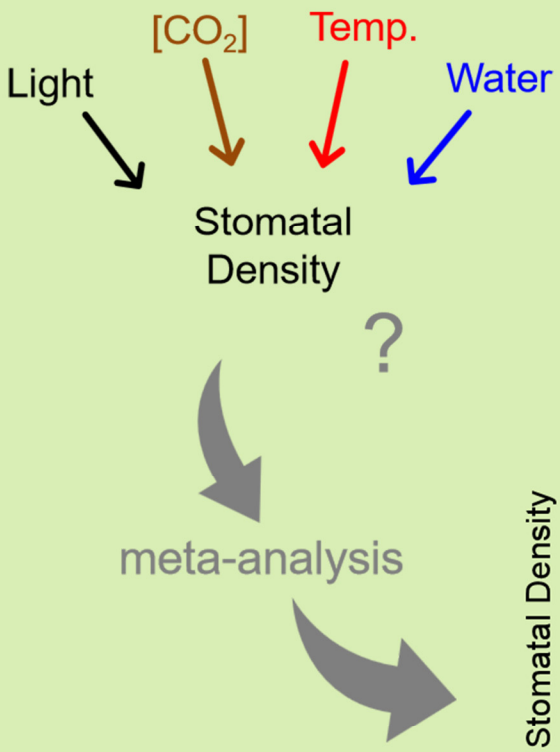

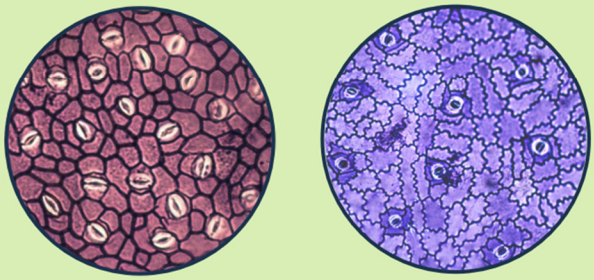

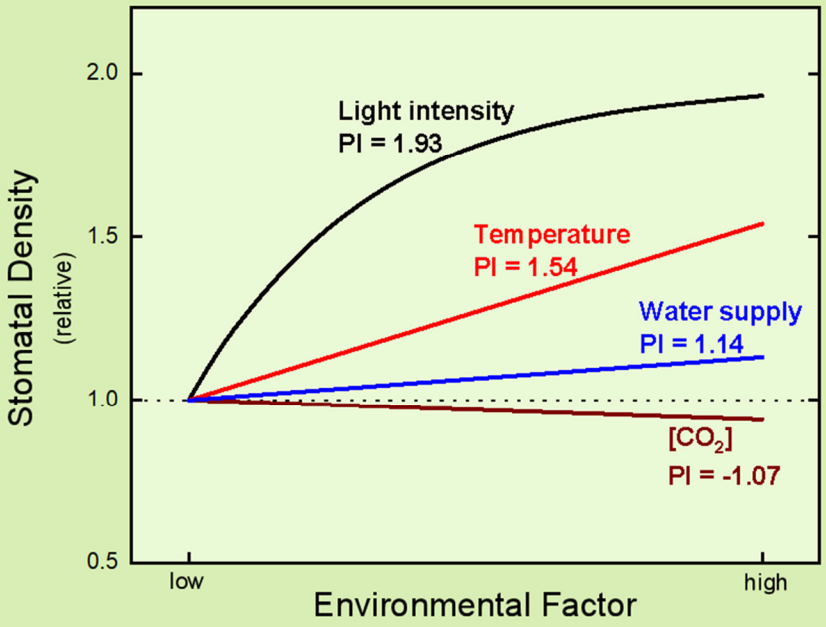

Stomatal Density and Index Are More Responsive to Light Intensity than to [CO_2]: A Meta-Analysis and Implications for Paleo-CO_2 Reconstruction

Scilight

Aims & Scope

Aims:

Plant Ecophysiology (PlantEcophys) aims to be a premier journal for disseminating research in the field of plant ecophysiology (considered synonymous with physiological plant ecology, plant physiological ecology and plant autecology, and including crop physiology, among other related terms with similar meanings), welcoming diverse types of contributions, as long as they are conducted with rigor and offer a significant advancement in knowledge. Contributions may include, but are not limited toSpecific subject areas may include, but not limited to:

- Cutting-edge research and innovative findings.
- Experimental, theoretical, and confirmatory studies, including negative results. Descriptive works are also welcome, provided they have a sound hypothesis and well-explained implications. Merely descriptive studies are excluded.
- Studies encompassing fundamental or applied research.
- Studies performed in either natural or managed systems, including crops.
- Non-traditional outputs such as new methodologies, datasets, models, tools, etc.

Scope:

The scope of the journal encompasses the broad and intricate relationship between plant physiology and environmental factors across all levels of organization. This includes a diverse range of study subjects, spanning from molecules to ecosystems, and encompassing various temporal scales. The journal welcomes any methodologies employed to explore plant responses and their mechanisms in the face of environmental challenges. It invites also contributions from all 'omics' fields (e.g., genomics, metagenomics, transcriptomics, ionomics, metabolomics, and phenomics) and traditional ecophysiological methods (e.g., 'classic' traits - LMA, dry matter content and tissue density, leaf area and shape, venation… stem & root traits, etc. - , microscopy, water relations, gas exchange, chlorophyll fluorescence, and isotopic analysis). Additionally, the journal is open to larger-scale investigations of functional traits, biomass, yield and its components, eddy covariance fluxes, and more, as well as studies that integrate multiple techniques.

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Editorial

On the Neglected Importance of Plant Ecophysiology: Time to Say We Are Here!

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'*For those about to rock … we salute you*!' I haven't found any better introduction to a new Aussie-based journal than the title of a song from the most famous Aussie rock & roll band: AC/DC. The cannonball sound at the end of the song adds solemnity, which is also appropriate because—let me state it from the very beginning—this is not only (although actually it is) the launching of a new scientific journal, a step forward for a young new scientific publisher as it is *Scilight Press*, a new opportunity for publishing good ecophysiology papers … yes, for sure, it is all of this and more, but most specially it is an EXULTATION and an EXALTATION. It is my exultation for having the opportunity of exalting a scientific discipline that possesses a ca. bi-centenary tradition, which is nowadays somehow—or perhaps even *more* than 'somehow'—neglected: *Plant Ecophysiology*, synonymous of Physiological Plant Ecology, Plant Physiological Ecology and, somehow, Plant Autoecology, which Margalef (1991) defined as 'outdoors physiology' meanwhile stating that 'a substantial part of Ecology is outdoors Physiology'. And because of this, the present Editorial aims being as well an exaltation of the great researchers who have kept this discipline alive over these ca. two centuries '*Against the wind*'—this time the title is from Bob Seger—i.e., even though it has seemingly lost 'fashion' over the last decades. To some extent, 'phenomics' may just be another word for something that was sinking into oblivion: high-throughput, but *Plant Ecophysiology* at the end. Some years ago, I quantified with Xurxo Gago this 'loss of fashion' by showing how some of the top journals where we the ecofizzers traditionally publish our results had monotonically decreased the % of papers published on *Plant Ecophysiology* over the last decades while, in parallel, they have almost exponentially increased the % of published works on different 'omics' (Flexas and Gago, 2019). Of course, this is just a verification not neglecting the fact that 'omics' can indeed be an amazing tool for *Plant Ecophysiology*. In fact, combining traditional and newly developed ecophysiological with omic tools is highlighted as the best strategy to advance on understanding plants. This—which, in simple wording, means coupling 'upgraded technology' with 'multidisciplinarity'—is as well the spirit of *Plant Ecophysiology*, *the journal*. The question arises as to whether a new journal on *Plant Ecophysiology* is necessary. And, most importantly, is *Plant Ecophysiology—the discipline*, not the journal—still necessary and important nowadays? Spoiler: I'm completely convinced that *Plant Ecophysiology* is currently more necessary than ever in the past, otherwise, I wouldn't have accepted helping to launch such a new journal.

To shed light on this issue, let me start with the origins of *Plant Ecophysiology*, a long-standing discipline very likely having its roots in the observation of morphological convergence among trees of different continents and of diverse phylogenetic backgrounds. This was already noticed by Fray Bernardino de Sahagún as early as in 1579, when he wrote about America "*Hay pinos en esta tierra como los de España. Hácense en ellos piñas y piñones. Sácanse de ellos las teas y la pez y la resina. Son muy poblados de hojas o de cabellos. Hacen un crujido con el aire como los de España*" (De Sahagún, 1579). Such kind of convergence was later notably highlighted by von Humboldt after his expedition spanning the transition years between the XVIII and XIX centuries, establishing the foundations of biogeography (Von Humboldt, 1805, 1859). The roots of *Plant Ecophysiology* can be traced from these early basements to intermediate necessary inputs in the late XIX century by people such as Gregor Mendel, Charles and Francis Darwin or Julius von Sachs. However, its definitive academic foundations were established on the very late XIX and early XX century. As early as in 1898, A.F.W. Schimper published a compendium book relating plant biogeography to physiology (Schimper, 1898). Almost coetaneous to Schimper was Blackman (1919), who established the 'limitation factor' concept. By the mid-XX century, Mason and Stout (1954), Thomas (1955), Walter (1955) and Billings (1957), among others, published important works of synthesis that helped reinforcing the roots for *Plant Ecophysiology*. They prepared the field for a visible flourishment of *Plant Ecophysiology* in the 60's of the past century, followed by its massive bloom in the 70's and 80's.

To quantify as many traits as possible for a complete vision of plant responses to the environment, methodological developments have been crucial for, and parallel to, the advance of the discipline. To mention just a few examples, advances in the design of new micrometeorological instruments were stimulated by Geiger's (1957) conceptual synthesis effort, and the development of pioneering portable systems for measuring leaf gas exchange (Gaasstra, 1959; Bosian, 1960; Eckardt, 1966) was an important impulse for field-based ecophysiology, as it was the building of pressure chambers for measuring water potential (Scholander et al., 1964). But for a discipline focusing on physiological processes and their response to the environment, based on well-established physical and chemical laws, besides developing suitable instruments it is also important to develop conceptual frameworks to integrate theories with empirical assessment, i.e., testable models. In this sense, and again to mention just some examples, Monsi and Saeki (1953) made the earliest efforts to describe and model light distribution within canopies, Gaastra (1959) established the conceptual basis of photosynthetic gas exchange under fluctuant environments, Slatyer (1967) developed the basis for the understanding of plant water relations and, retrieving and expanding early pioneer work by Blackman (1919), plant growth models were developed for both crops (Brouwer & de Wit, 1969) and wild vegetation (Miller & Tieszen, 1972), which were later strongly consolidated by e.g., Penning de Vries (1975).

As said, in the 70's and 80's there was a bloom of the discipline, and some recognizable groups or 'schools', which had been born in the previous decades, were especially visible by that time—please, allow me this license, keeping in mind that the different 'schools' I will mention are subjective and not watertight compartments. One of them is what I would call the 'German-Austrian' school, having been pioneer, since Schimper (1898), in the physiological analysis as a tool to interpret worldwide plant species distribution. Hence their focus had roots in Geobotany and Biogeography, and they provided *Plant Ecophysiology* scientists so much recognized as e.g., Otto L. Lange, Walter Larcher, R. Pisek, K. Raschke, O. Stocker, Walter Tranquillini, Heinrich Walter or H. Ziegler, among many others (see Larcher, 1977, for a detailed bibliography about these authors), plus more recent ones as Christian Körner, Ulrich Lüttge, E.-Detlef Schulze, Ulrich Schreiber, John D. Tenhunen, Klaus Winter, … It is to Heinrich Walter to whom we are probably indebted to the term "*Plant Ecophysiology*". In one of his early studies about the use of plant hydration measurements as physiological indicators, he used the term 'ecological physiology' ("*physiologisch-ökologische*", Walter, 1931). But later, Walter (1964) himself introduced the term 'ecophysiology' ("*ökophysiologischer*"). Parallel to the 'German-Austrian' group, a 'Scandinavian school' used a similar geobotany-rooted approach in, for instance, studies on the differentiation of ecotypes—introducing the concept 'ecotype' for the first time—in response to environmental conditions (Turesson, 1922) or the carbon economy in plant communities (Boysen-Jensen, 1932).

The 'Brittish school', on the contrary, originally focused mostly on crop physiology, contributing to knowledge on soil-plant relationships (Rorison, 1969) and to the development of rigorous micro-climatic assessment (Monteith, 1957, 1972, 1973). Some classical *Plant Ecophysiology* textbooks arose from this community (Milthorpe & Moorby, 1974; Bannister, 1976; Etherington, 1978; Jones, 1983, 2014; Gardner et al., 1985; Fitter & Hay, 1987; Hay & Walker, 1989; Willey, 2016), which have been and still are among the most used textbooks by *Plant Ecophysiology* researchers, teachers and students, alongside those of Larcher (1977, 1995), Nobel (1991, 2020), Lambers and co-workers (Lambers et al., 1998; Lambers and Oliveira, 2019), Atwell et al. (1999) and, of course, the four volumes of the Encyclopedia of Plant Physiology dedicated to Physiological Plant Ecology (2011, edited by Lange, Nobel, Osmond and Ziegler). The influence of British textbooks was so high that, in my own experience, in the late 80s and early 90s 'crop physiology' was very near to being synonymous with '*Plant Ecophysiology*'.

Additionally, it is possible to identify a 'US school' which, at its origins, was concentrated and recognizable in the '*Carnegie Institution desert group*' (Mooney et al., 1987). Somehow merging the geobotany-rooted and the micrometeorology approaches of the above communities, they developed an ample study of ecophysiological adaptations to the environment, together with a true exploring of the unknown with special emphasis to the desert and mediterranean biomes, without neglecting crops. The list of well-known US-based ecofizzers, many of them still active, is really ample. Just to mention just some amongst the most influencing, likely 'belling' your mind after hearing their names: W.W. Adams III, D. Baldocchi, J.A. Berry, O. Björkman, W.D. Billings, J.S. Boyer, M.M. Caldwell, T.E. Dawson, B. Demmig-Adams, C.B. Field, J.A. Gamon, A. Gibson, N.M. Holbrook, R.B. Jackson, J. Keeley, P.J. Kramer, R. Monson, H.A. Mooney, P.S. Nobel, C.B. Osmond, R.W. Pearcy, P.B. Reich, P. Rundel, J.S. Sperry, M.T. Tyree, M.H. Zimmermann, …. I could keep citing more for days!

Without pretending to be exhaustive in listing past and current notorious ecofizzers, still it is worth highlighting the 'Australian ecophysiology hub'—with many excellent ecofizzers across the country (W.J.S. Downton, B.R. Loveys, Hans Lambers, Mark Westoby, Ian Woodrow …)—but with a remarkable concentration in Canberra (Research School for Biological Sciences; Australian National University) which, in the late 70's and along the 80's, encompassed a huge amount of well-recognized plant physiologists and ecofizzers: Jan Anderson, T.J. Andrews, Suzanne von Caemmerer, Wah Soon 'Fred' Chow, Ian Cowan, John Evans, Graham Farquhar, Paul Kriedemann, again Barry Osmond after his US years, John Passioura, Stephen Powles, Richard Richards, Neil Turner, Suan Wong … and many others, including *Plant Ecophysiology* Associate Editors Marilyn Ball and Rana Munns! If the list of 'resident' researchers was absolutely impressive, not least was the one of celeb visitors being around in those years: Bill Adams, Joe Berry, Gabriel Cornic, Barbara Demmig-Adams, Gunnar Öquist, Tom Sharkey, Ichiro Terashima … 'not bad' for a just 26 million people country nowadays! Later, in the late 90's, I had the great fortune and pleasure of developing a significant part of

my PhD at RSBS-ANU and, even then, that place was still the best environment that one could imagine for developing new scientific ideas and establishing collaborations and fruitful discussions. For instance, Steve Long was there for a short visit, and I could attend his vivid seminar talk together with just another 20 persons! While I used to visit Marylin Ball's lab to share coffee with her group—thanks Marylin for those coffee breaks, Katalapi times and for agreeing to become associate editor of the journal—at that time I was too shy for interacting with CSIRO researchers without having been introduced to them, even if I just needed to cross a single road from the apartment where I was living (yellow-crested cockatoo on the balcony included, courtesy—the apartment, not the cockatoo—of and likely the result of tedious paperwork and calls by Barry Osmond). Indeed I crossed that road several times to reach their offices' doors without having spirit enough to knock at them—Neil Turner, John Passioura and my nowadays associate editor, Rana Munns —thanks, thanks and thanks for accepting! Despite of it, I still interacted there at ANU with many incredible scientists. For sure with Barry Osmond, my scientific light and master, but also with many more, not necessarily 'pure ecofizzers', but certainly well-integrated with those (Murray Badger, Marylin Ball, 'Fred' Chow, Graham Farquhar, Adam Gilmore, Hideo Yamasaki, Tom Wydrzynski …). All these people were definitive crucial to me for mutating from a promising student into a true ecofizzer. Of course, such a mutation would have never happened without a previous and continued relationship with Hipólito Medrano, the person who introduced me the word 'ecophysiology' while teaching me an entire course on this subject, who supervised my undergraduate projects and my PhD thesis and who, now in his 'wine-making retirement', is still an ecophys and science reference to me and a good friend of mine. Or without the 'Orsay group', another mix of multi-disciplinary scientists including Ismael Moya—who was part of my 'PhD trinity' with Hipólito and Barry—Zoran Cerovic, Yves Goulas and Jean-Marie Briantais (who, strictly, was perhaps not an ecofizzer but he certainly was the best of persons) and, again 'just crossing the road', also Gabriel Cornic, Bernard Genty and others. My formation was completed thanks to interactive debates with Serge Rambal from France, Tom Sharkey from the US, Francesco Loreto and Mauro Centritto from Italy, Ichiro Terashima from Japan, Ülo Niinemets from Estonia, Manuela Chaves from Portugal … Those were the times—late nineteens of the past century—when *Plant Ecophysiology* was a mature, solid discipline, with multiple networking amongst its scientists and with other disciplines like remote sensing, ecology, molecular biology, biophysics and so on. Thirty years later I can still not imagine a better environment to grow as a scientist and as a person.

But even nowadays, I'm sure that most of us, if not all, are pretty convinced that *Plant Ecophysiology* is certainly a well-defined scientific discipline with deep roots and solid foundations, but still let me come back to 'emotional' Ecophysiology for another while. To me at least, as it is likely evident from previous paragraphs, the ecophysiology people is also a family. This feeling is reinforced by my devotional belonging to two standing well-defined *Plant Ecophysiology* communities: the Katalapi community in Chile and the Coloquio community in Spain. The Katalapi Colloquium,named after its hosting institution, the Parque Katalapi, (https://english.parquekatalapi.cl/, accessed on 20 May 2025) has been held without interruption (except for the pandemic years) since 2008 thanks to the generosity of an outstanding ecoffizzer (formerly a plant biochemist!): Luis Corcuera, '*el Doc*'. I am glad to have attended this colloquium many years, meeting there outstanding Chilean ecoffizers—many of them having become our most frequent scientific collaborators—as Lucho Corcuera himself, Luisa Bascuñán, León Bravo, Lohen Cavieres, Rafa Coopman, Nicolás Franck, Enrique Ostria-Gallardo, Claudio Pastenes, Frida Piper, Alejandra Zúñiga, and so many others, including, of course, our *Plant Ecophysiology* Associate Editor Patricia 'Patty' Sáez! Sorry for not mentioning each and every one, the amazing thing is that we are so many people that I can easily miss some. The Katalapi Colloquium is international, and it has hosted relevant international ecofizzers such as John Bishop, Tim Colmer, Ingo Ensminger, Norman Huner, Alex Ivanov, Christian Körner, Adrienne Nicotra, Ülo Niinemets, Rafael Oliveira, Mark Olson, Michael Shane, Robert Turgeon, Matthew Turnbull, and many others (I met there another of my current Associate Editors, Paulo Marchiori, thanks for being there and here!), together with many Spanish researchers including Pere Aguiló-Nicolau, Marc Carriquí, Xisco Castanyer, María J Clemente, Antonio Diaz-Espejo, Xurxo Gago, Jeroni Galmés, Leopoldo García-Sancho, Conchi Íñíguez, Melanie Morales, Miquel Nadal, Alicia Perera-Castro, Miquel Ribas-Carbo, …. and the 'almost every-year' participants, Nacho Garcia-Plazaola, Bea Fernandez-Marin and myself. Besides the three of us, and among the 'internationals', I must highlight another three people that have repeated many times, becoming 'whole-right-Katalapiers': Marylin Ball, Hans Lambers and Bruce Osborne. Judging just by the names and the year-to-year fidelity of quite some of the participants, you can imagine how special is the Katalapi Colloquium for creating a real community and pushing up *Plant Ecophysiology*, from which many international collaborations have emerged. So special that Nacho, Bea and myself decided years ago to plagiarize the idea and create a similar one in Spain, i.e., to bind a restricted group of plant ecoffizers that meet every year for several days, in this case in rotating remote places in Spain, sharing not only science but also excursions (most to the field, but also to the closest pubs, I must say). This one, the 'Coloquio', is mostly Spanish-based and hosted in Spanish, but still you can find there—some of them more usually, and some more sporadically—well-reputed ecofizzers as the Chilean Daniela Aros, Luis Corcuera, Rafa Coopman and Enrique Ostria-Gallardo, and the Spanish Ismael Aranda, Javier Cano, Marc Carriqui, Miquel A. Conesa, Antonio Diaz-Espejo, Raquel Esteban, Bea Fernandez-Marin, Xurxo Gago, Jeroni Galmés, Nacho Garcia-Plazaola, Eustaquio Gil-Pelegrin, Águeda González, Javi Gulías, Rosana López, Jordi Martinez-Vilalta, Enrique Mateos-Naranjo, Fermín Morales—the only one having attended every single edition!—Sergi Munne-Bosch, 'Jota'

Peguero-Pina, Nacho Querejeta, Fernando Valladares, Albert Vilagrosa, ... Starting from the pioneering introduction of *Plant Ecophysiology* in Spain by few people like e.g., Enar Alegre, José Luis Araus, Luis Ayerbe, Carles Gràcia, Maria Soledad Jimenez, José Alberto Pardos, Manuel Sanchez-Diaz, Robert Savé, Arturo Torrecillas or Hipólito Medrano, the Coloquio has now created a real scientific community around *Plant Ecophysiology*, from which many collaborations and visitor exchanges have arisen and, most importantly, it has generated in all of us the awareness of belonging to a family.

After this very personal and emotional dissertation, please understand that I was not trying to be exhaustive in listing prominent ecofizzers, as certainly each of you may have your own list of 'inspiring ecofizzers'. Besides my personal feelings, my intention was to simply make visible a sufficiently large list of well recognizable names that most of us, current ecofizzers, have in mind as acknowledged references for our own work, to demonstrate that *Plant Ecophysiology* do has solid foundations and for reminding plant scientists that providing a genome or a transcriptome is undoubtfully a powerful analytical tool, but is not the only way of making good science, advancing in our understanding of how plants function, acclimate and adapt to a continuously changing environment.

Nevertheless, and while certainly it is not compulsory to do molecular and genetical analysis for producing good *Plant Ecophysiology*, perhaps it is in general a good idea! In fact, from the late XX century and through the present *Plant Ecophysiology* has incorporated more and more molecular biology knowledge and techniques. According to De Lucia et al. (2001), this approximation of *Plant Ecophysiology* to molecular biology (and, particularly, to molecular genetics and phylogenetics) was what defined the most the evolution of our discipline over the late decades of the XX century. Together with the increasing interest for ecolophysiological approaches to assess whole plant, population and community processes—aided again by technological developments such as Eddy covariance or satellite- and drone-based remote sensing, to mention just spread ones—they have consolidated plant ecolophysiology as a discipline, in De Lucía et al. (2001)'s own words, "*linking the organism to scales above and below*". Years later, Xurxo Gago and myself were further to propose that nowadays ecophysiology is so impregnated of molecular biology that we are entering an "*ecophysiolomics era*" (Flexas & Gago, 2018). And, in fact, as I stated earlier, combining traditional and newly developed ecophysiological with omic tools is envisaged as the best strategy to advance on understanding plants and this is as well the spirit of *Plant Ecophysiology*, the journal.

Yes, we still can publish our work in top plant science journals—and it is something that for sure we must keep on doing. Additionally, some journals are actually quite focused on ecophysiology studies, at least in specific aspects of ecophysiology (e.g., *Journal of Plant Hydraulics*) or specific groups of species (e.g., *Tree Physiology*). But up to now many of us have been missing a journal focused on the globality of aspects of the discipline. This is why I believe *Plant Ecophysiology* deserves to start its journey "*from Mulga to Mangoes*" (as Aussie's songwriter John Williamson would say), a journey that many of us believe should have started decades ago! Therefore, it is for strenghthen the discipline, but also for honoring the ecofizzers' family, that following *Scilight* offer I decided to place 'oh, no! another journal in the science publishing market!'. But this is not 'another journal', this is *Plant Ecophysiology*! It is born to honor the pioneers like Walter, Gaastra or Mooney; to recognize those who consolidated the discipline like Berry, Farquhar or Osmond; and—following the spirit of the Katalapi and Coloquio 'tribes'—to regroup the community around a new fire where to share our stories. This is why I am convinced that *Plant Ecophysiology* is here for lasting.

I am proud of initiating the very first scientific journal fully focused on *Plant Ecophysiology*, and the very first one (to the best of my knowledge) that aims to economically compensate reviewers for they work. It aims providing the plethora of "heirs" of the pioneering ecofizzers a high standard journal that they can consider THEIR journal.

I acknowledge *Scilght* for hosting this initiative, including accepting all my business-breaking crazy initiatives. I also acknowledge the many ecofizzers who interacted with me upon my initial consultation to ca. 350 potential users of the new journal. A particular acknowledgement to Hendrik Poorter and Diego Marquez for their continuous dedication to help improving this journal's manuscript and web designs, and to Diego also for his detailed decalogue of reviewers' good practices, which we have simply adopted without any change. And to the very few (you know who you are) that strongly advertised me against creating a new journal: you are among the most admired ecofizzers to me, and I honestly hope I will keep your friendship even if I did not follow your advice.

You may have noticed that I have mentioned many names—and very likely missed many other important ones—but I have referred briefly to just some of the journal's initial Associate Editors—a list that I expect to enlarge in the next years. This is not because they are not among the most excellent plant ecofizzers, it is because I wanted to very specially highlight them here at last, but for sure not least: Marylin Ball, Sigfredo Fuentes, Diego Márquez, Paulo R. Marchiori, Rana Munns, Miquel Nadal, Patty Sáez, Lou Santiago, Erik Veneklaas and Dongliang Xiong. Isn't it an impressive list? You ARE among the greatest ecofizzers, and I'm so proud and thankful of having you on board. Thank you guys for getting involved in this adventure!

And a very last consideration for everyone: remember, this is not a new journal, this is just the journal we all ecofizzers had in mind for long and were somehow astonished that it did not exist in practice. It is simply that now, YES, it exists. Hope to meet you all here!

Conflicts of Interest

The author declares no conflict of interest.

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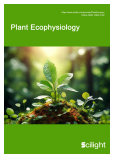

Review

Stomatal Density and Index Are More Responsive to Light Intensity than to [CO_2]: A Meta-Analysis and Implications for Paleo-CO_2 Reconstruction

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Abstract: Stomatal density is one of the plant traits influencing leaf gas exchange and is known to be affected by the plant's environment. Understanding its degree of plasticity to various abiotic factors is therefore important. We conducted a meta-analysis of a wide range of experiments in which plants were grown under different levels of CO_2, light, temperature, and water availability, and derived generalized dose-response curves. Although both stomatal density and stomatal index showed a significant negative correlation with CO_2 levels, these relationships were weak and only marginally consistent across the analyzed experiments. In contrast, the effect of growth light intensity was positive, highly consistent, and substantially stronger than the impact of atmospheric CO_2. Temperature also positively influenced stomatal density, while water availability showed no consistent effects. Based on these dose-response curves, we highlight several caveats when using stomatal density or stomatal index for paleo-CO_2 reconstruction. The weak CO_2 response, coupled with the strong confounding impact of light intensity, poses significant limitations to the accuracy of such estimates.

Keywords: CO_2; daily light integral; light intensity; meta-analysis; paleoclimatology; stomatal density; stomatal index

1. Introduction

There are probably more stomata on Earth than grains of sand on all the world's beaches. A single leaf can contain hundreds of thousands of stomata (Ciha & Brun, 1975), making the total number of stomatal pores on a single tree—or across an entire forest - staggeringly immense. Stomata act as critical gateways for carbon dioxide uptake while limiting water loss, thus playing a central role in Earth's carbon and water cycles (Berry, Beerling, & Franks, 2010). At the leaf level, gas and water fluxes are co-regulated by various stomatal characteristics. One extensively studied trait is stomatal conductance, which can respond relatively quickly (within minutes to hours) to changes in light intensity, CO_2 concentration, or the leaf's water status (Lawson & Vialet-Chabrand, 2019). Over longer time frames (days to months), plants can further adjust their gas and water fluxes by producing new leaves with different stomatal sizes, or by altering the number of stomata per unit leaf area. This latter trait, known as 'stomatal density', forms the central focus of this paper.

Stomatal density (SD) is known to vary systematically within a plant. Typically, SD is higher on the abaxial (lower) side of a leaf than on the adaxial (upper) side, and it increases from the base to the tip of the leaf, as well as from the midrib toward the leaf margin (Salisbury, 1927). SD may also increase with a leaf's position on the plant, and can vary with genotype or species (Wall et al., 2023). These factors must be considered when examining how SD responds to environmental conditions (Körner, 1988; Woodward, 1993; Roth-Nebelsick, 2005). Our study investigates how SD is influenced by four important abiotic factors: ambient [CO_2], light intensity, temperature and water availability. To this end, we conducted a meta-analysis focusing on the longer-term effects of these factors on SD. A few years ago, Yan, Zhong, and Shangguan (2017) conducted a similar analysis and found that the response ratio of SD between high and low CO_2 levels was not significantly different from 1.0, suggesting no overall effect of

ambient [CO_2]. They also found that both higher temperature and lower water availability increased SD. However, their analysis did not consider the effects of light intensity, a gap that we aim to address in this study.

A standard meta-analysis typically categorizes the levels of an environmental factor or treatment in each experiment as 'high' and 'low', and then calculates the relative response by comparing the ratio of the phenotypic variable of interest between these two categories (Gurevitch et al., 2018). However, the observed response can also depend on the specific levels of the environmental factor applied, and may saturate within certain ranges. To gain more comprehensive and generalizable insights, it is beneficial to derive dose-response curves, which describe the response of a phenotypic variable across a broad range of levels for the environmental factor of interest (Poorter et al., 2022a). In this study, we adopt this approach to derive dose-response curves for the four environmental factors under consideration. Since plants typically respond more to the cumulative light flux received over time rather than the instantaneous light intensity present at a given moment in time (Kelly et al., 2020), we represent light availability in our analysis using the Daily Light Integral (DLI). DLI quantifies the total number of quanta in the photosynthetically active range (400–700 nm) received per unit ground area per day. This metric generally provides a more biologically relevant measure of light availability for plants (Poorter et al., 2019).

The application of dose-response curves for SD has proven significant in paleoclimatology. As atmospheric CO_2 concentrations continue to rise, understanding Earth's climate sensitivity to CO_2 is of critical importance. Examining past variations in atmospheric CO_2 and the corresponding climate changes can provide valuable insights (Hönisch et al., 2023). CO_2 levels from the past 800,000 years can be measured directly from air trapped in Antarctic ice (Higgins et al., 2015). However, for periods prior to 800,000 years ago, direct measurements are not possible, making it necessary to rely on proxy estimates (Royer, 2001). One such method of reconstruction involves comparing the SD of well-preserved fossilized leaves to that of the same or closely related species growing today (e.g., McElwain & Chaloner, 1996; Kürschner, 1997; Rundgren & Beerling, 1999; Kürschner, Kvaček, & Dilcher, 2008). This approach was pioneered by Woodward (1987), who showed that SDs sampled in the 1980s were generally lower than those of herbarium specimens collected 200 years earlier. A functional explanation for the observed negative relationship between SD and atmospheric [CO_2] is that higher CO_2 levels allow plants to maintain sufficient CO_2 uptake, while decreasing water loss through transpiration by decreasing the number of stomata per unit area (Royer, 2001). Assuming that the sensitivity of these plants to CO_2 has remained constant over time, SD in fossilized leaves offers a proxy for estimating atmospheric CO_2 levels in past eras.

Not all findings have been unequivocal, though. Herbarium material of various species collected over an 80–110 year span did not reveal any consistent trends over time (e.g., Hu et al., 2015; Ydenberg et al., 2021). Over a span of 70–90 years, Körner (1988) observed *increases* in SD rather than decreases, be it that there was considerable variation among species and the overall response was not statistically significant. Experimentally, the negative relationship between SD and [CO_2] has also been inconsistent, with various studies failing to replicate it (e.g., Apel, 1989; Reid et al., 2003). A complication that soon became apparent, is that SD not only depends on the number of stomatal cells initiated, but also on the degree of expansion of the surrounding epidermal cells. This issue has prompted researchers to adopt the stomatal index (SI) as an alternative proxy. SI represents the percentage of stomata relative to the total number of stomata and epidermal cells (Salisbury, 1927). In the paleobotanic literature it is generally assumed that SI is more strongly influenced by ambient [CO_2] than by other environmental factors, such as water availability or light intensity (Royer, 2001), making it a potentially more reliable indicator for ancient CO_2 levels than SD. As a result, SI has become the more dominant metric in this field (see compilation by Hönisch et al., 2023), although its underlying premise is still not well constrained. To address this, we also have derived dose-response curves for SI in relation to environmental variation, as far as data were available.

In this paper, we examine the effects of CO_2 concentration, daily light integral, temperature, and water availability on both stomatal density (SD) and stomatal index (SI). Using a meta-analysis of studies in which plants were experimentally exposed to varying levels of these environmental factors, we derive generalized dose-response curves wherever possible. We evaluate the consistency of the data, determine the form of the dose-response curve that best represents the generalized relationships, and quantify the overall plasticity. Finally, we assess the generalizability of the CO_2 dose-response curve and compare our results with functions currently used to estimate past CO_2 concentrations based on fossil SD and SI.

2. Materials & Methods

We analyzed compiled data from experimental treatments where plants were grown for a minimum of two weeks and at least one-third of their actual lifespan, under varying levels of CO_2, light intensity, temperature, or water availability, and where stomatal density (SD) and/or stomatal index (SI) was reported for leaves that had developed under these conditions. These experiments were conducted in growth chambers, glasshouses, open-top chambers or free-air CO_2 enrichment (FACE) facilities, provided that the plants were grown individually or in mono-specific stands. Unlike the meta-analysis by Yan et al. (2017), we excluded data from herbarium specimens and from field-grown plants in CO_2 springs or along natural gradients of light, temperature, water availability or altitude, as other environmental factors may have co-varied with the factor of interest.

Stomatal density exhibits significant variation across plant species. Many tree species lack stomata on the adaxial (upper) surface of their leaves, while numerous herbaceous species exhibit higher stomatal densities on the abaxial

(lower) side compared to the adaxial side (Salisbury, 1927; Körner, 1988). However, in some cases, stomata are more abundant or exclusively present on the adaxial surface (Kaul, 1976). When SD data were reported for both leaf surfaces, we summed the values from the abaxial and adaxial sides, as this provides the most comprehensive measure relevant to gas exchange. For SI, we averaged the values from both surfaces. If data for only one side was reported (typically the abaxial side), we used that value as SD or SI estimate, assuming that researchers considered that to be the most relevant surface for their species of interest. To standardize the data, phenotypic values in a given experiment and species were subsequently scaled to the values observed at a reference [CO_2] of 450 ppm, a Daily Light Integral (DLI) of 8 mol m^{-2} d^{-1}, an average temperature level over the full diurnal cycle of 20 °C. For water availability, drought severity was estimated by scaling the biomass or leaf area of drought-stressed plants relative to control plants grown under optimal water conditions. We therefore excluded papers where no quantification of plant size was made. For [CO_2] and DLI we expanded the datasets reported by Poorter et al. (2019, 2022a, 2022b), incorporating approximately 50% more data to the compilation.

After scaling the phenotypic responses for each species and each individual experiment separately, we fitted four types of curves describing different potential relationships in the data: (a) no relationship, (b) linear regression, (c) a saturating curve and (d) a quadratic polynomial. To determine the best-fitting curve, we applied the Akaike Information Criteria (AIC). Although an assessment across different environmental factors to some extent is a comparison between different entities, we used the data and the resulting curve fits to summarize all observations through three key indices.

(a) A **Plasticity Index (PI)**: This index represents the ratio of SD or SI derived from the fitted relationships at CO_2 concentrations of 1200 and 200 ppm, a DLI of 50 and 1 mol m^{-2} day^{-1}, or temperatures of 35 and 5 °C, respectively. For drought stress, we considered SD and SI values under optimal watering conditions relative to those at 10% of the optimal plant biomass. In cases of negative responses, we calculated the inverse of the ratio and denoted this with a minus sign, to maintain comparable scales.

(b) A **Consistency Index (CI)**: This index reflects the percentage of experiments in which plants treated with the highest levels of CO_2, light, temperature or water exhibited higher SD or SI compared to those treated with the lowest levels. A value of 100% indicates fully consistent increases across experiments, while 0% indicates fully consistent decreases. A value of 50% suggests either random variability, or strong contrasting responses between species.

(c) A **Reliability Index (RI)**: This index assesses the robustness of the selected form of the dose-response curve, the PI and the CI, on a scale from 0 to 9. It accounts for the number of experiments, the number of species studied, the variability between observations, and the range of the environmental factors over which experimental data are available. A higher value indicates a lower likelihood of changes in results with the addition of new data. While this index is particularly useful for comparing different plant traits in response to the same environmental variable, it can also be used - albeit with caution - to compare the reliability of PI and CI across different environmental factors.

For a more detailed description of the analysis, readers are referred to Poorter et al. (2022a) and Supporting Info S1.

3. Results & Discussion

3.1. Stomatal density

In total, we compiled data from 245 papers, with references listed in Supporting Info S2. The analysis of these data reveals a significant and overall negative response of stomatal density (SD) to ambient CO_2 levels: as CO_2 concentration increases, SD decreases (Figure 1A). Median values calculated for each subsequent 10% of the data suggest a steeper slope in the low-CO_2 range compared to the high-CO_2 range, consistent with the 'ceiling' discussed by Roth-Nebelsick (2005), where SD becomes less responsive to further increases in [CO_2]. However, the data show considerable variability, and the Akaike Information Criterion identified a linear relationship as the simplest model to describe the trend. The slope of this regression line is very modest, with a Plasticity Index (PI) of -1.07 (Table 1), indicating a marginal decrease of 7% in SD across the CO_2 range of 200–1200 ppm. Moreover, the consistency of the response across the compiled experiments is relatively low: when comparing the treatments with the highest and lowest CO_2 levels within each experiment and for each species, 40% of the studies report increases in SD, while 60% report decreases (Table 1). This distribution is close to what would be expected by chance, where increases and decreases would occur in approximately 50% of the experiments.

What might cause such large variability? Firstly, methodology may play a role, as SD can vary substantially within a single leaf, between different leaves, and even between the adaxial and abaxial sides (Salisbury, 1927; Körner, 1988; Poole et al., 1996; Yan et al., 2017). Therefore, care must be taken to sample the same part of the same leaf in the analysis (Woodward, 1993), or, ideally, to systematically sample various parts of the leaf to account for spatial variability. Secondly, intrinsic differences among species, functional groups or phylogenetic clades may contribute to the observed variability. The results suggest that herbaceous C_4 species, in general, show no response to [CO_2], whereas both herbaceous and woody C_3 species exhibit a slightly negative response (Table 2). However, none of these species contrasts is statistically significant. Thirdly, strong interactions between [CO_2] and other environmental conditions could lead to variations among experiments. Currently, however, there are too few data in our dataset to derive dose-response surfaces, which would enable quantification of the strength of these interactions and offer a more detailed understanding. Finally, maternal effects may also influence SD (Vráblová et al., 2018). However, nearly all experiments in our compilation used seeds from plants grown at control CO_2 levels, likely limiting this source of variation.

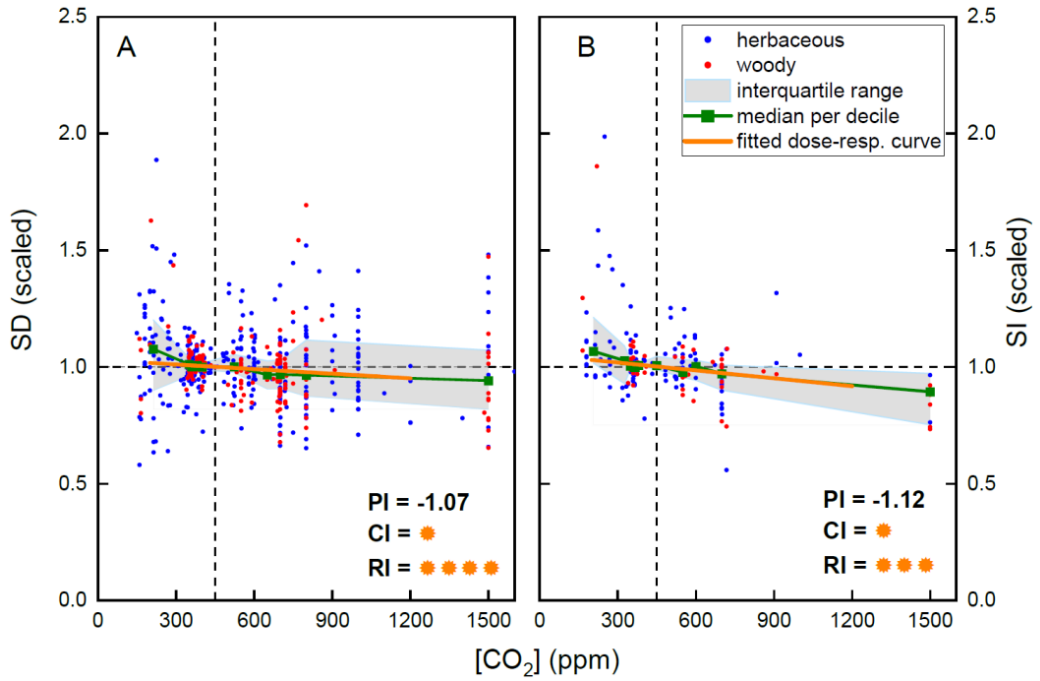

Figure 1. Dose-response curves for (**A**) Stomatal Density (SD) and (**B**) Stomatal Index (SI) as functions of ambient CO_2 concentration. Data points represent scaled mean values per species and experiment, relative to a reference [CO_2] of 450 ppm. Data for herbaceous plants are in blue, for woody species in red. Green squares indicate the median scaled trait value and [CO_2] per decile of observations, or per group of 10 observations when fewer than 100 observations are available. The shaded area represents the interquartile range (25th and 75th percentiles). The thick orange line shows the fitted relationship across all data points. Calculated Plasticity Indices (PI) are provided, along with a visual indication of the Consistency index (CI) and Reliability Index (RI). The strength of the Consistency Index is indicated by the number of orange symbols: none: % increases in the trait value with an increase in the abiotic environmental factor: 40–60%; ✸: 30–40% or 60–70%; ✸✸: 20–30% or 70–80%; ✸✸✸: 10–20% or 80–90%; ✸✸✸✸: 0–10% or 90–100%. The strength of the Reliability Index: no symbol: 0–1; ✸: 2–3; ✸✸: 4–5; ✸✸✸: 6–7; ✸✸✸✸: 8–9. For more detail see Tables 1 and 2 and Supporting Info S3 (Figures S1–S8).

Table 1. Summary of the dose-response curve analysis for Stomatal Density (SD) and Stomatal Index (SI) in relation to four environmental factors: (1) ambient CO2 concentration (2) Daily Light Integral (DLI), (3) average daily temperature and (4) water availability during growth.

Env. Factor	Trait	Range in Env. Factor	# of Observations	# of Species	Fit	Pseudo r^2	Plasticity (PI)	Consistency (CI)	Reliability (RI)	*p*	a	b	c
[CO_2] (ppm)	SD	150–3200	660	180	L***	0.02	−1.07	40	8	ns	1.031	-6.53×10^{-5}	
	SI	165–2000	220	80	L***	0.14	−1.12	32	7		1.053	-1.12×10^{-4}	
DLI (mol m^{-2} day^{-1})	SD	0.4–72	360	100	S***	0.51	**1.93**	**94**	7	ns	1.477	0.526	6.69×10^{-2}
	SI	0.9–64	130	30	S***	0.31	**1.96**	**92**	4		1.285	0.553	0.1147
Temp. (°C)	SD	5–38	150	35	L***	0.31	**1.54**	68	4		0.710	1.40×10^{-2}	
	SI	13–30	10	5	nd	nd	nd	57	1				
Water (Rel. units)	SD	0.15–1	110	35	-	0.00	1.14	47	3		0.8709	0.1291	
	SI	0.25–1	30	10	nd	nd	nd	62	2				

Columns 1 and 2 indicate the environmental factor under consideration and the traits analyzed. For temperature, the average temperature over the full day/night cycle during active growth was used. Water stress was assessed as the relative biomass of water-stressed plants compared to well-watered plants in the same experiment. Columns 3 and 4 show the range of the environmental factor for which data are available in the database, as well as the total number of observations (i.e., number of mean values per species and level of the environmental factor of interest; rounded to the nearest 10). Column 5 indicates the number of species for which observations are available for the various traits. Column 6 refers to the form of the dose-response curve. Fitted equations were categorized as follows: no relationship (-; Y = a where Y is the scaled value of the phenotypic trait and a is the overall average of Y values); linear (L; Y = a + bX where X is the environmental factor), or saturating (S; $Y = a\,(1 - b \cdot e(-c^X))$). No fit was determined (nd) with fewer than 30 datapoints, Column 7 shows the fraction of variability explained by the fitted curve. Column 8 lists the Plasticity Index (PI) calculated as the fitted value at [CO_2] = 1200 divided by the fitted value at [CO_2] = 200; or the fitted value at DLI = 50 divided by the fitted value at DLI = 1. Positive values indicating positive trends with the environmental factor of interest, while negative PI values indicate decreasing trends; bold numbers indicate a |PI| ≥ 1.5. The Consistency Index (column 9) represents the percentage of cases (species x experiment combinations) where the phenotypic value at the highest level of the experimental factor considered was greater than at the lowest level. Values lower than 15 or larger than 85 signify highly-consistent positive or negative responses and are indicated in bold. Column 10 shows the Reliability Index (RI), based on the number of records in the database for that trait, the number of different species, the range of levels for the environmental factor, and the average deviation from the median response. The RI is on a relative scale from 0 (low) to 9 (high reliability level). Column 11 shows the significance of a bootstrap test comparing differences in PI for stomatal density and stomatal index. The last 3 columns provide the values for parameters a, b and, if relevant, c for the equations mentioned above.

Table 2. Variation in Plasticity Index (PI) for Stomatal Density (SD) and Stomatal Index (SI) among functional groups, for four environmental factors.

Env. Factor	Trait	C_3 Woody			C_3 Herb.		C_4 Herb.		
		PI	n	*p*	PI	n	PI	n	*p*
$[CO_2]$	SD	−1.14	220	ns	−1.06	320	−1.01	90	ns
	SI	−1.13	60	ns	−1.04	100	−1.01	30	ns
DLI	SD	1.95	170	ns	2.27	150	-	20	-
	SI	2.54	30	ns	1.79	80	-	0	-
Temperature	SD	1.88	50	ns	1.41	70	-	0	-
	SI	-	0	-	-	0	-	0	-
Water	SD	1.32	40	ns	1.09	50	-	0	-
	SI	-	10	-	-	10	-	0	-

PI data were analyzed based on dose-response curves for three distinct functional groups: C_3 woody species, C_3 herbaceous species, and C_4 herbaceous species. The number of data points available for each group is also provided, rounded down to the nearest 10 (n). Each data point represents the average value per treatment for each experiment and species or genotype. PI's and significance values were not calculated for groups with fewer than 30 data points. To assess statistical significance, we tested whether the PI of C_3 woody species and C_4 herbaceous species differed significantly from that of C_3 herbaceous species, by means of bootstrapping (5000 repetitions). None of the contrasts between functional groups showed significant differences.

The results for light intensity contrast sharply with those for CO_2, as light exerts a strong influence on SD (Table 1). Numerous studies have reported positive responses to higher light intensity (e.g., Cooper & Qualls, 1967; Valladares et al., 2002; Wang et al., 2020b), a trend that is evident in our meta-analysis as well (Figure 2A). The response of SD is most pronounced at low DLI levels, and saturates above 35 mol m^{-2} d^{-1}. When considering the range of 1–50 mol quanta m^{-2} d^{-1}, which encompasses DLI's from the shaded forest floors to low-latitude deserts exposed mostly to full sunlight, the Plasticity Index is 1.93, indicating nearly a doubling of SD over this range. This value is intermediate compared to the responses of 85 ecophysiological traits to DLI, but comparable in size to the well-known increases in leaf thickness and photosynthetic capacity (Poorter et al., 2019; Poorter et al., 2022b). The increase in SD is highly consistent, with a Consistency Index of 94%, indicating that nearly all experiments and species exhibit increases in SD with higher light levels. The Reliability Index indicates an intermediate level of confidence. As with $[CO_2]$, we analyzed whether responses to light intensity varied among species groups. While herbaceous C_3 species may exhibit slightly stronger responses than woody C_3 species, these differences are small and statistically non-significant (Table 2). For each of the groups, the effect of light on SD is markedly stronger than CO_2.

Information on SD responses to temperature and water availability is much scarcer compared to responses to CO_2 and light. The dose-response curve for temperature indicates a positive association, best described by a linear relationship (Figure 3A). The Plasticity Index for temperature is 1.54 over the 5–35 °C range, with a Consistency Index of 68%. While the Plasticity Index is higher for woody C_3 species compared to herbaceous C_3 species (Table 2), also this species contrast is not significant. Water availability or stress was quantified by comparing the size of water-stressed plants to control plants, assuming that control plants in the compiled experiments were adequately watered. No significant relationship was found (Figure 1B), which aligns with a low Consistency Index of 47% (Table 1). Further separations in species subgroups are given in supporting Info S4. We did not analyze responses to varying levels of nutrient availability or relative humidity. However, small data compilations conducted so far indicate that responses to these factors are also mixed (Bertolino, Caine, & Gray, 2019; Fanourakis et al., 2020).

3.2. Stomatal index

While SD is functionally linked to gas exchange, stomatal index (SI) provides greater insight into the developmental process of stomatal initiation (Royer, 2003). Similar to SD, SI generally exhibits a negative correlation with $[CO_2]$, but with a slightly steeper slope, indicating greater plasticity (more negative PI; Figure 1B). Unlike SD, however, the median values for each consecutive 10% of the data do not indicate saturation at higher CO_2 levels. Therefore, these experimental data do not support the concept of a 'ceiling' in SI at CO_2 concentrations above current levels, as proposed by Woodward (1987) and Roth-Nebelsick (2005). The Consistency Index for SI deviates further from the neutral 50% than that for SD, suggesting a slightly more consistent relationship with $[CO_2]$. Nevertheless, with a Consistency Index of 32%, this relationship remains weak and far from universal. On average, herbaceous C_3 and C_4 species, as well as young woody plants, exhibit similar Plasticity Indices (Table 2).

Information on the effect of light on SI is less abundant. However, similar to SD, the response of SI to daily light integral (DLI) contrasts sharply with its response to $[CO_2]$, both in direction and strength. The dose-response curve for DLI is positive, and follows a saturating pattern (Figure 2B). The Plasticity Index for SI is 1.96, and the results exhibit high consistency (>90%). Intriguingly, these findings contrast with earlier studies by Salisbury (1927) and Poole et al. (1996), which were not included in our dataset because they compared sun and shade leaves within individual trees. Those studies observed differences in SD that largely diminished or disappeared when SI was analyzed. We have no clear explanation for this discrepancy, but it is possible that the regulation of SI at the whole-plant level differs from that within a single plant. Additional independent research on this contrast would be valuable.

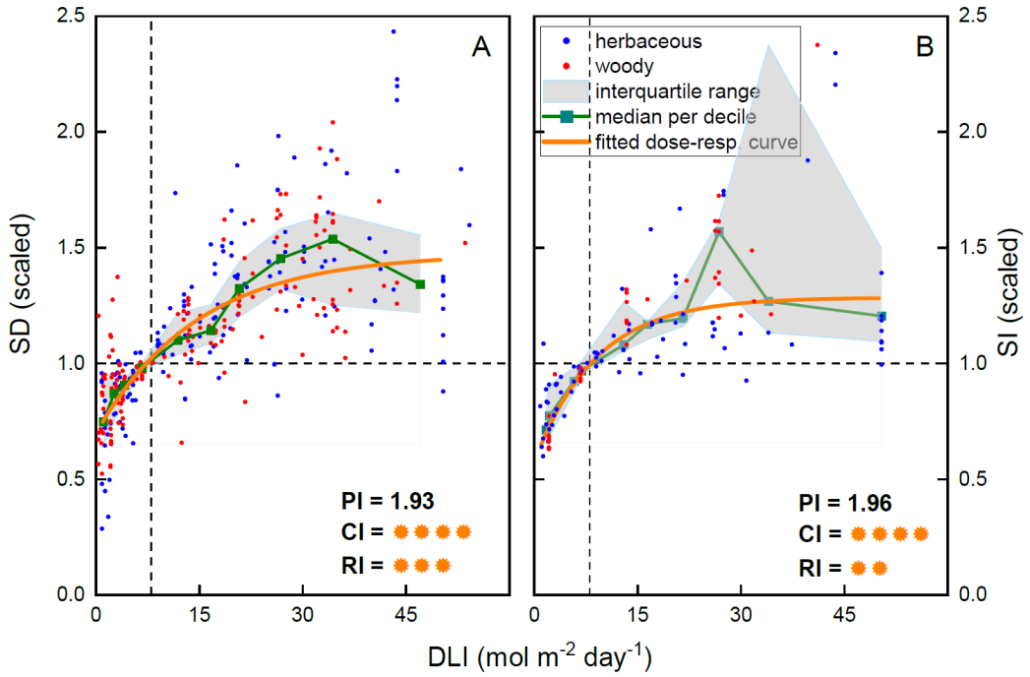

Figure 2. Dose-response curves for (**A**) Stomatal Density (SD) and (**B**) Stomatal Index (SI) as functions of Daily Light Integral (DLI). Data points represent scaled mean values per species and experiment, relative to a reference DLI of 8 mol m^{-2} d^{-1}. Two and four datapoints, respectively, with values exceeding 2.5 are not shown in this graph, but can be inspected in Figs. S03 and S04. For further details on data scaling, symbols, and indices, see the legend of Figure 1.

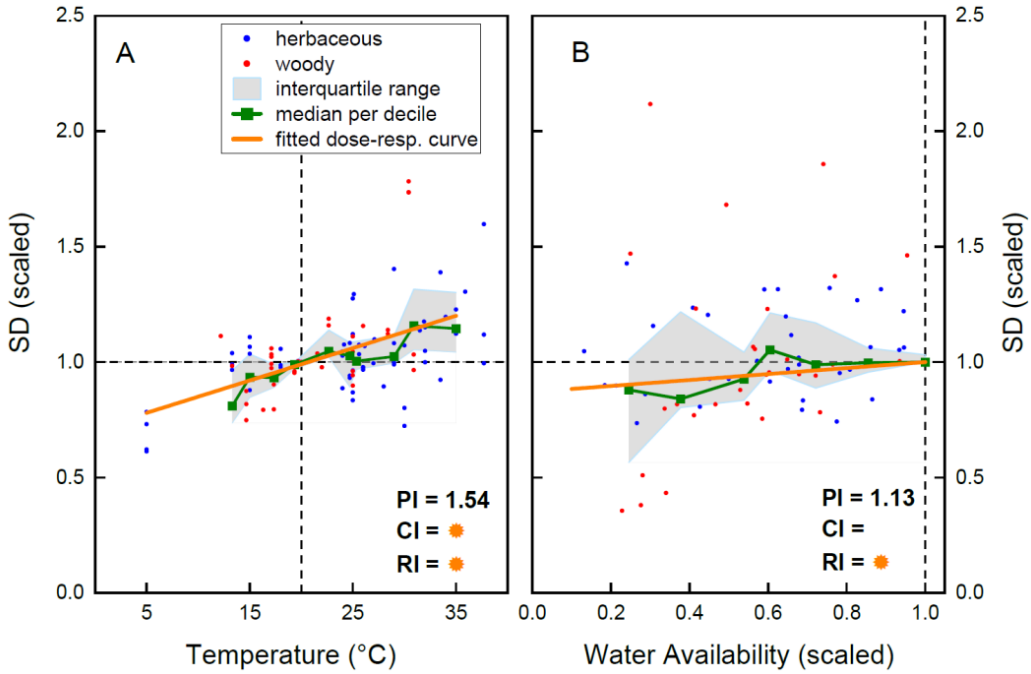

Figure 3. Dose-response curves for Stomatal Density (SD) in relation to (**A**) Temperature and (**B**) Water Availability. In (**A**), data points represent scaled mean values per species and experiment, normalized to values at a mean daily temperature of 20 °C. In (**B**), SD values are scaled relative to control plants grown under optimal water availability, with drought stress severity inferred from the biomass or leaf area of plants of drought-stressed plants compared to controls. For more information see the legend of Figure 1.

Another potential explanation is that one or more of our assumptions may not (fully) hold. Our approach aims to integrate as much information as possible, often combining data from different subfields of plant biology (Poorter et al., 2022a). Unfortunately, this information is highly scattered, and we rely on the assumption that compiling sufficient data for all species or subgroups thereof will allow to establish the proper dose-response curves. However, the available data remain limited, and to some extent reflect experiments where SD was measured for species A and B, whereas others focused only on SI for species C and D, rather than determining both traits for all species. We therefore also

analyzed data for those literature sources where both SD and SI were determined for leaves exposed to different light intensities. For those cases, we found a PI of 2.39 for SD, and 1.78 for SI, indicating a weaker response for SI. This aligns with the general observation that the size of stomatal complexes and pavement cells decreases under higher light intensities (Rahim & Fordham, 1991; Thomas, Woodward, & Quick, 2004; Oh & Kim 2010). Consequently, SD tends to increase with light intensity not only because there are relatively more stomatal complexes formed at high light, but also because the epidermal cells are smaller in size. Regardless of which data are included, the overarching conclusion remains that both SI and SD are far more sensitive to prevailing light conditions than to ambient CO_2 concentration.

We found insufficient data to construct dose-response curves for SI in response to temperature and water availability. The limited experimental data available showed no significant differences in SI between high-temperature and low-temperature treatments, nor between plants exposed to low and high water-availability. In both cases the Consistency Index was close to 50% (Table 1), indicating no clear pattern. Although these findings are based on limited data, they align with Royer's (2003) proposition that SI is largely independent of temperature and water availability. However, the results also highlight that light intensity is a far more significant modulator of SI than previously recognized.

3.3. The value of SD and SI for paleo-reconstruction

Stomatal density (SD) and stomatal index (SI) can both be measured from fossil leaves with a well-preserved cuticle. Of these two traits, SI is currently preferred to estimate CO_2 concentrations over geological timescales (Royer, 2003). However, based on the results of the meta-analyses discussed above, we would like to highlight several areas of caution regarding the use of either of these proxies.

1. Canopy position of leaves. Across the environmental ranges analyzed, the response of SD to [CO_2] is relatively modest compared to its response to temperature, and only slightly greater than its response to water availability (Table 1). Both SD and SI show weak responses to [CO_2] when compared to their much stronger responses to variations in light intensity. Given the high sensitivity of both traits to daily light integral (DLI), it is essential to account for the canopy position of fossilized leaves when interpreting stomatal data (Poole et al., 1996). In paleo-botanical studies, it is often assumed that most leaves in fossil assemblages are canopy leaves from light-saturated environments. This assumption is based on the idea that upper canopy leaves are more abundant, and more likely to be transported by wind to the actual deposition sites (Ferguson, 1985; Greenwood, 1991). However, light levels in a tree canopy can easily decrease by half within the top 4 m of a tree crown (Fauset et al., 2017). Assuming a DLI of 30 mol m^{-2} d^{-1} above the canopy, and that sun and shade leaves of trees follow the same trends as the fitted curve in Figure 2B, we calculate that leaves 4 m below the top of the tree canopy would have approximately 6% lower SI. This reduction represents half the 12% variation in SI observed across the CO_2 range of 200–1200 ppm range (Table 1), underscoring the importance of light gradients within the canopy (Poole et al., 1996).

Several proxies can help distinguish sun leaves from shade leaves in fossil specimens. Sun leaves typically have smaller epidermal cells, less undulated cell walls and greater ^{13}C discrimination compared to shade leaves (Kürschner, 1997; Graham et al., 2014; Šantrůček et al., 2014; Dunn et al., 2015; Poorter et al., 2022a). Although some studies indicate that the majority of fossil leaves were likely exposed to high-light conditions (Ferguson, 1985; Greenwood, 1991; Kürschner, 1997), others indicate considerable variation in the light environments experienced by fossil leaves (Bush et al., 2017). Therefore, it is prudent to infer the original canopy position of fossil leaves when using them for paleo-CO_2 reconstructions. For example, Reichgelt et al. (2020) estimated CO_2 levels from early Miocene fossil leaves by selecting those with relatively high cell density, minimal cell undulation and high leaf $\delta^{13}C$. Since cell density and leaf $\delta^{13}C$ values are already key input parameters in current gas-exchange models for CO_2 estimation (Franks et al., 2014), incorporating these criteria does not require much additional analytical work. Consequently, this approach should be considered standard protocol when interpreting fossil leaves for CO_2 reconstructions.

The above analysis relies on the assumption that the dose-response curve for SD and SI, as determined for whole plants grown at different DLIs, is also applicable to leaves that experience varying light availability within a tree. While this assumption holds for a range of leaf-level traits (cf. Niinemets, Keenan, & Hallik, 2015; Poorter et al., 2019), the few studies comparing the SI of sun and shade leaves in individual trees have reported much larger differences in SD than in SI (Salisbury, 1927; Poole et al., 1996; Kürschner, 1997). As noted earlier, this aspect warrants further investigation.

2. Above-canopy light availability. The issue of DLI related to canopy position extends to broader above-canopy light conditions. Assuming a fixed level of DLI for canopy leaves across geological eras overlooks uncertainties introduced by variables such as cloud cover, which can vary and affect above-canopy light availability (Stephens, 2005). Based on the saturation observed in the dose-response curves (Figure 2A,B), we would expect the SD or SI of plants in locations with minimal cloud cover, such as desert areas at low latitudes, to be relatively unaffected. However, fossil leaves are often better preserved in wetter areas, where cloud cover can substantially influence DLI, and consequently SD and SI. Of particular interest are environments with few or no modern analogues, such as temperate polar forests during the hothouse climates of the Cretaceous and Eocene (e.g., Herman & Spicer, 2010; West, Greenwood, & Basinger, 2019). The above-canopy light conditions in these environments are difficult to reconstruct and the associated ecophysiological adaptions are challenging to constrain

(Brentnall et al., 2005; Konrad, Roth-Nebelsick, & Traiser, 2023). Nonetheless, fossil leaves from these environments do get used in paleo-CO_2 reconstructions (Wolfe et al., 2017; Wang et al., 2020c).

3. Between and within-species specificity. We established generalized dose-response curves based on the compiled data, showing consistent positive responses to light, but variable responses to [CO_2] (Table 1). This variability poses a challenge for accurately estimating paleo-CO_2 levels. A key question is whether this variation stems from between or within species differences in stomatal responses. Although we found slightly stronger responses to [CO_2] in C_3 herbaceous and woody species compared to C_4 species, these differences were not statistically significant. Similarly, there were no significant differences in plasticity between C_3 herbs and woody species (Table 2). We also tested whether SD responses were different between deciduous and evergreen tree species. For CO_2, PI was marginally and non-significantly different (−1.05 and −1.14, respectively), but for light intensity, evergreens showed higher plasticity than deciduous species (2.20 and 1.56, respectively, respectively), with $0.05 < p < 0.10$. Since most fossilized leaves stem from woody species, and have a better chance to be preserved when the leaves are sturdy and therefore of evergreen nature, this may aggravate the problems with interpretation mentioned above.

In paleobotany, researchers calibrate absolute values of SD and SI in fossil leaves with those of their nearest-living relatives growing under known CO_2 concentrations (McElwain & Steinthorsdottir (2017). However, substantial species-level differences in SD and SI, as well as variations between genotypes, have been reported (e.g., Christophel & Rowett, 1996; Hovenden & Schimanski, 2000; Wall et al., 2023). Consequently, selecting a genotype from a nearest-living relative introduces additional uncertainty into CO_2 estimates. Another source of uncertainty stems from within-species variability in the relative CO_2-response observed across experiments. In our compilation, such repetition was available for only a few species, and primarily in sufficient numbers for SD. In Figure 4, we show the responses for the most-frequently studied species, *Triticum aestivum*, and the 'living fossil' *Ginkgo biloba.* For both species, studies report both positive and negative responses to increasing [CO_2]. However, the overall pattern is not very different from the generalized dose-response curve shown in Figure 2A. This suggests that relying on data for a given species from only one or two experiments may not produce a robust calibration curve. To better capture these species-specific or genotypic responses, more comprehensive datasets are required. Nonetheless, given the within and across species variation of SI and SD in response to [CO_2], it is unlikely that modern living species, such as *Ginkgo biloba*, can be used to estimate CO_2 concentrations in deep time, as the genetic and ecophysiological variability cannot be constrained.

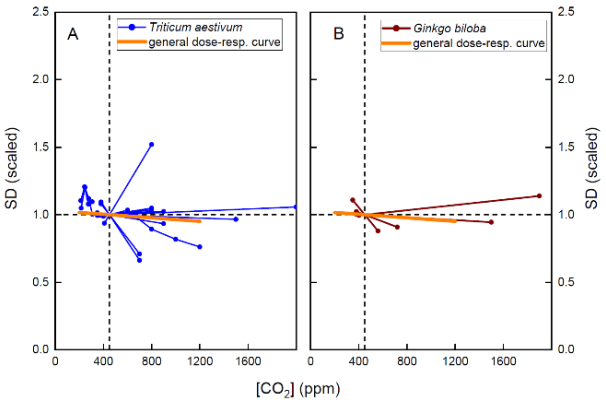

Figure 4. Effect of ambient CO2 on Stomatal Density (SD) as reported in different experiments for (A) *Triticum aestivum,* and (B) *Ginkgo biloba.* The orange line represents the overall fit from Figure 1A. Data points connected by a line are mean values per experiment. All data are scaled relative to the phenotypic values at a reference [CO_2] of 450 ppm.

4. Experimental data vs using historical leaves. To estimate paleo-CO_2 levels, transfer functions can be established between CO_2 concentration and SI or SD. These functions are analogues to the dose-response curves we previously discussed, but with inverted axes. Several approaches have been used to derive these transfer functions. These include, from shorter to longer timespans: (1) plants grown experimentally under various CO_2 concentrations, (2) herbarium leaves collected during periods with known CO_2 levels, and (3) leaves from sub-recent sediments calibrated against CO_2 data from ice-core records. We compared five transfer functions derived from herbarium records and sedimentary leaves, converting them into CO_2 dose-response curves and scaling them similarly as our general dose-response curve (Figure 5). The difference in slope between the generalized dose-response curve on the one hand and those derived from the published transfer functions on the other is striking. The latter exhibit much steeper slopes compared to the curve based on controlled experiments. This was previously noted by Beerling & Chaloner (1992) and Royer (2001). They postulated that long-term genetic pressures on stomatal initiation may outweigh the more immediate, modest plastic response. They further suggested that it might take 100–1000 years for plants to fully adjust to new atmospheric CO_2 levels. For trees this would imply adaptation over 2–10 generations. From an ecophysiological perspective, reduced stomatal density under elevated CO_2 seems plausible, as a lower SD could maintain sufficient conductance for CO_2 diffusion. It is not easy to experimentally substantiate this thesis, but the scarcely-available evidence is not supportive. Yang et al. (2023) conducted an experiment where rice was grown over five consecutive generations under either control or elevated CO_2. They found a marginal *increase* in SD due to [CO_2], in both the 1st and 5th generation, rather than the anticipated decrease. Their study, the first of its kind to assess SD across so many generations, offers little support for a substantial and negative generational effect.

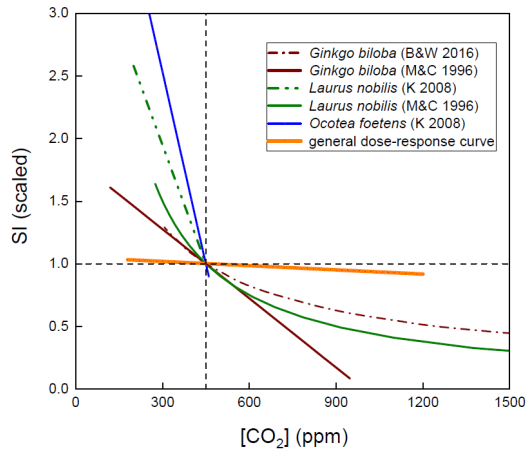

Figure 5. Comparison of the generalized dose-response curve for Stomatal Index (SI) with respect to [CO_2] (shown in Figure 1B) with transfer functions derived for *Ocotea foetens* and *Laurus nobilis* (Kürschner, Kvaček, & Dilcher, 2008), *Laurus nobilis* and *Ginkgo biloba* (McElwain & Chaloner, 1996), and *Ginkgo biloba* (Barclay & Wing, 2016).

Alternatively, the marginal change observed in experimental plants (Figure 5) could be attributed to the fact that they all originated from seeds of plants grown under present-day CO_2 levels, and were often pre-grown under control CO_2 levels during seedling establishment. Studies show that older leaves signal their growth conditions to younger leaves, influencing SD and SI in newly-developing leaves in response to both CO_2 concentration (Lake et al., 2001) and light intensity (Thomas, Woodward, & Quick, 2004). This suggests that a temporary carry-over effect from past CO_2 conditions might lead to an underestimation of the CO_2 effect in experimental approaches. However, the median duration of CO_2 experiments in our compilation was 98 days. For comparison, even a single day of exposure to different light conditions already affects the final SD (Schoch et al., 1980). Thus, assuming similar dynamics, we expect 98 days of CO_2 exposure to be sufficient to eliminate legacy effects. Another possible explanation for the observed discrepancy is that stomatal development in field-collected plants over extended time periods may be influenced by co-varying environmental factors, such as cooler temperatures during periods of lower [CO_2] (Figure 3A). Finally, publication bias could play a role, with studies reporting transfer functions with shallow slopes being less likely to be submitted or accepted for publication. In any case, understanding the mechanisms driving the difference in sensitivity between contemporary experimental data and field-collected historical measurements would be helpful to improve confidence in transfer functions based on field data.

5. Extrapolating transfer functions. A final consideration is that transfer functions derived from herbarium or sediment leaves, and calibrated using CO_2 concentrations from ice cores, are only validated within the range of 280–400 ppm, as these are the data for which we have independent CO_2 measurements. Any values beyond this range represent extrapolations, which complicates the use of these transfer functions for periods when CO_2 levels exceeded current values.

The toolbox available to paleobotanist is limited, and their methodologies cannot be as refined as those employed by ecophysiologists studying living plants. Nonetheless, the questions paleobotanists address are crucial, and reliable proxies for past CO_2 concentrations are indispensable for understanding system Earth. Unfortunately, stomatal density and stomatal index responses to CO_2 suffer from considerable variability and inconsistency. New approaches that integrate (eco)physiological and morphological traits with modeling techniques (Franks et al., 2014; Konrad et al., 2017) hold greater promise. These methods are increasingly replacing SD- and SI-based approaches due to their improved reliability. Despite this progress, it remains prudent to base paleo-CO_2 reconstructions on a diverse array of proxies. As demonstrated effectively by Hönisch et al. (2023), combining multiple lines of evidence enhances confidence in estimates and provides a more comprehensive understanding of past atmospheric conditions.

4. Conclusions

We developed generalized dose-response curves for stomatal density (SD) and stomatal index (SI) in response to [CO_2] and light intensity, along with additional curves for SD as dependent on temperature and water availability. Although both SD and SI exhibited negative correlations with [CO_2], these responses were relatively small and inconsistent. In contrast, their responses to changes in Daily Light Integral (DLI) were significantly stronger and more consistent, emphasizing the dominant influence of light in shaping SD and SI. Consequently, the position of leaves within the canopy or variations in light availability across different eras introduces significant complexity, further challenging the reliability of fossil leaf stomata as robust paleoproxies for reconstructing past atmospheric CO_2 concentrations.

Supplementary Materials

The following supporting information can be downloaded at: https://www.sciltp.com/journals/PlantEcophys/2025/1/514/s1. S1. Extended Materials & Methods. S2. References used for the meta-analyses, listed per environmental factor. S3. Detailed figures of the responses of stomatal density and index to [CO2], Daily Light Integral, Temperature and Water availability. S4. Responses of various functional groups of species.

Author Contributions

H.P. and T.L.P. conceptualized the idea and carried out the data collection. H.P. calculated and analyzed the data. H.P. TL.P. and T.R. wrote the ms, with H.P. and T.L.P. covering the ecophysiological aspects, and TR the paleobotanical aspects.

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Data Availability Statement

Data are available upon request from Hendrik Poorter.

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Conflicts of Interest

The authors have no conflict of interest.

Peer Review Statement

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Abbreviations: CI, Consistency Index; DLI, Daily Light Integral; PI, Plasticity Index; RI, Reliability Index; SD, Stomatal Density; SI, Stomatal Index

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References marked with an asterisk indicate studies included in the meta-analysis.

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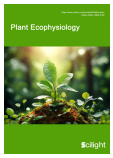

Review

Navigating Challenges in Interpreting Plant Physiology Responses through Gas Exchange Results in Stressed Plants

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Abstract: This paper explores the challenges that arise when performing and interpreting leaf gas exchange measurements in plants subjected to abiotic stress. It highlights how factors such as cuticular fluxes, stomatal closure, and common assumptions about gas exchange can lead to errors, especially under stress conditions. Key phenomena such as substomatal cavity unsaturation and stomatal patchiness during water stress are discussed in detail, as they significantly complicate the calculation of gas exchange parameters under stress. The paper also addresses the importance of other factors, including steady-state conditions, the differences between adaxial and abaxial surface responses, and boundary layer effects, all of which play critical roles in influencing the accuracy of measurements. Important physiological indicators—such as intrinsic water-use efficiency, minimum leaf conductance, substomatal CO_2 concentration, and mesophyll conductance—are analysed in the context of how stress-induced discrepancies in data often result from measurement artefacts rather than true physiological differences. To address these challenges, the paper outlines practical approaches to improving measurement accuracy, offering insights on standardising experimental conditions and minimising errors. By recognising these issues, gaps in current knowledge are identified, providing a comprehensive overview of the challenges in interpreting leaf gas exchange data under stress conditions and suggesting areas for further study.

Keywords: plant stress; cuticular conductance; stomatal patchiness; unsaturation; abaxial; adaxial; leaf gas exchange

1. Introduction

Leaf gas exchange measurements have been instrumental in plant physiology research, underpinning critical aspects of photosynthesis and transpiration (Long & Bernacchi, 2003; Sharkey, 2016). Gas exchange involves the movement of carbon dioxide (CO_2), oxygen (O_2), and water vapour (H_2O) into and out of the leaf. A significant area of scientific interest has been understanding the mechanisms governing the ratio of CO_2 uptake to water loss (Cowan & Farquhar, 1977; Deans et al., 2020), i.e., assimilation (A) and transpiration (E) rates. Over the past century, these measurements have been used to explore plant-environment interactions at the leaf level, with methods evolving from early porometer techniques to today's sophisticated systems such as the LI-6800 (LI-COR, Lincoln, NE, USA), Walz GFS-3000 (Walz, Effeltrich, Germany), CIRAS-4 (PP Systems, Amesbury, MA, USA), among others. From the early stages of using these techniques, one of the aims was to estimate the internal leaf microenvironmental conditions that would allow us to better scrutinise the plant's physiological responses related to photosynthesis. Over time, reliable models were developed to estimate internal leaf conditions from external measurements, leading to the definition of key physiological parameters that encapsulate biological meaning (Gaastra, 1959; Moss & Rawlins, 1963; von Caemmerer & Farquhar, 1981; Márquez, Stuart-Williams, & Farquhar, 2021). Physiological parameters such as stomatal conductance to water (g_{sw}) and internal CO_2 concentration (c_i) have become standard and common language in plant sciences, as they serve as the cornerstone for comparing trends, checking for improvement, or evaluating performance *in planta*.

External measurements, combined with modelling, enable us to explore *in planta* physiological responses to varying environments, growth conditions, and broader ecophysiological trends (Wong, Cowan, & Farquhar, 1979; Farquhar, von Caemmerer, & Berry, 1980; Farquhar & Richards, 1984). These approaches have provided a wealth of information linking biochemical and biophysical knowledge in plant sciences with leaf- and plant-scale phenomena. Thus, leaf gas exchange

measurements have been pivotal in numerous areas of plant science research (see, for example Wong, Cowan and Farquhar (1979); von Caemmerer and Farquhar (1981) and Long and Bernacchi (2003)) and are often the benchmark for assessing treatment effects and evaluating genetically modified organisms *in planta* (von Caemmerer, 2000; Long & Bernacchi, 2003; von Caemmerer, 2013).

The physical principles—including diffusion theory, Fick's laws of diffusion, and mass conservation—underlying the estimation of gas exchange parameters and the interpretation of these calculated values are well-established and scientifically sound. These principles are based on assumptions that have been developed from studies conducted on healthy, well-watered plants, which typically exhibit high assimilation rates and stomatal conductances (Gaastra, 1959; Scholander et al., 1965; von Caemmerer & Farquhar, 1981). While these assumptions work reliably for unstressed plants, they were not initially designed for application to stressed plants. As a result, when transitioning to stress conditions, such as drought or extreme temperatures, the robustness of these assumptions is called into question (Turner, Schulze, & Gollan, 1984; Boyer, Wong, & Farquhar, 1997; Boyer, 2015a; Yan, Zhong, & Shangguan, 2016; Cernusak et al., 2018; Buckley & Sack, 2019; Cernusak et al., 2019). This raises important concerns about the reliability of gas exchange data collected under stress conditions and whether the interpretations drawn from these measurements remain valid.

Some notable examples of these assumptions during gas exchange measurements include the assumption that leaf cuticle conductance to water (g_{cw}) is negligible. This assumption is critical for calculating g_{sw}, assuming that transpiration passes only through the stomatal pores (von Caemmerer & Farquhar, 1981). However, under water stress conditions, the cuticle can become a more significant pathway for water loss, invalidating this assumption and potentially leading to errors in the estimation of gas exchange parameters (Boyer, Wong, & Farquhar, 1997; Boyer, 2015a; Tominaga & Kawamitsu, 2015; Márquez, Stuart-Williams, & Farquhar, 2021). Another key assumption is that the internal leaf gas space is saturated with water vapour (Gaastra, 1959), which is a crucial assumption for determining the driving force for water vapour diffusion from the leaf to the atmosphere. However, evidence suggests that the saturation assumption may no longer hold under mild to severe water stress conditions, resulting in inaccuracies in gas exchange measurements and interpretations (Cernusak et al., 2018; Wong et al., 2022; Márquez et al., 2024). Additionally, there is the assumption that leaf surface properties, such as stomatal aperture and stomatal conductance, are uniform across the leaf (Moss & Rawlins, 1963). While this assumption may be valid for unstressed plants, it does not always hold for stressed plants (Laisk, 1983; Downton, Loveys, & Grant, 1988). Stressed plants frequently exhibit substantial heterogeneity in stomatal behaviour within a single leaf and among different leaves on the same plant (Mott, Cardon, & Berry, 1993; Cardon, Mott, & Berry, 1994; Mott & Buckley, 2000). These inconsistencies can introduce significant errors in gas exchange calculations and complicate the interpretation of the data, potentially undermining our calculations or even making them essentially wrong.

> "*Lo que por sabido se calla, por callado se olvida*"
> — Spanish Proverb
> (Translation: "What is assumed to be known and left unspoken, by remaining unspoken, falls into oblivion.")

Over the past century, scientists have extensively documented the weakening of fundamental assumptions in gas exchange measurements when the conditions required to uphold these assumptions are unmet. It has been noted that the robustness of these assumptions is closely linked to the presence of an unstressed plant during measurement. This is especially important considering the increasing interest in assessing how stressful conditions affect plant physiology, particularly in light of the anticipated challenges posed by climate change (Grossiord et al., 2020; Fu et al., 2024). Addressing these uncertainties in a practical manner, rather than merely theoretically, is essential, but they are often overlooked in many studies. This mirrors the old Spanish proverb, "*Lo que por sabido se calla, por callado se olvida*:" despite general awareness of these uncertainties, they often remain implicit, theoretical, and largely unspoken in the context of plant physiology. However, we must critically examine and challenge these underlying assumptions to fully capitalise on our understanding of gas exchange in stressed plants. This requires investigating when, why, and how these assumptions fail and determining how we can account for or bypass these weaknesses to improve the reliability of our measurements.

Importantly, we are not starting from scratch. Numerous researchers have already explored the weakening of these assumptions in stressed plants, offering potential solutions, alternative methods, and strategies to address various aspects of the problem. Building on this foundation, our aim here is to identify the challenges encountered during gas exchange measurements of stressed plants, discuss the causes of these challenges, and highlight the remaining gaps that still need to be addressed for a better understanding of leaf gas exchange under stressed conditions. Additionally, when possible, we seek to provide practical solutions to mitigate or avoid these problems to ensure that results extracted from experiments under stressed conditions are robust and reliable.

2. Gas Exchange Measurements

Let us briefly address the calculations involved in common leaf gas exchange measurements. Gas exchange systems typically include a chamber that encloses an area of leaf in an open gas system, where a reference gas is introduced (Gaastra, 1959). The entering gas (reference) and the gas exiting the chamber (sample) are analysed for water vapour and CO_2 concentrations (Figure 1a). Additional measurements are taken from the chamber and leaf, including leaf and air temperature, flow rate into the chamber, and light intensity.

The mass balance obtained from the flow rate, and the reference and sample concentrations, permit to derive the following expression for the transpiration rate (E):

$$E = \frac{\mu_{out} w_a - \mu_0 w_0}{s} \quad (1)$$

where μ_{out} is the flow rate exiting the chamber, μ_0 is the flow rate entering the chamber, w_0 is the water vapour mole fraction entering the chamber, w_a is the water vapour mole fraction exiting the chamber, and s is the projected leaf surface area within the chamber. The flow rate exiting the chamber is approximated as $\mu_{out} \approx \mu_0 + sE$, neglecting CO_2 uptake due to its significantly lower magnitude compared to water release. Consequently, the equations for E become:

$$E = \frac{\mu_0 \left(w_a - w_0 \right)}{s\left(1 - w_a\right)} \quad (2)$$

Analogous for CO_2 assimilation rate (A)

$$A = \frac{\mu_0}{s}\left[c_0 - c_a \left(\frac{1 - w_0}{1 - w_a} \right) \right] \quad (3)$$

where c_0 is the CO_2 mole fraction entering the chamber and c_a is the CO_2 mole fraction exiting the chamber.

These equations allow us to estimate A and E based on the known leaf surface area within the chamber. While the degree of control over gases entering or exiting the chamber, temperatures, light intensity, and other factors varies across different commercial and in-house developed gas exchange systems, the fundamental objective remains the same. These systems aim to estimate the same gas exchange parameters, the ease with which water escapes the leaf and the CO_2 concentration within the leaf, from the above mass flow balances.

Gas exchange parameters are defined using an analogy to electrical resistances, with the boundary layer, stomata, and cuticle acting as resistors for water vapour and CO_2 diffusion (Figure 1b) or their inverses, conductances. The most recent theory for estimating these parameters was presented by Márquez, Stuart-Williams and Farquhar (2021), representing water vapour diffusion from the leaf surface and substomatal cavity to the atmosphere as:

$$r_{tw} = \frac{1}{\frac{1}{r_{cw}} + \frac{1}{r_{sw}}} + r_{bw} = \frac{1}{\frac{1}{\left[\frac{w_c - w_s}{E_c}\right]} + \frac{1}{\left[\frac{w_i - w_s}{E_s - E_s \overline{w}_s}\right]}} + \left[\frac{w_s - w_a}{E - E\overline{w}_b}\right] \quad (4)$$

and

$$\overline{w}_s = \frac{w_i + w_s}{2}$$

$$\overline{w}_b = \frac{w_s + w_a}{2}$$

where r_{tw}, r_{cw}, r_{sw}, and r_{bw} are the total, cuticular, stomatal, and boundary layer resistances to water vapour diffusion, respectively. E_c, and E_s are the cuticular and stomatal transpiration rates such that $E = E_s + E_c$, and w_c, w_s, and w_i are the cuticular, leaf surface, and substomatal cavity mole fractions of water vapour, respectively. Parameters $\overline{w}_s$ and $\overline{w}_b$ are associated with ternary corrections through the stomata and boundary layer. The aim of water measurements is usually to obtain r_{sw}, which from Equation (4) is

$$r_{sw} = \frac{w_i - w_s}{E_s - E_s \overline{w}_s} \quad (5)$$

To solve Equation (5) the values for r_{bw}, w_s, r_{cw}, and E_c ($E_s = E - E_c$) are needed.

Empirical values of r_{bw} for the gas mixing system in the chamber are typically embedded in the instrument's calculations. Given r_{bw}, w_s can be estimated as,

$$w_s = \frac{r_{bw} E \left(1 - \frac{w_a}{2}\right) + w_a}{1 + r_{bw}\frac{E}{2}} \quad (6)$$

There are independent methods that allow for the estimation of r_{cw}, which should be conducted either before or after the experiment at hand. These estimates assume r_{cw} remains constant as long as the leaf is turgid, with w_c generally considered equal to w_i, thereby allowing for the estimation of E_c (from Equation (4): $E_c = (w_i - w_s)/r_{cw}$). Details of the available techniques and the assumption of constant r_{cw} will be discussed later in the text.

The common assumption is that there are vapour water-saturated conditions within the leaf ($w_i = w_{sat}$), so that w_i can be estimated from the dew point at leaf temperature:

$$w_{sat} = \frac{0.611121 e^{\frac{17.502 T_l}{240.97 + T_l}}}{P_{atm}} \quad (7)$$

where T_l is leaf temperature in Celsius, and P_{atm} is atmospheric pressure (kPa). Then, all the parameters to obtain r_{sw} (Equation (5)) are estimated.

Analogous to Equation (4), for CO_2 the resistance becomes:

$$r_{tc} = \frac{1}{\frac{1}{r_{cc}} + \frac{1}{r_{sc}}} + r_{bc} = \frac{1}{\frac{1}{\left[\frac{c_{cut} - c_s}{A_c}\right]} + \frac{1}{\left[\frac{c_i - c_s}{A_s + E_s \overline{c}_s}\right]}} + \left[\frac{c_s - c_a}{A + E\overline{c}_b}\right] \quad (8)$$

and

$$\overline{c}_s = \frac{c_i + c_s}{2}$$

$$\overline{c}_b = \frac{c_s + c_a}{2}$$

where r_{tc}, r_{cc}, r_{sc}, and r_{bc} are the total, cuticular, stomatal, and boundary layer resistances to CO_2 diffusion, respectively. A_c and A_s are the cuticular and stomatal assimilation rates, and c_{cut}, c_s, and c_i are the cuticular, leaf surface, and substomatal. cavity mole fractions of CO_2, respectively. The aim of CO_2 measurements is usually to obtain c_i.

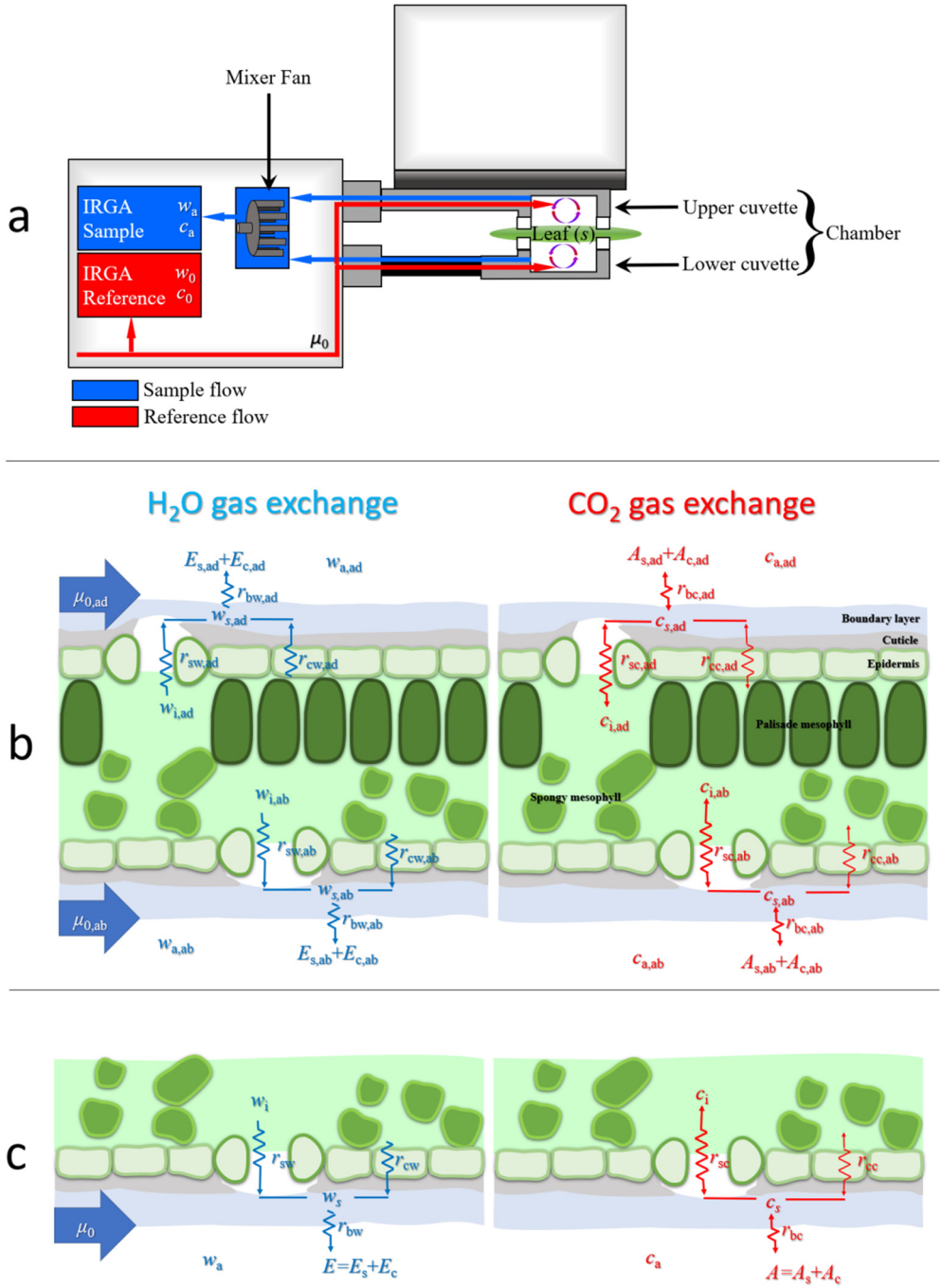

Figure 1. Schematic of gas exchange system setup and illustration of H_2O and CO_2 gas exchange. (**a**) Diagram of a typical gas exchange chamber system, illustrating the flow of the sample (blue lines) and reference (red lines) air through the chamber, which houses the leaf. Infrared gas analysers (IRGA) measure water vapour and CO_2 concentrations in the sample and reference flows to estimate bulk transpiration (E) and assimilation (A) rates. The upper and lower cuvette fluxes are typically mixed in standard gas exchange systems, resulting in bulk measurements of gas exchange parameters. (**b**) Detailed representation of H_2O gas exchange (**left**) and CO_2 gas exchange (**right**) at the adaxial (upper) and abaxial (lower) leaf surfaces in an amphistomatous leaf. This panel illustrates the separate gas exchange processes for each leaf surface, which can be measured individually if the upper and lower cuvettes are analysed separately—though this setup is not common in commercial gas exchange systems. Stomatal, cuticle, and boundary layer resistances are depicted for both surfaces, along with the mole fractions of water vapour and CO_2. (**c**) Simplified depiction of overall H_2O and CO_2 gas exchange as in most commercial systems. A bulk measurement of transpiration (E) and CO_2 assimilation (A) rate is obtained, representing the combined contributions of stomatal and cuticular components from adaxial and abaxial surfaces alongside a bulk boundary layer conductance. Parameters: w_i is the water vapour mole fraction in the substomatal cavity, w_s is the water vapour mole fraction at the leaf surface, w_a is water vapour mole fraction exiting the chamber (i.e., atmospheric), w_0 is the water vapour mole fraction in the reference gas, c_i is the CO_2 mole fraction in the substomatal cavity, c_s is the CO_2 mole fraction at the leaf surface, c_a is the CO_2 mole fraction exiting the chamber (i.e., atmospheric), c_0 is the CO_2 mole fraction in the reference gas, r_{sw} is the stomatal resistance to water vapour, r_{bw} is the boundary layer resistance to water vapour, r_{sc} is the stomatal resistance to CO_2, r_{bc} is the boundary layer resistance to CO_2, r_{cw} is the cuticular resistance to water vapour, r_{cc} is the cuticular resistance to CO_2, μ_0 is the flow rate of the reference gas, ad and ab subscripts refer to the adaxial and abaxial leaf surfaces, respectively.

Table 1. Summary of the key challenges in gas exchange measurements under stress conditions.

Category	Key Challenges
Small fluxes (Leaf cuticle)	**Neglecting g_{cw}:** Ignoring cuticular conductance when stomatal conductance is low can result in significant overestimations of g_{sw} and c_i.
	Variability in g_{cw}: Differences in g_{cw} across species and under stress conditions complicate the universal application of a value for the parameter.
Unsaturation in the substomatal cavity	**Saturation assumption ($w_i = w_{sat}$):** Assumption of water vapour saturation fails under moderate to high VPD, resulting in underestimation of g_{sw} and c_i.
	Non-stomatal control of transpiration: Often overlooked, it introduces errors in physiological interpretations, including g_{sw} and c_i.
Patchiness	**Stomatal spatial and temporal heterogeneity:** Uneven stomatal behaviour across the leaf surface distorts gas exchange parameters such as c_i and g_{sw}.
	Unpredictability: Limited understanding of the drivers of patchiness and lack of methods to account for its effects in gas exchange measurements.
Adaxial and abaxial flux differences	**Combining fluxes:** Mixing fluxes from both leaf surfaces may obscure important stress responses and result in misinterpretations.
Steady-state conditions	**Complexity of defining:** Fluctuations and transient responses under stress make it challenging to define and evaluate steady states in gas exchange measurements.
Technical and calibration errors	**Unreliable raw data:** Sensor drift, condensation, lack of equipment maintenance, and unstable environmental conditions can compromise the accuracy of gas exchange measurements (not discussed in detail here, but a key challenge overall).
Flow rate adjustments	**Signal-to-noise ratio:** Balancing the signal-to-noise ratio with maintaining environmental conditions is challenging, particularly for low gas exchange rates under stress.
Practical constraints in fieldwork	**Complexity in measurements:** Translating lab insights, such as the implementation of advanced measurement techniques or specific conditions, to field settings is challenging due to environmental variability and logistical constraints.

To obtain r_{sc} and r_{bc}, the estimates of r_{sw} and r_{bw} are used with the ratio of water over CO_2 diffusivity coefficients ($D_{H_2O,air}/D_{CO_2,air}$), which range around 1.58 ± 0.04, (Massman, 1998), usually taken as 1.6. Then, $r_{sc} = 1.6 r_{sw}$ and from Cowan (1972) $r_{bc} = 1.6^{2/3} r_{bw} = 1.37 r_{bw}$. Then, c_s can be estimated,

$$c_s = \frac{c_a\left(\frac{1}{r_{bc}} - \frac{E}{2}\right) - A}{\frac{1}{r_{bc}} + \frac{E}{2}} \tag{9}$$

Unlike r_{cw}, reliable techniques to estimate r_{cc} are scarce due to the difficulty of obtaining accurate A_c measurements. Currently, r_{cw} is used as a reference with a diffusion coefficient ratio for CO_2 and water through the cuticle between 20 and 40 (Boyer, 2015b; Márquez, Stuart-Williams, & Farquhar, 2021). Regardless, assuming A_c equals 0 and neglecting r_{cc} introduces negligible error in gas exchange calculations (Márquez, Stuart-Williams, & Farquhar, 2021).

Finally, c_i can be estimated as,

$$c_i = \frac{c_s\left(\frac{1}{r_{sc}} + \frac{1}{r_{cc}} - \frac{E_s}{2}\right) - A}{\frac{1}{r_{sc}} + \frac{1}{r_{cc}} + \frac{E_s}{2}} \approx \frac{c_s\left(\frac{1}{r_{sc}} - \frac{E_s}{2}\right) - A}{\frac{1}{r_{sc}} + \frac{E_s}{2}} \tag{10}$$

From the equation presented above, it is evident that the assumptions discussed earlier significantly influence our estimations and propagate through subsequent calculations if incorrectly applied (see Table 1). This highlights how errors in parameter assumptions can undermine the reliability of measurements under stress conditions, ultimately leading to misinterpretations of physiological responses.

3. Risk of Misreading Physiological Trends in Stressed Plants

It is essential to recognise that technical and calibration issues are a significant source of error in gas exchange measurements. Common problems include erroneous calibrations leading to physically impossible values, such as negative c_i, and technical issues related to temperature fluctuations, condensation, or measurements taken during transition states. These errors can compromise the accuracy of raw data, making proper instrument calibration and operation critical before proceeding to parameter estimations. While technical and calibration issues are fundamental and warrant detailed exploration, they are not the focus of this manuscript. Other publications, manuals and reviews, such as Flexas et al. (2007a), Kitao, Harayama and Uemura (2017), LICOR (2020), Garen et al. (2022), Busch et al. (2024) among others, have addressed these topics comprehensively.

Leaving aside possible technical errors and calibration issues that could arise from the instruments and sensors during gas exchange measurements, the main source of error is usually the erroneous estimation of r_{sw} or its inverse stomatal conductance to water ($g_{sw} = 1/r_{sw}$) (Laisk, 1983; Mott, 1995; Boyer, 2015a; Márquez, Stuart-Williams, & Farquhar, 2021; Wong et al., 2022; Márquez et al., 2023a; Hussain et al., 2024). This error can emerge from neglecting some relevant fluxes, such as cuticular transpiration, erroneously assigning values to parameters indirectly estimated, such as w_i, or a more general problem with the used model, such as assuming even stomatal behaviour on the

leaf surface (Table 1). Regardless of the source, any parameters derived from r_{sw} and the physiological trends based on those parameters will be compromised, with the likelihood of these errors increasing when dealing with stressed plants.

In this section we explore the potential for misinterpretations of commonly used parameters and physiological trends derived from leaf gas exchange measurements in plant stress research. These parameters are often employed to compare treatments in experiments and to draw conclusions in studies. Thus, misinterpretations can result in inaccurate assessments and potentially flawed conclusions.

3.1. Intrinsic water-use efficiency interpretation

Intrinsic water-use efficiency (*i*WUE = A/g_{sw}) provides a simple yet effective means for evaluating plant water-use efficiency by combining the rate of carbon assimilation with stomatal conductance to water into a single, easily comparable ratio (Flexas et al., 2013; Leakey et al., 2019). This approach is often preferred over direct flux measurements (A/E) as it accounts for the influence of atmospheric vapour pressure deficit (VPD), normalising the data in relation to atmospheric demand. Such normalisation is particularly advantageous in drought and stress research, where it is believed to assist in identifying species or genotypes better adapted to water-limited environments.

Moreover, *i*WUE, combined with techniques like carbon isotope discrimination, enables comparative analyses across plant species and environmental conditions. This makes it valuable for breeding programs, ecological studies, and agronomic research focused on optimising water use (Condon et al., 2004). In this regard, recent studies have gone even further by correlating long-term *i*WUE with nonphotosynthetic $^{12}C/^{13}C$ fractionation in carbon isotope discrimination data from plant biomass. Yu et al. (2024) showed that correcting *i*WUE estimates for this fractionation helps to reconcile discrepancies between isotope-based *i*WUE and those derived from gas exchange measurements, giving a practical approach for long-term *i*WUE estimations.

It is important to note that the strengths of *i*WUE can also be a limitation. While *i*WUE is closely linked to stomatal conductance, it provides a limited snapshot that may not fully capture the plant's overall water use strategy (Liang et al., 2023). Factors such as VPD can influence stomatal conductance and transpiration, creating feedback loops that complicate the assumption of a steady-state relationship among these variables. Additionally, other factors beyond stomatal behaviour can influence transpiration at any given time, potentially leading to incorrectly estimating g_{sw} and biasing *i*WUE analysis, for instance, if factors such as patchiness or unsaturation in the substomatal cavity are neglected.

In this regard, another important consideration beyond the calculation of *i*WUE is its interpretation, which inherently assumes that stomatal aperture is the sole regulator of plant transpiration (Farquhar & Raschke, 1978). However, even if g_{sw} is accurately estimated while accounting for non-stomatal control of transpiration, the interpretation of *i*WUE becomes problematic. Non-stomatal control of transpiration allows for reductions in transpiration rate without impacting carbon gain, meaning transpiration can decrease without stomatal closure. This decoupling makes the traditional definition of *i*WUE (*i*WUE = A/g_{sw}) less straightforward in conditions of substomatal cavity unsaturation (Márquez et al., 2024). Further research is necessary to fully understand the role of non-stomatal factors in regulating transpiration and how these factors may influence the interpretation of *i*WUE measurements.

3.2. Minimum leaf conductance

Minimum leaf conductance to water ($g_{lw,min}$) is a measurement that reflects the lowest conductance to water loss through the leaf surface, typically observed in the dark when stomata are likely closed (Duursma et al., 2018). This conductance is a composite value that reflects both cuticular and minimum stomatal conductance and should not be attributed as a proxy of either (Márquez et al., 2021). The importance of studying $g_{lw,min}$ lies in the fact that plants continue to lose water at night without any corresponding carbon gain, and nocturnal respiration further contributes to carbon depletion (Caird, Richards, & Donovan, 2007; Resco de Dios et al., 2019). In stressed plants, such as those under soil moisture depletion, where water use efficiency becomes even more critical, $g_{lw,min}$ can account for a significant portion of total water use and influence the plant's overall carbon balance.

Under stress conditions, minimum leaf conductance has been observed to vary in response to environmental factors such as low relative humidity and variation in temperature (Duursma et al., 2018; Wang et al., 2024). This raises important questions about the respective contributions of stomatal and cuticular conductance under these conditions and how each responds to environmental stress. Research has shown that the proportion of water transpired through the cuticle relative to that lost through the stomata can vary widely among species and is likely influenced by growth conditions (Caird, Richards, & Donovan, 2007; Márquez et al., 2021). However, detailed information on these responses remains limited and warrants further investigation.

Cuticular conductance can fluctuate when leaves lose turgor (Boyer, 2015b), and there is speculation that it may also change under extreme heat, based on isolated cuticle temperature permeability evaluation (Burghardt & Riederer, 2006). However, existing *in planta* measurements (Márquez, Stuart-Williams, & Farquhar, 2021) do not support this proposition. It is important to note that these measurements have not been extensively conducted across a broad range of species and temperature ranges, highlighting the need for further research on the topic. On the other hand, stomatal conductance is known to vary even in the dark, but the physiological significance of this response is not yet fully understood (Caird, Richards, & Donovan, 2007; Resco de Dios et al., 2019). The physiological role of minimum stomatal conductance also requires more attention to fully understand

its impact on plant water and carbon dynamics. Still, in this context, cuticular conductance remains the most elusive and least understood parameter for studying $g_{lw,min}$.

3.3. CO_2 concentration in the substomatal cavity (c_i)

The concentration of CO_2 within the leaf's air spaces, particularly in the substomatal cavity (c_i), serves as a crucial gateway for understanding the intricacies of photosynthesis and CO_2 diffusion within the leaf (von Caemmerer & Farquhar, 1981; Long & Bernacchi, 2003). Accurately estimating c_i is paramount for plant stress research, as it directly influences the interpretation of physiological responses and the calculation of critical parameters such as photosynthetic capacity (Busch et al., 2024) and mesophyll conductance (Márquez & Busch, 2024).

One of the primary tools for assessing the photosynthesis response under varying conditions is the A-c_i curve, which plots the rate of photosynthesis (A) against the intercellular CO_2 concentration (c_i). This curve is instrumental in diagnosing limitations to photosynthesis, whether they are biochemical (e.g., limitations in the Calvin cycle) or physical (e.g., limitations due to stomatal conductance) (Farquhar, von Caemmerer, & Berry, 1980; von Caemmerer & Farquhar, 1981; Farquhar & Sharkey, 1982; Busch & Sage, 2017). Additionally, the ratio of c_i to ambient CO_2 concentration (c_a) is frequently used to infer the efficiency of CO_2 uptake relative to the external environment, providing a window into the plant's physiological state under stress conditions. Typically, a c_i/c_a ratio of 0.6–0.7 for C_3 plants and 0.3–0.4 for C_4 plants is regarded as optimal or indicative of unstressed conditions (Wong, Cowan, & Farquhar, 1979).

However, the accurate estimation of c_i is fraught with challenges, particularly under stress conditions. The standard approach to estimating c_i involves the calculation of g_{sw} and assumes that this is the sole pathway for gas exchange. This approach, however, overlooks other potential factors, such as cuticular conductance to water (g_{cw}) or the uneven closure of stomata across the leaf surface (stomatal patchiness) (von Caemmerer & Farquhar, 1981). During stress conditions, failing to account for g_{cw} or stomatal patchiness can lead to an overestimation of c_i (Mott, 1995; Boyer, 2015a). Conversely, c_i can be underestimated if the assumption of saturated water vapour in the substomatal cavity, which is typically assumed to be saturated at leaf temperature, does not hold. Under certain stress conditions, such as high temperature or low humidity, the water vapour in the substomatal cavity may not be fully saturated, which can lead to an overestimation of the diffusion gradient and, consequently, an underestimation of c_i (Wong et al., 2022; Cernusak et al., 2024).

Moreover, the internal CO_2 concentration is not uniform throughout the leaf. Gradients of CO_2 are likely to form, particularly in amphistomatous leaves, which have stomata on both the adaxial (upper) and abaxial (lower) surfaces. In such leaves, each surface may have a distinct c_i, influenced by different rates of stomatal conductance and photosynthetic activity (Wong, Cowan, & Farquhar, 1985c; Wong, Cowan, & Farquhar, 1985b; Parkhurst et al., 1988; Wall et al., 2022; Márquez et al., 2023a). Most gas exchange measurements, however, combine signals from both leaf surfaces, resulting in a single, averaged c_i value (Figure 1c). This average c_i is a weighted value, reflecting the relative contributions of the adaxial and abaxial surfaces to the overall assimilation rate (Márquez et al., 2023a), which can obscure the underlying physiological differences between the two surfaces.

Given the critical role of c_i in understanding plant physiology, particularly under stress conditions, it is imperative to ensure that its estimation is accurate and precise. Inaccuracies in c_i estimation can lead to the misinterpretation of physiological trends and erroneous conclusions about a plant's response to stress. Therefore, researchers must carefully consider the potential sources of error, including the effects of cuticular conductance, stomatal patchiness, and unsaturation of water vapour in the substomatal cavity, when interpreting c_i data from experiments in plants under stress.

3.4. Mesophyll conductance

Mesophyll conductance (g_m) refers to the ease with which CO_2 moves from the substomatal cavity to the sites of carboxylation in the chloroplasts (Evans et al., 1986). It plays a crucial role in determining the efficiency of photosynthesis and overall plant productivity. In studies of plants under stress, g_m has gained importance due to the suggested interaction between mesophyll cell wall thickness and composition with water stress tolerance and the potential trade-off that could occur with a reduction in g_m (Clemente-Moreno et al., 2019; Roig-Oliver et al., 2020).

The common calculations of g_m rely on c_i measurements, meaning that any errors in c_i can lead to inaccurate g_m values. Even when c_i is accurately estimated, measuring and comparing g_m under stress conditions presents additional challenges. A key issue arises when measurements are conducted under constant atmospheric CO_2 concentration (c_a) rather than constant c_i (Márquez & Busch, 2024). This approach can introduce inconsistencies when comparing g_m across treatments, as g_m is known to respond to variations of c_i (Flexas et al., 2007b; Vrábl et al., 2009; Márquez & Busch, 2024), potentially compromising the robustness of the analysis and leading to misleading conclusions.

There is substantial evidence that CO_2 concentration affects g_m measurements, although the underlying mechanism is still debated (Flexas et al., 2007b; Hassiotou et al., 2009; Tazoe et al., 2011; Xiong et al., 2015; Busch et al., 2020; Márquez & Busch, 2024). Typically, g_m reaches a maximum at specific c_i levels and decreases with either increasing or decreasing c_i. This variability highlights the difficulty of accurately correlating a single g_m measurement with treatment effects without additional data. Given the impact of CO_2 concentration on g_m estimations, the practice of using constant c_a in experiments should be approached with caution, as it risks leading to incorrect conclusions. Instead, methodologies that account for varying c_i should be employed to ensure more

accurate and reliable assessments of g_m variations associated with specific treatments, especially under stress conditions.

4. Small Fluxes (Leaf Cuticle)

Accounting for small fluxes in leaf gas exchange measurements is crucial for accurately calculating gas exchange parameters such as c_i and stomatal conductance (Boyer, 2015a; Márquez et al., 2021). This accuracy becomes particularly important under conditions of low stomatal conductance, such as during drought or low light conditions when stomata are mostly closed. The small fluxes in question primarily refer to the fluxes of water vapour and CO_2 through the cuticle of the leaf (Hanson, Stutz, & Boyer, 2016). Although these fluxes are typically much smaller than those through the stomata, they can become significant when stomatal conductance is low. Neglecting these small fluxes can lead to significant errors in gas exchange calculations, such as miscalculation of stomatal water and CO_2 fluxes, thereby leading to erroneous estimates of c_i (Márquez, Stuart-Williams, & Farquhar, 2021) (Figure 2). Figure 2 presents the differences between two approaches for estimating c_i—the commonly used vCF theory (von Caemmerer & Farquhar, 1981), neglecting cuticular conductance, and the Márquez, Stuart-Williams and Farquhar (MSF) theory (Márquez, Stuart-Williams, & Farquhar, 2021), which includes it. The data show that in conditions of high total leaf surface conductance to water ($g_{lw} = g_{sw} + g_{cw}$), dominated by stomatal conductance, the vCF theory approaches the estimations provided by the MSF theory. The larger the g_{lw}, the closer the results from both theories become, but the MSF theory continues to provide more accurate estimations of c_i, with differences still observed, particularly at moderate g_{lw} values.

The key issue with neglecting g_{cw} arises when g_{sw} is not largely dominating transpiration, specifically when cuticular conductance represents 8% or more of the total conductance in these calculations (Márquez, Stuart-Williams, & Farquhar, 2021; Hussain et al., 2024). In these cases (Figure 2), the vCF theory fails to produce accurate c_i values, with errors reaching up to 100 µmol mol^{-1}. This discrepancy is significant enough to interfere with measurements that rely on precise c_i values, such as A-c_i curves and g_m calculations. Studies have shown that cuticular conductance can vary between species, generally ranging from 5 to 20 mmol m^{-2} s^{-1} (Holmgren, Jarvis, & Jarvis, 1965; Kerstiens, 1996; Boyer, Wong, & Farquhar, 1997; Márquez et al., 2021; Slot et al., 2021). This means that depending on the species, cuticular conductance can become a critical factor when g_{lw} falls below 160 mmol m^{-2} s^{-1}, and in some cases, even when g_{lw} is as high as 250 mmol m^{-2} s^{-1}. Thus, incorporating cuticular conductance into calculations is essential for more accurate c_i estimations, particularly in species with lower stomatal conductance under natural (uncontrolled) or stress conditions where the contribution of the cuticle to gas exchange is substantial.

Additionally, there is the importance of $g_{lw,min}$ and nighttime transpiration leading to water loss without carbon gain (Coupel-Ledru et al., 2016; Resco de Dios et al., 2019; Yu et al., 2019). For accurate analysis, it is essential to separate residual stomatal conductance from cuticular conductance (Márquez et al., 2021), especially in studies focused on plant water relations under stress conditions such as drought. Distinguishing g_{sw} and g_{cw} from $g_{lw,min}$ is crucial for understanding plant behaviour under varying environmental conditions (Duursma et al., 2018). Also, it is important to note that $g_{lw,min}$ is neither equivalent to minimum stomatal conductance nor cuticular conductance, and should not be used as a proxy for either (Márquez et al., 2021). This distinction is critical for assessing how plants regulate water loss by balancing cuticular permeability and stomatal closure, particularly under stress conditions, and provides valuable insights into their adaptive responses to environmental challenges.

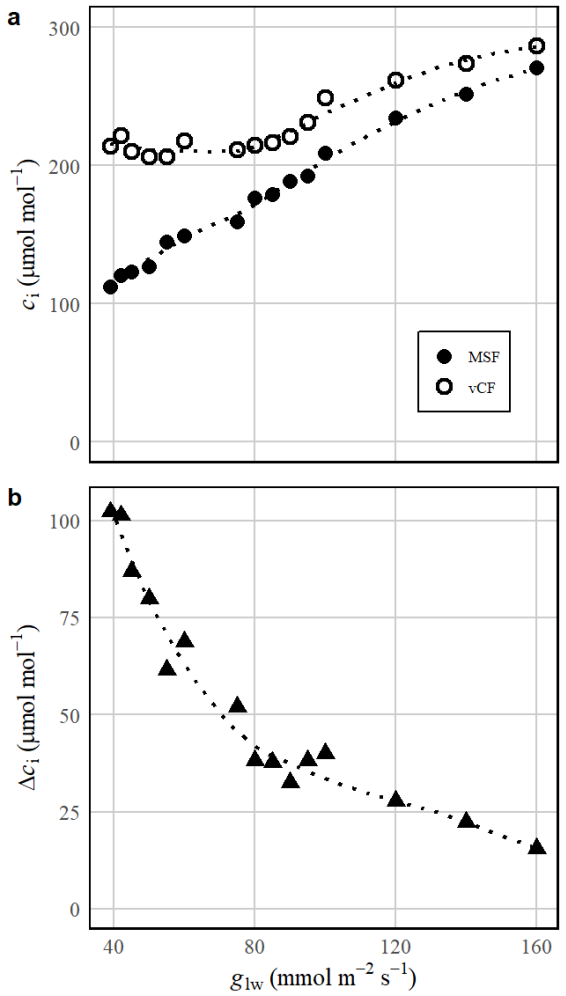

Figure 2. Estimation of substomatal CO_2 concentration (c_i) as a function of total leaf surface conductance to water (g_{lw} = stomatal conductance + cuticular conductance). (**a**) Substomatal CO_2 concentration (c_i) plotted against changes in g_{lw}, which follow the leaf's circadian rhythm. The comparison includes two models to estimate c_i: one that accounts for small fluxes (MFS, solid circles) and one that does not (vCF, open circles). (**b**) The difference in c_i (Δc_i = vCF-MSF, solid triangles) between the two models is plotted against g_{lw}. Measurements were conducted under constant light, vapour pressure deficit, and ambient CO_2 concentration. The estimated cuticular conductance for this leaf is 10 mmol m^{-2} s^{-1}. Data sourced from Márquez, Stuart-Williams and Farquhar (2021). Each point represents a single measurement, calculated independently using the vCF and MFS models.

4.1. Practical approaches

To account for small fluxes and avoid the issues discussed earlier, it is essential to use a model that includes cuticular conductance. The most updated approach is the MSF theory (Márquez, Stuart-Williams, & Farquhar, 2021), which applies ternary corrections to stomatal and boundary layer fluxes while recognising the independence of cuticular fluxes. Although the MSF model is not yet integrated into most commercial gas exchange systems, an add-on script is available for the LI-6800 system (https://github.com/PlantPhysiologist/Add-on-MSF-calculations-for-LI6800 (accessed on 6 of January of 2025)), allowing easy integration with a LI-6800 to obtain real-time parameter recalculation. Additionally, a post-analysis tool recently published (Tholen, 2024) includes the MSF model and can be adapted to various instruments, enabling an analysis of small fluxes from the raw data.

To effectively account for g_{cw} in models, it is essential to accurately estimate it. This estimation is not straightforward; however, a few techniques have been developed to facilitate this measurement. A non-destructive method, the Red-Light method (Márquez et al., 2021), offers a practical solution by measuring gas exchange as the leaf transitions from darkness to red light. This technique enables the estimation of g_{cw} on an attached leaf, and it is particularly useful for continuous *in planta* experiments, as it does not interfere with ongoing measurements (Hussain et al., 2024). Alternatively, traditional methods can be employed (Kerstiens, 1996), such as measuring leaf transpiration in the dark after detaching the leaf and estimating minimum leaf surface conductance ($g_{lw,min}$). However, these methods usually include residual stomatal and cuticular conductance, so they should be used with caution and only when no other options are available.

In field experiments, performing specific analyses, such as the red-light method, can be challenging due to the requirement for dark-acclimated leaves, which complicates measurements when experiments are conducted outdoors during the day. As a result, full quantification of g_{cw} may not always be feasible under field conditions. However, the method introduced by Laisk (1983), which involves comparing the behaviour of the A-c_i curve under different conditions, provides a practical alternative for detecting issues in gas exchange measurements (hereafter referred to as the Laisk method). This approach enables researchers to identify problems such as excessively small fluxes, stomatal patchiness, or general inconsistencies in measurements by comparing observed A-c_i curves based on previously measured under non-stressful conditions to the relation of A and c_i observed during the experiments.

While the Laisk method is highlighted here and in the following sections as a diagnostic tool tailored to field conditions, it is important to note that, although effective in detecting the presence of issues, it does not pinpoint or account for the specific cause of the problem. Despite this limitation, the Laisk approach has been successfully employed to verify the reliability of gas exchange measurements under stressful field conditions. For example, Grassi and Magnani (2005) used the method to ensure measurement reliability while identifying various limitations to photosynthesis in field settings. Thus, the Laisk method provides a valuable strategy for validating gas exchange data in challenging experimental setups.

Finally, if direct measurements of cuticular conductance are not feasible, a tentative correction or estimate of uncertainty can be incorporated by assuming a value of 5 to 10 mmol m^{-2} s^{-1}, which provides a reasonable estimate of uncertainty. However, this should be used with caution, as g_{cw} can vary significantly across species. While g_{cw} seems to remain stable under a range of conditions, including temperatures between 15 °C to 30 °C, g_{cw} may change if the leaf loses turgor or is exposed to extreme temperatures (Schreiber, 2001; Boyer, 2015b). While more research is needed to explore variability across species and conditions further, this practical approach provides a reliable means of accounting for small fluxes and enhancing the accuracy of gas exchange measurements.

5. Unsaturation in the Substomatal Cavity

Unsaturation in the substomatal cavity is the consequence of a recently recognised plant adaptation non-stomatal control of transpiration (Wong et al., 2022), allowing for more nuanced water regulation under high evaporative conditions. Research has shown that non-stomatal control of transpiration plays a vital role in maintaining a favourable microclimate with higher CO_2 concentrations in the mesophyll air space, supporting high carbon fixation under vapour pressure deficit (VPD) stress (Márquez et al., 2024). This mechanism, previously overlooked, causes the water vapour concentration of the substomatal cavity (w_i) to decline below saturation (w_{sat}), contrary to traditional assumptions used in gas exchange measurements (Cernusak et al., 2024).

Traditionally, during gas exchange measurements, it is assumed that the internal leaf air space remained fully saturated with water vapour due to rapid evaporation from the mesophyll surface, with stomatal conductance considered the main regulator of water loss (Gaastra, 1959). This translated into the leaf temperature used as a proxy to estimate w_i, assuming $w_i = w_{sat}$. However, recent studies have shown that unsaturation is a common phenomenon in plants (Cernusak et al., 2018; Wong et al., 2022; Márquez et al., 2024), particularly under moderate to high VPD. The assumption of full saturation has long been a fundamental premise in plant physiology research, but it leads to significant errors when unsaturation is not accounted for.

The error arises from assuming $w_i = w_{sat}$ when, in reality, w_i is below the saturation point. When this is an incorrect assumption, it leads to an underestimation of the vapour gradient that drives transpiration, which in turn results in an underestimation of g_{sw} and c_i (Figure 3). The errors become especially pronounced at higher VPD, where w_i deviates significantly from w_{sat}. This discrepancy can result in underestimating g_{sw} by as much as 20% to 30% and c_i by 50 μmol mol^{-1} or more, being particularly significant in C_4 plants (Márquez et al., 2024). Cotton is used as an example in Figure

3, but studies have shown that substomatal cavity unsaturation can vary between species, with the degree of unsaturation typically increasing under moderate to severe VPD stress (Cernusak et al., 2018; Wong et al., 2022; Márquez et al., 2024).

This variation underscores the importance of incorporating a more reliable estimation w_i into the gas exchange calculations, particularly in water-stressed environments, where plants may experience significant deviations from saturation. These deviations can impact physiological interpretations, such as A-c_i curves, iWUE, and g_m, among many other estimations, making accurate accounting of w_i essential for reliable results in the contexts of stressed plants.

Further research is necessary to fully understand the non-stomatal control of transpiration and the role of unsaturation. Current gas exchange models do not account for this process, leaving a gap in our ability to accurately predict or estimate the effects of unsaturation during routine measurements.

5.1. Practical approaches

There are currently four methods to evaluate actual w_i during gas exchange measurements. Three of these methods require a dual chamber setup, which enables independent control and measurement of gas exchange on the upper and lower surfaces of the leaf. (1) The dual chamber using the CO_2 Gradient Method, which creates a CO_2 concentration difference between the two leaf surfaces to assess unsaturation (Wong et al., 2022); (2) the dual chamber using the c_w Correction Method, which focuses on the assimilation rate in response to changes in the minimum CO_2 concentration (c_w) in the leaf airspace (Márquez et al., 2023a); (3) the Inert Gas Method that uses inert gases like nitrous oxide or neon to refine assessments of w_i (Jarvis & Slatyer, 1970; Wong et al., 2022); and (4) the Stable Isotope Method that leverages the equilibrium reached by the exchange of ^{18}O between CO_2 and H_2O in the liquid volume of mesophyll cells to infer w_i (Cernusak et al., 2018). Lastly, a fifth promising technique worth mentioning is the AquaDust Method (Jain et al., 2021), which offers a potential real-time measurement alternative using a fluorescent reporter, making it independent of gas exchange measurements. However, further validation of external measurements with AquaDust is required to ensure consistency compared to more direct techniques. Below is a discussion of each method in more detail.

The dual chamber using the CO_2 Gradient Method leverages reducing the CO_2 concentration on one side of the leaf until the assimilation rate on that side of the leaf is zeroed, creating a CO_2 gradient between the adaxial and abaxial substomatal cavities under benign conditions (assuming saturation). This gradient serves as a baseline to study unsaturation under stress conditions to compute the expected CO_2 gradient during increased stress or to compute the expected resistance to CO_2 diffusion within the leaf (Wong et al., 2022; Márquez et al., 2024). Even though this CO_2 gradient is an artificial setup, it encourages stomatal opening, allowing us an easier study of the non-stomatal control of transpiration and the effects of unsaturation. This trade-off is valuable for studies aimed at isolating non-stomatal mechanisms, but it may not be suitable for more standard measurements that require natural conditions.

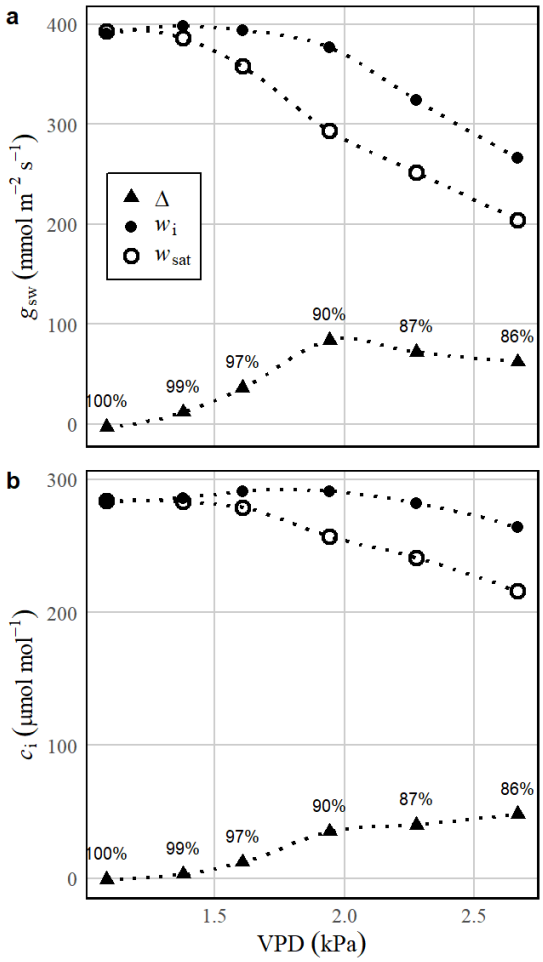

Figure 3. Computation of stomatal conductance (g_{sw}) and substomatal CO_2 concentration (c_i) assuming saturation in the substomatal cavity (w_{sat}) and accounting for actual vapour concentration (w_i) as a function of atmospheric vapour pressure deficit (VPD). (**a**) g_{sw} plotted against VPD, comparing the calculations for w_{sat} (open circles) and w_i (filled circles). The triangles represent the difference (Δ) between these calculations. (**b**) c_i plotted against VPD, with the same model comparisons as in panel (**a**). The percentages at the top of the triangles indicate the relative humidity (RH) in the substomatal cavity calculated as the ratio of w_i/w_{sat}. The species used in this example is cotton. Data sourced from Wong et al. (2022).

The dual chamber using c_w Correction Method monitors the assimilation rate as it responds to changes in the minimum CO_2 concentration (c_w) within the leaf airspace. This approach requires performing a CO_2 response curve under benign conditions (assuming saturation), relating c_w to the measured assimilation rate, and then the rest of the measurements can be taken under any desired atmospheric condition (Márquez et al., 2023a). It does not require zeroing the assimilation rate on either leaf side, allowing measurements to be conducted under standard conditions (i.e., both cuvettes present the same atmospheric conditions). This method also helps identify other stress responses, such as patchy stomatal closure, which might otherwise obscure the effects of unsaturation.

The Inert Gas Method also employs dual chambers but introduces inert gases such as neon (Ne) or nitrous oxide (N_2O) into one of the cuvettes and analyses its diffusion through the leaf to the other cuvette of the system (Jarvis & Slatyer, 1970). Then, in a similar approach as the CO_2 Gradient Method, the apparent changes in inert gas diffusion in relation to vapour diffusion are analysed, and the changes are used to compute w_i (Wong et al., 2022). These gases diffuse differently from atmospheric gases, providing additional constraints that improve the accuracy of w_i estimations in the substomatal cavity. Although this method can offer refined measurements, it requires complex calibration and remains resource-intensive, making it less practical for large-scale or routine studies.

The stable isotope method leverages the measurement of stable oxygen isotope (^{18}O) compositions in CO_2 and H_2O vapour entering and exiting the leaf gas exchange chamber. This technique assumes isotopic equilibrium in the liquid volume within mesophyll cells, such as in the cell wall or chloroplasts, which are closely linked to transpiration and assimilation processes (Cernusak et al., 2018). By using the isotopic composition of transpired water and assimilated CO_2, we can independently assess the expected isotopic composition of both gases. In practice, w_i is adjusted in the calculation until the measured isotopic compositions of CO_2 and H_2O reach a calculated equilibrium. This allows for precise estimation of w_i. A key advantage of this method is that, unlike the dual chamber techniques, it does not rely on the estimation of gas diffusion through the leaf. This opens the door to studying hypostomatous species, where stomata are only present on one side of the leaf, or species with challenging leaf shapes, such as conifers (Cernusak et al., 2024). While the stable isotope method requires specialised equipment and technical expertise, often limiting its application to controlled laboratory environments, it presents a valuable alternative for measuring species and leaf types where other methods face practical limitations.

The AquaDust method offers a new approach to monitoring leaf water potential in real time by injecting a fluorescent reporter into the leaf mesophyll airspace and measuring fluorescence from outside the leaf. AquaDust has shown promising results in tracking changes in leaf water potential using an external probe (Jain et al., 2021). In more specialised setups involving confocal microscopy, it has been able to detect changes in the water potential within the liquid of mesophyll cell walls (Jain et al., 2023; Jain et al., 2024), which can be translated into an estimation of w_i. However, there are important distinctions to consider. While confocal microscopy setups show potential for directly measuring w_i, the need for such specialised equipment and the requirement to remove the leaf from its gas exchange conditions make this approach impractical for routine gas exchange measurements. On the other hand, AquaDust's external probe approach, which measures the average water potential across a section of the leaf, seems to be much more compatible with normal gas exchange setups. It should be noted, however, that this method does not specifically target the substomatal cavity and instead provides a more generalised measurement of water potential across the mesophyll of the leaf. To make probe measurements using AquaDust a reliable tool for estimating w_i, further validation is needed using more targeted techniques such as dual-chamber methods or stable isotope analysis. This validation may need to be species-specific, as differences in leaf structure, such as stomatal density, could influence the relationship between AquaDust's averaged water potential measurements and the actual w_i in the substomatal cavity. Consequently, a universal relationship between AquaDust probe readings and w_i may not apply across all plant species.

The techniques described above present varying levels of difficulty for implementation under field conditions. In practice, either new techniques or instrument adaptations, such as an integrated double chamber, are required to meet these demands. However, such techniques or easily integrated solutions are not yet available. There is evidence that the Laisk method may still be effective in C_4 plants for corroborating whether data align with expected A-c_i curves (Márquez et al., 2024). However, Márquez et al. (2023a) tested this method in C_3 plants and found it inconsistent in detecting unsaturation. The physiological reasons why the method appears reliable in C_4 plants but not in C_3 plants remain to be analysed in detail.

If there is no direct way to quantify w_i during gas exchange measurements, it becomes essential to analyse the uncertainty associated with the estimates by imposing ranges of w_i within known or measured values, such as assuming relative humidity (RH) values between 100% and 80%. While this can offer a useful perspective on the uncertainty involved, it is not sufficient to draw definite conclusions. The need for a direct, simple method to measure w_i or a well-established mechanistic model to estimate it remains a priority. Until such measurements become standard for gas exchange measurements for plants under stress, uncertainties in w_i will limit the robustness of gas exchange analyses when w_i is not measured, making it difficult to assess the physiological responses of plants under stress fully.

6. Patchiness

The phenomenon of patchiness in stomatal movements refers to the spatial and temporal heterogeneity in the behaviour of stomata across the leaf surface. Rather than acting uniformly, stomata in discrete areas or "patches" may respond differently to environmental stimuli than those in adjacent regions. This can result in small, localised groups of stomata exhibiting distinct patterns of opening and closing that are not mirrored across the entire leaf.

Patchiness is particularly pronounced in plants under stress, such as during water deficit conditions or fluctuations in light intensity. Under such stresses, stomatal movements become more erratic and less synchronised across the whole leaf. For example, water stress can induce non-uniform stomatal closure, leading to areas with significantly different gas exchange capabilities within the same leaf (Downton, Loveys, & Grant, 1988). It is speculated that this heterogeneity represents an adaptive strategy, allowing parts of the leaf to maintain gas

exchange and photosynthesis while other areas are conserving water by closing their stomata (Mott, Cardon, & Berry, 1993).

In gas exchange measurements, it is typically assumed that stomatal conductance is uniform across the leaf surface, allowing a single parameter calculation to represent the leaf surface, such as g_{sw}, c_i, assimilation rate, etc. However, patchy stomatal behaviour can significantly impact gas exchange estimates. Non-uniform stomatal responses may introduce artefacts in the data, complicating the accurate interpretation of whole-leaf gas exchange measurements (Cardon, Mott, & Berry, 1994; Mott & Buckley, 2000). For instance, when patchiness is present, the assumption of uniform conductance can lead to misestimation of c_i and *i*WUE (Laisk, 1983; Mott, 1995). Figure 4 illustrates the distortion of the A-c_i relationship caused by patchy stomatal closure. Panel (a) shows time-lapse data from flowering crabapple (*Malus dolgo*) after ABA treatment, and panel (b) presents steady-state measurements in sunflower (*Helianthus annuus*) under elevated VPD, both highlighting the effects of patchiness on gas exchange dynamics.

A significant challenge in understanding and modelling patchy stomatal behaviour is that the drivers of patchiness are not well understood, and the evidence explaining its occurrence remains limited. Patchiness is highly variable, not only between species and environmental conditions but also between different leaves on the same plant (Kaiser & Kappen, 1997, 2000; Mott & Buckley, 2000). While some models, such as those proposed by Mott and Buckley (2000) and Cardon, Mott and Berry (1994), suggest hydraulic interactions leading to coordinated stomatal opening and closing within patches, this remains speculative. In fact, much of the available evidence suggests a lack of consistent coordination, particularly between the two leaf surfaces.

For example, studies such as Mott, Cardon and Berry (1993) have shown that stomata on the adaxial and abaxial surfaces can behave independently, responding asymmetrically to environmental stimuli such as under stress conditions like low humidity. However, they also found that stomata can respond semi-symmetrically under certain conditions, such as when humidity is decreased on one surface, suggesting some level of communication between the two sides. This inconsistent variability in stomatal responses complicates the modelling of patches responses and raises important questions about how these erratic behaviours can be accounted for in gas exchange studies (Lawson, Weyers, & A'Brook, 1998; Grantz, Karr, & Burkhardt, 2020).

There is even less evidence to support the idea of coordination between stomatal patches. While patches might independently respond to the same environmental cues, potentially resulting in similar overall patterns, the behaviour of one patch does not seem to significantly influence another distant patch (Cardon, Mott, & Berry, 1994). Lawson, Weyers and A'Brook (1998) observed erratic stomatal responses within individual patches, both spatially and temporally, further suggesting that the behaviour of one patch is largely independent of others. This lack of coordination is likely due to the spatial separation and the distinct micro-environments that different patches experience. As Cardon, Mott and Berry (1994) and Kaiser and Kappen (2000) suggested, possibly the physical distance between patches decreases the likelihood of direct hydraulic or chemical signal transference, making it highly unlikely that patches would act in a coordinated fashion.

Overall, the evidence points to more erratic and independent behaviour of stomata both within and between patches. This unpredictability poses a significant challenge for modelling and interpreting gas exchange, as assumptions of uniformity or coordination are unlikely to hold true (Downton, Loveys, & Grant, 1988). Given this complexity, the most reliable approach at present is to use the available techniques that can directly measure patchiness or track its effects on gas exchange, allowing us to detect its occurrence. More research is necessary to better understand the mechanisms behind patchiness, its variability across species, and its implications for physiological measurements, especially in stressed plants.

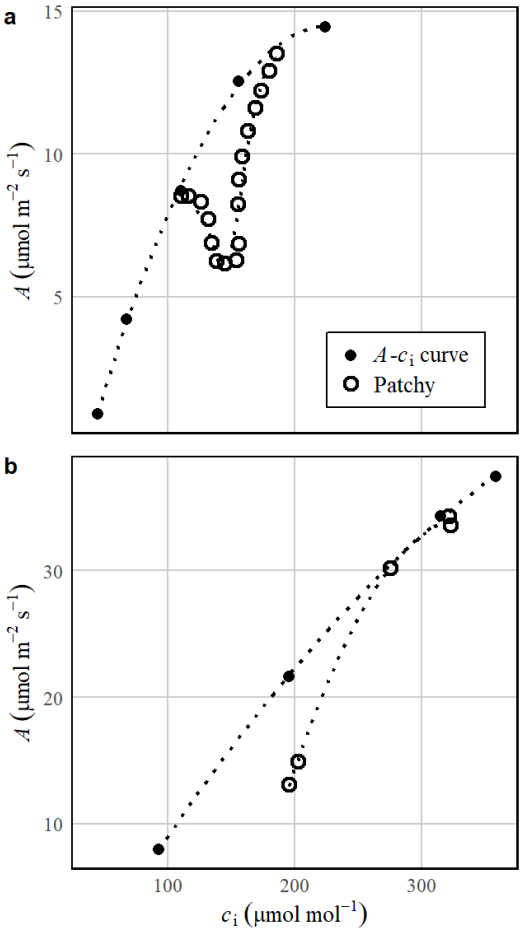

Figure 4. The effect of stomatal patchiness on the assimilation rate (A) vs. CO_2 concentration in the substomatal cavity (c_i) relationship (A-c_i). (**a**) Filled circles represent the A-c_i curve under steady-state and benign conditions. Open circles show how patchy stomatal closure distorts this curve over time after ABA application to induce patchiness in *Malus dolgo*. Time progression in the open circles moves from right to left. Data from Mott (1995). (**b**) Filled circles represent the A-c_i curve under steady-state and benign conditions, while open circles show steady-state measurements at elevated VPDs to induce patchiness in *Helianthus annuus*. Data sourced from Márquez et al. (2023a). The VPD increase imposed for each open circle in panel (b) was 1, 1.2, 1.5, 2, and 2.5 kPa, progressing from right to left as depicted in the figure.

6.1. Practical approaches

Detecting patchiness in stomatal movements during gas exchange measurements requires various sophisticated techniques, each with strengths and limitations.

Chlorophyll fluorescence imaging visualises the spatial distribution of photosynthesis, providing an indirect measure of stomatal patchiness by highlighting areas of varying photosynthetic activity (Mott, 1995). Although informative, this method does not directly quantify the impact of patchiness on gas exchange, limiting its ability to provide comprehensive insights into gas exchange data. Microscopic observation offers direct measurements of individual stomatal apertures, revealing detailed variability in stomatal behaviour (Mott & Buckley, 2000). However, it is labour-intensive, limited to small areas of the leaf, and does not provide information about whole-leaf gas exchange (Lawson, Weyers, & A'Brook, 1998). It is, therefore, not ideal for large-scale measurements or for capturing the full complexity of stomatal responses across the leaf. Similarly, infrared thermography detects temperature variations across the leaf surface, which can be indicative of differences in transpiration rates and thus offer a broader view of patchiness (Mott & Buckley, 2000). While useful for detecting regional differences in water loss, it is sensitive to external temperature fluctuations, which can compromise its accuracy under varying environmental conditions.

Most common techniques, such as chlorophyll fluorescence imaging and infrared thermography, provide insights into the occurrence of patchiness but do not directly inform gas exchange measurements unless specialised (often custom-built) equipment is used to integrate these techniques with gas exchange setups. Therefore, while some methods allow researchers to track the effects of patchiness, others focus on measuring its direct impact on gas exchange, highlighting the need for more advanced and integrative approaches.

Real-time monitoring of stomatal movements using microscope videos, as presented by Sun et al. (2021), provides continuous, high-resolution data that detects dynamic changes in stomatal behaviour. When combined with gas exchange, this method allows for some degree of accounting for the effects of patchiness. However, it requires sophisticated equipment and advanced analytical tools, making it less accessible for routine use.

Márquez et al. (2023a) introduced a technique to evaluate the effects of patchiness and unsaturation by comparing CO_2 response curves under benign and stressful conditions (the previously mentioned c_w Correction Method). While this method helps detect the occurrence of patchiness and account for unsaturation in gas exchange, it does not quantify the level of patchiness or directly observe the spatial behaviour of patches.

In general, current techniques allow us to detect the occurrence of patchiness and quantify its degree in an area but do not provide a way to account for this variability directly in gas exchange measurements. To address patchiness effectively with gas exchange measurements, a model that relates effective surface area and conductances under gas exchange would be needed. One potential approach could involve tracking the mismatch of the A-c_i relation (Mott, 1995), using the Laisk method, giving an apparent conductance reduction, which would allow corrections but will not give information about the patches.

It has been suggested that new models could account for the variability of patches as independent sections of the leaf, with multiple fluxes and resistances in parallel (Laisk, 1983; Rockwell et al., 2022). This would allow for a more nuanced representation of stomatal patchiness by treating different patches as separate entities within the leaf. However, this approach would not be directly compatible with current systems and methods of gas exchange measurements. For now, the best approach remains directly measuring patchiness or tracking its effects on gas exchange, which in practice is detection rather than fully accounting for it in gas exchange measurements. Continued research into methods for more effective modelling and compensating for patchiness will be critical for improving the accuracy of gas exchange studies in plants under stress.

7. Other Important Considerations

7.1. Standardising conditions for valid comparison

Ensuring that environmental conditions are standardised across treatments is critical for valid comparisons in gas exchange studies, particularly in stress conditions where the target variable is often linked to multiple physiological effects. Stomatal conductance, which governs gas exchange, is sensitive to changes in temperature, light intensity, CO_2 availability, and vapour pressure deficit (VPD), among other factors (Cowan & Farquhar, 1977). In parallel, biochemical processes involved in photosynthesis and respiration are primarily influenced by temperature, light intensity, and CO_2 availability but are not directly affected by VPD (Wong, Cowan, & Farquhar, 1985a; Wong, Cowan, & Farquhar, 1985c; Wong, Cowan, & Farquhar, 1985b; Busch et al., 2013). These distinctions are important because gas exchange measurements integrate stomatal and biochemical responses. As a result, isolating their individual effects on the net gas exchange requires careful control of experimental conditions.

Since stomatal conductance and biochemical processes respond to different environmental factors, controlling interrelated variables becomes essential. Such is the case of temperature, RH, and VPD, which are closely interrelated, and while gas exchange systems allow for their control, adjusting one parameter often influences the others. Similarly, leaf temperature, transpiration rate, and light intensity are interdependent, particularly when stomatal conductance is low. In such conditions, reduced transpiration cooling increases the leaf-to-air temperature difference. As light intensity increases, it can further raise leaf temperature, and if stomatal conductance is low, this heat builds up and can increase the leaf-to-air temperature gradient.

A prime example of these interactions is the relationship between temperature and VPD. Stomatal conductance

responds to both temperature and VPD, while the biochemical processes in photosynthesis, particularly enzyme activity, are primarily influenced by temperature. As temperature rises, VPD typically increases, which can induce partial stomatal closure and reduce CO_2 diffusion into the leaf. Without careful control of these variables, the effect of temperature on gas exchange can be misinterpreted, as changes in temperature may also involve indirect effects from VPD. It is important to note that maintaining constant RH does not ensure a constant VPD, since VPD is a function of temperature and vapour concentration, not the relative vapour concentration difference. For example, increasing the temperature from 25 °C to 30 °C while holding RH constant at 70% causes the VPD to rise from 0.95 kPa to 1.27 kPa—a 34% increase, even though RH remains unchanged.

Due to this interdependence, it is essential to standardise environmental conditions in order to isolate the variable of interest for the study at hand. Failure to account for the complex interactions between temperature, VPD, RH, light intensity, and CO_2 availability can obscure the physiological processes being studied, potentially leading to misleading conclusions.

7.2. Steady-state conditions

Under stress conditions, fluctuations over time, irregular behaviours, transient responses, and downward trends in gas exchange parameters, such as stomatal conductance, assimilation rate, and transpiration, become more common (Mott, Cardon, & Berry, 1993; Cardon, Mott, & Berry, 1994; Wong et al., 2022). As a result, special care must be taken when defining what constitutes a steady state under these circumstances or how researchers define "steady state" in their work.

Finding a single, universal definition of gas exchange steady-state conditions is challenging, as it often depends on the specific objectives of the experiment and the physiological response being studied. While many publications implicitly assume a shared understanding of steady-state conditions—usually described as "steady for this experiment"—this can be problematic when working with stressed plants, where gas exchange tends to fluctuate more erratically. In such cases, the definition of steady-state needs to be clearly articulated to ensure accurate interpretation of results, particularly when analysing the complex dynamics of stressed plants.

It is essential to define how stability was determined in the study clearly. This includes describing the acceptable range within which the input and output gas concentrations (CO_2 and H_2O) in the gas exchange chamber were allowed to fluctuate. Also, the criteria for evaluating consistency in key physiological parameters, such as stomatal conductance, assimilation rate, and transpiration rate. A practical approach would be to report the stability criteria in terms of:

1. *Duration of the stability period:* The length of time the leaf remained stable to be considered a steady state.
2. *Input/output gas concentration fluctuations:* The allowable variability of CO_2 and H_2O concentrations entering and exiting the chamber during the stability period.
3. *Variability in stomatal conductance:* The peak-to-peak variation in stomatal conductance (g_{sw}) within the defined stability period.
4. *Variability in assimilation rate:* The peak-to-peak variation in the assimilation rate (A) over the same period.
5. *Variability in transpiration rate:* The peak-to-peak variation in the transpiration rate (E) within the stability period.

By providing these specific criteria, researchers can ensure that stability is clearly defined and reproducible across experiments.

7.3. Adaxial and abaxial gas exchange

When analysing gas exchange in plants, it is recommended to consider the adaxial (upper) and abaxial (lower) surfaces independently, especially under stress conditions. While it is common practice to combine adaxial and abaxial fluxes due to the limitations of standard commercial instruments, this approach may overlook important details, particularly in stressed plants.

Under stress conditions, the stomatal responses of the adaxial and abaxial surfaces often behave independently or respond differently to the same stimuli. Studies have shown that the two sides of the leaf can react in distinct ways to environmental stressors, such as water deficit or changes in light intensity (Wong, Cowan, & Farquhar, 1985c; Wong, Cowan, & Farquhar, 1985b; Wall et al., 2022; Márquez et al., 2023a). By mixing fluxes from both surfaces, part of the plant's stress response can be missed, potentially leading to an incomplete understanding of the physiological adaptations.

While combining fluxes might not necessarily result in incorrect conclusions, it could mask the more subtle differences in how each surface adapts to stress (see for example Márquez et al. (2023a) and Collaviti et al. (2024)). Therefore, for a more accurate and detailed analysis of gas exchange, especially under stress conditions, it is recommended to measure the adaxial and abaxial surfaces independently to capture these independent responses. Custom-built or adaptations of commercial instruments exist in the literature to incorporate independent measurements of leaves' adaxial and abaxial surfaces (e.g., Márquez et al. (2023b) for LI-6800 or Wall et al. (2022) for LI-6400).

7.4. Incoming flow rate

Researchers may need to modify the chamber's flow rate depending on their experimental objectives when studying plants under stress, carefully balancing the signal-to-noise ratio with the need to maintain specific environmental conditions. Decreasing the flow rate can help detect smaller changes in gas exchange, particularly when gas exchange rates are low under stress conditions. A lower flow rate increases the concentration differential between incoming and outgoing gases, capturing subtle changes more effectively. However, this also introduces certain limitations. Reducing the flow rate increases the time required to stabilise conditions within the chamber, with this delay being proportional to the chamber

volume. Larger chambers and lower flow rates mean that it takes longer to reach desired environmental conditions, which may be a limiting factor (e.g., Farquhar, Griffani and Barbour (2021)). For example, achieving low RH becomes challenging when plant transpiration rate significantly affects humidity levels within the chamber, making it difficult to lower RH at reduced flow rates.

On the other hand, increasing the flow rate may be necessary when the aim is to create conditions like low RH in the chamber, particularly in stress experiments where controlling humidity is critical. Higher flow rates flush the chamber more quickly, preventing transpiration from raising RH to levels that would interfere with the desired conditions. While increasing the flow rate facilitates the creation of such conditions, it can also reduce the signal-to-noise ratio, as smaller changes in gas exchange become harder to detect due to the smaller concentration differential between incoming and outgoing gases.

Thus, researchers must carefully consider the objectives of their experiments and how flow rate adjustments will influence both the environmental conditions they wish to achieve and the precision of gas exchange measurements. Achieving accurate data collection while meeting the experimental goals requires careful management of the chamber's flow rate settings.

7.5. Boundary layer

The mostly unexplored effect of boundary layer conductance on stomatal behaviour adds further uncertainty to the heterogeneity and erratic patterns sometimes observed in stomatal responses, such as patchiness. Bridging insights from laboratory-controlled settings to natural environments requires addressing these interactions to mimic field conditions accurately. In this regard, boundary layer conductance plays an important role in gas exchange measurements, particularly when studying plants under stress. In natural conditions, the boundary layer surrounding a leaf is typically thicker than in the chamber of a gas exchange system and may interact with other stressors, such as water deficit. As a result, the leaf does not directly experience the ambient relative humidity and CO_2 concentrations, as the boundary layer acts as a buffer. This interaction between the boundary layer and stressors can influence how plants respond to their environment (Schuepp, 1993).

Researchers may wish to explore the effects of the boundary layer in their experiments, particularly to understand how a thicker boundary layer in natural settings influences plant stress responses. Adjusting the mixing fan speed in gas exchange chambers can modify boundary layer conductance. Increasing fan speed typically enhances boundary layer conductance, reducing noise in stomatal conductance calculations. On the other hand, decreasing the fan speed or modifying chamber conditions to simulate a thicker boundary layer may help mimic natural conditions more accurately.

It is crucial, however, to be cautious when making such adjustments. Commercial gas exchange instruments are calibrated to operate within specific boundaries, and modifying the boundary layer too drastically—such as by excessively reducing fan speed —can violate these specifications. Doing so may lead to unintended consequences, such as insufficient mixing, which can create unintended gradients within the chamber and result in inaccurate readings. To ensure data quality, researchers must either remain within the specified conditions of the instrument or find a way to account for the effect of altered boundary layer conductance without compromising the integrity of the measurements.

In summary, while exploring the interaction between boundary layer thickness and stress responses can provide valuable insights, it is important to balance these adjustments with the technical specifications of the instruments to maintain the accuracy and reliability of the data.

7.6. Thermodiffusion and its impact on transpiration

Thermodiffusion refers to the movement of water vapour driven by temperature gradients rather than solely by vapour concentration gradients, making it particularly relevant when there is a temperature difference between the leaf and the surrounding air. A recent study by Griffani, Rognon and Farquhar (2024) significantly advanced our understanding of the effects of thermodiffusion on transpiration rates. Thermodiffusion becomes particularly important when stomatal conductance is low, such as during abiotic stress (e.g., drought) or in darkness, where transpiration is already reduced. In extreme cases—such as when there is a significant temperature difference between the leaf and the air, combined with a small water vapour concentration difference across the boundary layer—thermodiffusion can account for more than 30% of total transpiration. This is particularly relevant when boundary layer conductance significantly exceeds stomatal conductance, a common scenario in gas exchange chamber experiments. While boundary layer conductance is typically larger than stomatal conductance in these chambers, the issue becomes problematic under specific, less common conditions, such as when the stomata are nearly closed, and there is a substantial temperature gradient between the leaf and the surrounding air.

Thermodiffusion can also contribute to reverse transpiration, where water vapour is absorbed by the leaf instead of being lost. This phenomenon occurs when external conditions, such as high humidity or cool temperatures, create a reverse water vapour gradient. While this is not common under normal conditions, it becomes relevant when there is a combination of large temperature gradients and high external humidity—conditions that may arise during extreme salt stress or dark periods. Understanding and accounting for thermodiffusion may be crucial in such cases to avoid misinterpreting water loss or gain in the leaf, particularly in experiments involving stressed plants where transpiration dynamics are altered.

8. Conclusions

Gas exchange measurements have long been a cornerstone of plant physiology research, providing critical insights into the interactions between plants and their environment. Their strength lies in the simplicity of the methodology and the flexibility of modern instruments, which enable precise control over experimental conditions. However, the ease with which these measurements are obtained can sometimes lead to overlooking the fundamental assumptions that underlie them—assumptions that often do not hold under stress conditions.

This manuscript has discussed and provided practical approaches to address these limitations, particularly under stress, which is summarised in Table 2. By identifying areas where traditional assumptions may falter, we offer strategies to improve measurement accuracy in stress and non-stress scenarios. Our focus has been on incorporating often overlooked elements such as small fluxes, cuticular conductance, substomatal cavity unsaturation, and stomatal patchiness—factors critical for accurate gas exchange data, especially in stressed plants. These approaches aim to refine the methods, ensuring more reliable results and a deeper understanding of plant physiological responses across diverse environmental conditions.

While this manuscript has addressed key challenges in gas exchange measurements, further research is needed in areas such as cuticular conductance and its relationship to leaf surface composition, the variability and mechanisms behind substomatal cavity unsaturation, and the complexities of non-uniform stomatal behaviour, including patchiness. By incorporating the methods and approaches discussed here, we aim to facilitate addressing these gaps through further research and thus advance our understanding of plant physiology.

Table 2. Overview of methods and techniques for addressing small fluxes, unsaturation, and patchiness in routine gas exchange measurements of stressed plants.

Approach	Addresses	Consideration	References
Accounting for small fluxes	Small Fluxes	**Pros**: Accurately includes cuticular conductance (g_{cw}) for improved estimates of g_{sw} and c_i. **Cons**: None. **Additional needs**: Requires previous measurement of g_{cw} (some alternative methods for estimating g_{cw}: Márquez et al. (2021) and Kerstiens (1996)).	Márquez, Stuart-Williams and Farquhar (2021)
Chlorophyll Fluorescence Imaging	Patchiness	**Pros**: Visualises photosynthesis distribution. **Cons**: Indirect measure; requires integration with gas exchange setups for complete analysis. **Additional needs**: Requires specialised or homemade setups to combine with gas exchange measurements.	Cardon, Mott and Berry (1994) Lawson, Weyers and A'Brook (1998)
Infrared Thermography	Patchiness	**Pros**: Broad detection of transpiration levels across the leaf. **Cons**: Indirect measure; Sensitive to external temperature changes, limiting reliability in variable environments. **Additional needs**: Integration with gas exchange systems is required to link temperature with gas fluxes.	Downton, Loveys and Grant (1988) Cardon, Mott and Berry (1994)
Microscopic Observation	Patchiness	**Pros**: Direct measurement of stomatal aperture variability. **Cons**: Limited to small leaf areas and labour-intensive. **Additional needs**: Can inform patchiness but lacks direct gas exchange data integration.	Mott, Cardon and Berry (1993) Lawson, Weyers and A'Brook (1998) Mott and Buckley (2000)
Real-time Monitoring via Microscopy	Patchiness	**Pros**: It allows for high-resolution dynamic observation of stomatal movements. **Cons**: It requires advanced equipment and data analysis tools. **Additional needs**: It can be combined with a gas exchange for detailed patchiness tracking.	Mott, Cardon and Berry (1993) Sun et al. (2021)
Dual-chamber CO_2 Gradient Method	Unsaturation	**Pros**: Effective in assessing unsaturation by measuring CO_2 difference between leaf surfaces. **Cons**: Imposes low CO_2 concentration during the measurements. **Additional needs**: Requires dual-chamber setup and careful calibration.	Wong et al. (2022)
Inert Gas Method	Unsaturation	**Pros**: It provides an unbiased analysis of gas flux through the leaf. **Cons**: It requires complex calibration and is resource-intensive. **Additional needs**: It requires a double chamber and instruments capable of measuring the concentration of the inert gas precisely, and it is not easily adaptable for large-scale use.	Jarvis and Slatyer (1970) Wong et al. (2022)
Stable Isotope Method	Unsaturation	**Pros**: it can be applied to any shape or leaf structure. **Cons**: it requires specialised and costly laboratory equipment. **Additional needs**: it requires instruments to measure the isotopic composition of ^{18}O in CO_2 and H_2O, and integration with gas exchange systems requires calibration.	Cernusak et al. (2018)
AquaDust Method	Unsaturation	**Pros:** Real-time, non-invasive tracking of leaf water potential via fluorescence. **Cons:** Provides an averaged water potential, which may not target the substomatal cavity specifically. **Additional needs:** Further validation is needed, especially correlating it with more targeted techniques like gas exchange systems or isotope analysis.	Jain et al. (2021)
Dual-chamber c_w Correction Method	Unsaturation and Patchiness	**Pros**: It is able to account for unsaturation and detect patchiness using the same setup and measurements. **Cons**: It does not directly quantify patchiness and lacks direct spatial analysis of patchiness. **Additional needs**: A double chamber is required.	Márquez et al. (2023a)
Laisk Method for Field Conditions	Checking the reliability of gas exchange data	**Pros:** Allows for evaluation of data reliability in field conditions in a relatively easy and practical way. **Cons:** Does not provide corrections, accountability, or identification of the source of unreliable data if found. **Additional needs:** Requires performing an A-c_i curve under a baseline condition to use as a reference.	Laisk (1983)

Author Contributions

D.A.M. and F.A.B. developed the initial concept of the manuscript. D.A.M. wrote the first draft and A.G. provided practical insights. All authors contributed to writing and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

All data discussed in this study are included in the main manuscript or referenced to publicly accessible sources. For further information or requests for additional materials, please contact D.A.M.

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Conflicts of Interest

The authors declare no competing financial interests.

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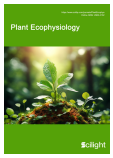

Article

Heat Stress Reduces Yield Through a Negative Effect on Radiation Use Efficiency during the Reproductive Phase in Cotton (*Gossypium hirsutum* L.) under Different Source Availabilities

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Abstract: Cotton is frequently exposed to high temperatures during the reproductive stage, which can negatively impact productivity. While previous research has shown that photosynthesis can decrease under heat stress, there is limited information on the effects of heat stress during the reproductive phase on crop variables such as radiation capture, use efficiency, and yield. This study aimed to: (i) assess the effect of heat stress on cumulative intercepted PAR radiation (IRcum), radiation use efficiency (RUE), harvest index (HI), and yield, and (ii) evaluate potential interactions between heat stress and source-sink relationships during the reproductive phase. Two field experiments were conducted, with heating treatments applied before and after flowering, and controls without temperature manipulation. In Experiment 1, two genotypes with contrasting growth cycles were compared, while Experiment 2 examined intact versus defoliated plants. Heat stress significantly reduced yield and HI, particularly during post-flowering. Source reduction (defoliation) further reduced yield, independent of temperature. Although IRcum was unaffected by treatments, RUE dropped sharply under heat stress in intact plants and was similarly low in defoliated plants under both control and heated conditions. These results suggest that heat stress, especially during post-flowering, exacerbates the effects on cotton productivity by reducing both total plant dry weight and HI. The study highlights that the relationship between RUE and yield strongly depends on the specific limiting factors, such as heat stress or source restrictions.

Keywords: cotton; heat stress; radiation interception efficiency; radiation use efficiency; harvest index; yield

1. Introduction

Climate change effects are observed across a wide range of ecosystems and species worldwide, associated with rising average temperatures and increasing annual fluctuations (Seneviratne et al., 2018). This process has been detected from pre-industrial times to the present, with an increase in heat stress periods and negative effects leading to crop yield losses (Bita & Gerats, 2013). In this context, high-temperature stress has a broad impact on plants in terms of physiology, biochemistry, and gene regulatory pathways, as temperature is a critical environmental factor controlling plant growth and development (Bhattacharya, 2019; Christiansen & Lewis, 1982). Thus, heat stress is linked to an increase in the maximum daily temperature above a threshold level sufficient to cause irreversible damage over the long term (Wahid et al., 2007).

Yield is the result of the product of cumulative intercepted photosynthetically active radiation (IRcum), Radiation Use Efficiency (RUE) (Andrade et al., 2005), and the harvest index (HI, the ratio between yield and total aboveground biomass). IRcum is the product of incident radiation and the crop's interception efficiency (IE). This is strongly influenced by the environment, not only through the amount of incident radiation but also through IE, as a consequence of the regulation of leaf area index (LAI, leaf area per soil area unit) (Naylor, 2012). Several studies conducted on annual crops such as wheat and maize suggest that temperature impact is associated with a decrease in

RUE and HI (Cicchino, Rattalino Edreira, & Otegui, 2010; Rattalino Edreira & Otegui, 2012). However, little information is available about its effects on herbaceous perennial commodities like cotton.

Cotton (*Gossypium hirsutum* L.) is a perennial plant, although it is grown as an annual crop in commercial systems through agronomic management practices, and it is characterized by an indeterminate growth habit. During the crop cycle, its productivity is sensitive to variations in environmental conditions, such as water availability and extreme temperatures (Welsh, Taschetto, & Quinn, 2022). The effects of heat stress on vegetative growth and development during this crop's cycle have been well documented (Burke & Wanjura, 2010; Hodges, 1991; Loka & Oosterhuis, 2016; Loka et al., 2020; Pettigrew, 2016; Virk et al., 2021). Other studies on this species have focused on the reproductive phase, with most of them examining pollination inhibition or fruit retention (Echer et al., 2014; Phillips, 2012; Snider & Oosterhuis, 2012; Snider et al., 2009, 2018; Van der Westhuizen et al., 2020) which reduces HI and, consequently, yield. Furthermore, recent research conducted at various developmental stages has highlighted diverse mitigation responses in cotton genotypes to heat stress, such as the accumulation of sugars, proline, phenolics, flavonoids, and heat shock proteins, which could aid in developing and selecting heat-tolerant cotton cultivars (Dev et al., 2024; Majeed et al., 2021).

The planophile structure of the cotton canopy (spreading horizontally rather than vertically) creates a sharp radiation gradient across the canopy profile, especially under high plant populations. In this case, little radiation reaches the leaves in the lower leaf layers (Dauzat et al., 2008; Hearn, 1976; Lv et al., 2013). In fact, studies have shown that artificially shading crops during the reproductive phase, with a 32% reduction in incident radiation, results in a 47% yield decrease in cotton (Eaton & Ergle, 1954; Sorour & Rassoul, 1974).

The majority of studies evaluating the impact of heat stress on cotton have been conducted in controlled environments, limiting their relevance to real agricultural conditions (Saini et al., 2023). A recent publication on cotton grown under field conditions revealed that leaf photosynthesis rates under saturated irradiance were reduced by up to 35% due to heat stress (as a consequence of negative acclimation), while no response to instant temperature changes in the 35 to 43 °C range was detected (Mercado Álvarez et al., 2022). However, no information is yet available about the impact of heat stress during the reproductive phase on crop variables related to radiation capture, use efficiency, and yield. Additionally, it is known that growth can be modulated by the influence of reproductive sinks in several crops (Lambers, Chapin, & Pons, 2008), suggesting that the detrimental effect of heat stress on biomass accumulation could be influenced by genotype differences in source-sink ratios at the time of stress. Current cotton genotypes typically have shorter cycles, thereby reducing the source (leaf area per plant)/sink (potential yield per plant) ratio (Baker & Baker, 2010; Casuso, Tarragó, & Galdeano, 2016). Furthermore, no information is available about the interactions between source/sink relationships and the effects of heat stress on the crop variables mentioned above.

Based on the above exposed, the objectives of this study are to evaluate (i) the impact of heat stress during the reproductive phase on IE, RUE, HI, and their contributions to cotton yield under field conditions and (ii) the possible interactions between heat stress and different source/sink relationships during the reproductive phase. The reproductive phase involves part of the critical period for yield determination in cotton, which ranges from the onset of floral bud development to 10 days after the end of the effective flowering stage (EEF) (Paytas et al., 2023).

2. Materials and Methods

2.1. Plant material and experiments

Two experiments were conducted under field conditions, with sowing dates of 24 November 2015, and 24 December 2016, for Experiments 1 and 2, respectively. Experiment 1 was conducted at the National Institute of Agricultural Technology (INTA) research station, located in Reconquista (29°15′ S, 59°44′ W, Santa Fe province, Argentina), and Experiment 2 at the experimental field of the Faculty of Agronomy, University of Buenos Aires, Argentina (34°35′ S, 58°29′ W). Two cotton genotypes (DP 402 BGRR, short cycle duration (from sowing to maturity), and DP 1238 BGRR, long cycle duration; INTA Reconquista, Argentina), hereafter referred to as Gs and Gl, respectively, were used in Exp. 1. Since no significant differences between genotypes were observed in this experiment, only the short-cycle genotype (DP 402) was used in Exp. 2. In both experiments, each experimental unit (microplot) consisted of an area of 3 × 1.56 m, and the experimental design was completely randomized, with 3 replicates for each treatment combination. Seeds were hand-planted in rows 0.52 m apart, using a stand density of 20 plants m^{-2}.

For Exp. 1, daily mean temperature and daily radiation integral records were taken from an automatic meteorological station located 420 m from the experimental site. In Exp. 2, weather data were recorded by a meteorological station located 300 m from the plots. Air temperature and relative humidity (RH) inside the portable structures described below were recorded using temperature sensors connected to two-channel data loggers (Cavadevices, Buenos Aires, Argentina) located in the upper part of the canopy. Crops were grown under optimal water and nutritional conditions, with soil irrigated weekly to ensure field capacity in the top meter of the profile throughout the growing season, and preventive agrochemical applications were used to control diseases and insects. Insect damage was prevented through regular crop monitoring and strategic insecticide applications. Plots were fertilized with 100 kg ha^{-1} of nitrogen, applied as urea at sowing. Herbicides were used for weed control (pendimethalin active ingredient, applied at 5 L ha^{-1} pre-planting, and glyphosate at 5 L ha^{-1} post-emergence).

For both experiments, high-temperature treatments were imposed starting at the flower-bud stage (FB, Figure 1). Two

heating treatments were applied: (i) a pre-flowering treatment of 14 days between FB and the flowering stage (FL) (H1), and (ii) a post-flowering treatment starting at FL and continuing until the end of the effective flowering stage (EEF) (H2). Control treatments were those without temperature manipulation during both phases (C1 and C2 for H1 and H2, respectively). Portable structures, consisting of an iron frame (3 m length, 1.3 m width, and 1.3 m height) covered with transparent polyethylene (150 µm thickness), were used for H1 and H2 treatments. The structures were closed on the top and sides, except for an aeration window of 20 cm at the base, to minimize potential artifacts associated with changes in humidity and carbon dioxide concentration (CO_2) resulting from transpiration and photosynthesis rates, respectively. The same structures were used for treatments C1 and C2, but in these cases, they were completely open on their sides, with the film deployed only on the roof. This setup was introduced to rule out possible artifacts associated with the structure itself and to quantify the extinction of radiation produced by the film.

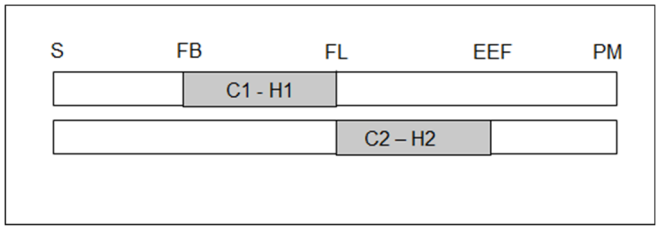

Figure 1. Schematic representation of the heating and control treatments conducted in Experiments 1 and 2 using control (C1-C2) and heating (H1-H2) portable structures during the pre-flowering (C1-H1) and post-flowering (C2-H2) phases. Gray bars indicate the periods when the corresponding treatments were applied. S = sowing, FB = flower-bud stage, FL = flowering stage, EEF = end of effective flowering stage or cut-out, PM = physiological maturity.

To examine the interactions between source/sink relationships and heat stress effects proposed in this work, an additional source-sink ratio manipulation was applied in Exp. 2. The source-sink ratio was adjusted through partial defoliation treatments. Specifically, a reduced source-sink ratio treatment (D-) was applied to 50% of the replicates for both C and H treatments by removing 50% of the total leaves per plant, interspersed along the stem, immediately before applying the heat treatment. The leaf area in the remaining experimental units was left intact (D0) as a treatment without defoliation.2.2. Measured variables

In both experiments, the measured and estimated variables included total and organ dry biomass, LAI, IE, and RUE. These traits were measured at the FB, FL, and EEF stages, for both control (C) and heated treatments (H). LAI was quantified by scanning the green leaves of the same harvested samples used for measuring dry weight, using a Portable Leaf Area Meter LI-COR LI-3000C (Li-Cor Inc., Lincoln, NE). Each sample consisted of 5 plants located within the plot, excluding those at the borders. Samples were divided into the following organs: leaf blades, stems (including petioles), flowers, and/or capsules (with seeds). LAI was estimated as the ratio of the sum of leaf area to the harvested soil area. Samples were dried at 60 °C for 7 days and weighed. Plant dry biomass measured at the end of the corresponding treatment (current dry weight, CDW) was estimated as the sum of all biomass fractions.

In each experimental unit, photosynthetically active radiation (PAR = 400–700 nm) incident and intercepted in lower strata was also measured using a 1-m long Li-Cor 191S line quantum sensor (Li-Cor Inc., Lincoln, NE, USA). IE was measured at solar noon by placing the linear sensor diagonally across the inter-row space, with the ends of the sensor window aligned with the center line of the rows. Five measurements were taken in each experimental unit, and the results were averaged.

RUE was initially estimated for each treatment using the overall reproductive phase (from FB to EEF) in which treatments were applied, as the slope of the biomass values from FB to EEF versus IRcum (Stöckle & Kemanian, 2009). RUE was also roughly estimated separately for the pre-flowering (from FB to FL) and post-flowering (from FL to EEF) phases, as the difference in biomass between two consecutive harvests divided by the corresponding amount of intercepted radiation during each phase (Sinclair & Muchow, 1999). IRcum was estimated by daily summing the product of daily PAR incident radiation and the IE estimated for the corresponding phase.

Raw yield (fiber + seeds) was estimated for each treatment after the plants reached 100% capsule opening, at 125 and 114 days after sowing (DAS) for Experiments 1 (Gs genotype only) and 2 respectively. Plants were harvested manually, and the raw yield was weighed. Additionally, in Experiment 2 only, seeds were separated from the fiber using a micro test gin with electric saws to determine fiber yield (kg ha^{-1}). Finally, for each treatment in that experiment, the harvest index (HI) was calculated as the ratio of raw yield to total aerial dry weight at harvest (DW).

2.3. Statistical analyses

Data were subjected to Analysis of Variance (ANOVA) using a two-way ANOVA (heat treatment x genotype for Exp. 1; heat treatment x defoliation for Exp. 2). Linear regressions were used to estimate RUE for the entire treatment phase as the slope of the cumulative total biomass versus cumulative intercepted radiation relationship. Statistical analyses were performed using Infostat software (Di Rienzo et al., 2011), and graphs were generated using GraphPad Prism 5 software for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com, accessed on 25 Feb 2025).

3. Results

3.1. Location and treatments

Environmental conditions at the two sites were described in detail in (Mercado Álvarez et al., 2022). Briefly, mean, minimum, and maximum outdoor air temperatures in

Experiment 1 (conducted in the northern subtropical location of Reconquista) were approximately 2 °C higher than those in Experiment 2, which was performed in Buenos Aires, Argentina. In both experiments, the average daily mean temperature during both heating phases (pre- and post-flowering) was 3.2 °C and 1.8 °C higher in the H treatment compared to the C treatment (Exp. 1 and 2, respectively; $p < 0.05$). Differences in average maximum temperatures between the H and C treatments (6.9 °C and 5.8 °C for Exp. 1 and 2, respectively; $p < 0.05$) were more pronounced, with the H treatment reaching a daily mean of 36.8 ± 0.8 °C and 37.9 ± 0.79 °C in Experiments 1 and 2, respectively (means ± standard errors). No differences in CO_2 concentrations at the top of the structures, leaf water potential, or vapor pressure deficit (VPD) were observed among treatments when measured at midday.

3.2. Leaf area index (LAI)

Although in Experiment 1 two genotypes with contrasting cycle duration between sowing and maturity were used, these differences were not reflected in the time from sowing to FB, as both reached this stage around 67 DAS. On the other hand, the duration of the same period in Experiment 2 was 50 DAS. LAI was affected in both experiments by heat (H), defoliation (D), and genotype (G) factors, with no interaction detected among these factors (Figure 2). In both experiments, initial values were lower than 1 in all treatments at FB, when the C1 and H1 structures were established (Figure 2A,C). In Experiment 1, LAI was unaffected by the H factor at FL, when heat treatments concluded (Figure 2A). However, the negative impact became evident later, with a significant reduction of around 20% detected in the H1 treatment at EEF, while C1 reached values up to 2.7 in the Gl genotype. A similar pattern was observed in pre-flowering in Exp. 2, where the negative impact of heat was detected in H1 immediately after the treatment concluded, regardless of the defoliation treatment (Figure 2C).

Curiously, in Experiment 1, the negative impact of the H factor was detected ($p < 0.05$) very early, near FL, when the temperature treatments were applied during the post-reproductive phase (between FL and EEF, Figure 2B). The measurements were taken only a few days after the heat treatments were implemented, and this trend persisted at least until EEF, when the post-flowering treatments were removed. In contrast, no differences were found in Exp. 2 between the H2 and C2 treatments in LAI when measured a few days after FL, though this trait also showed a reduction of around 25% by EEF (Figure 2D).

In addition, LAI was approximately 20% and 30% lower in the Gs genotype compared to the Gl genotype for the pre- (C1-H1, Figure 2A) and post-flowering (C2-H2, Figure 2B) heat treatments, respectively (G factor, $p < 0.05$). In Experiment 2, LAI was around 30% and 70% lower in the defoliated (D-) treatment compared to the intact (D0) treatment during EEF for the pre- (C1-H1, Figure 2C) and post-flowering (C2-H2, Figure 2D) treatments, respectively (D factor, $p < 0.05$).

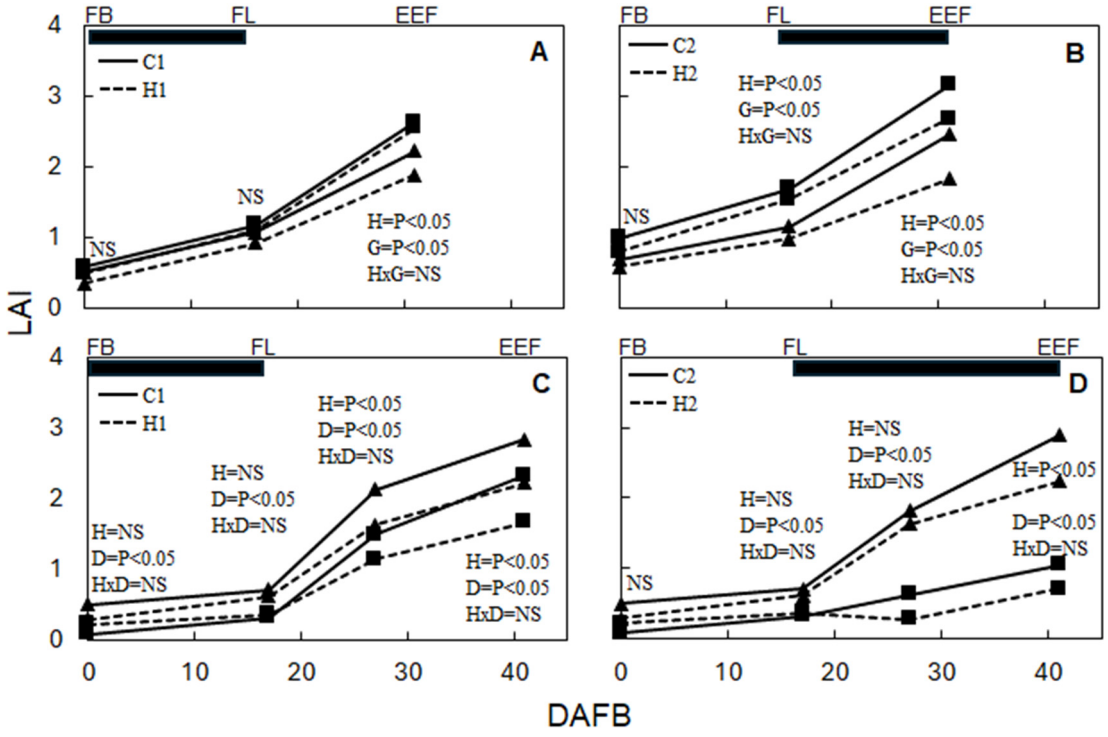

Figure 2. Leaf area index (LAI) as a function of the days after the onset of the flowering-bud stage (DAFB), for the short (Gs, ▲) and long (Gl, ■) genotypes, in Experiment 1 (**A**,**B**) and for the non-defoliated (D_0, ▲) and defoliated (D-, ■) treatments in Experiment 2 (**C**,**D**), subjected to control (entire lines) and heat (dotted lines) temperature treatments. Horizontal black bars indicate the period covered by the treatments between FB and flowering (FL) and between FL and the end of the effective flowering period or cut-out (EEF) for pre-flowering (**A**,**C**) and post-flowering (**B**,**D**) treatments, respectively. Two-way ANOVA analyses were performed for each measuring date and the results are indicate inside the Figure. H = temperature treatments, G = genotype, D = defoliation treatments, NS = non-significant.

3.3. Interception efficiency (IE)

There were significant differences between genotypes in IE during the overall pre and post reproductive phase in Experiment 1, regardless of the heat treatments (Figure 3A,B). IE showed a quick increase, with values from 60% in FB to 75% in EEF. In this phase, IE was slightly (although significantly) lower in the Gs than in the Gl genotype. An unexpected lack of a negative impact of heat treatment was detected when they were performed at pre-flowering (Figure 3A), while at post-flowering IE became significantly lower in the H treatment only at EEF (Figure 3B).

In contrast, strong temperature effects were detected in Exp. 2 for the H factor ($p < 0.05$). When treatments were applied during pre-flowering, significant effects were observed in H1 only at 27 DAFB, after the treatment was removed (Figure 3C). For the post-reproductive phase (C2-H2), this factor was significant at both the FL and EEF stages, with no interactions with the defoliation treatments (Figure 3D). Additionally, IE values were lower in D- compared to D0, as expected, although a clear recovery was observed in the former by EEF in the H1 treatment.

3.4. IR_{cum} and RUE estimated using slopes for the FB-EEF phase

Despite the negative effect of heat stress on IE shown in Figure 3, no differences in IRcum at EF were detected in either experiment compared to their respective controls, ruling out thermal stress effects on this trait (Tables 1 and 2). However, RUE was negatively affected by heat treatments in Experiment 1 during the pre-reproductive phase (Figure S1A,B,E,F; Table 1), regardless of genotype. For the post-flowering treatment, RUE also decreased, but this reduction was significant only in the Gl genotype (Figure S1C,D,G,H; Table 1). CDW was near 50% lower under heat stress in both genotypes, at both pre and post-flowering periods.

In contrast, in Experiment 2, RUE remained the same across all treatments, as neither the slopes nor the accumulated intercepted radiation and CDW differed among treatments when measured throughout the entire FB-EEF phase (Figures S2 and S3; Table 2).

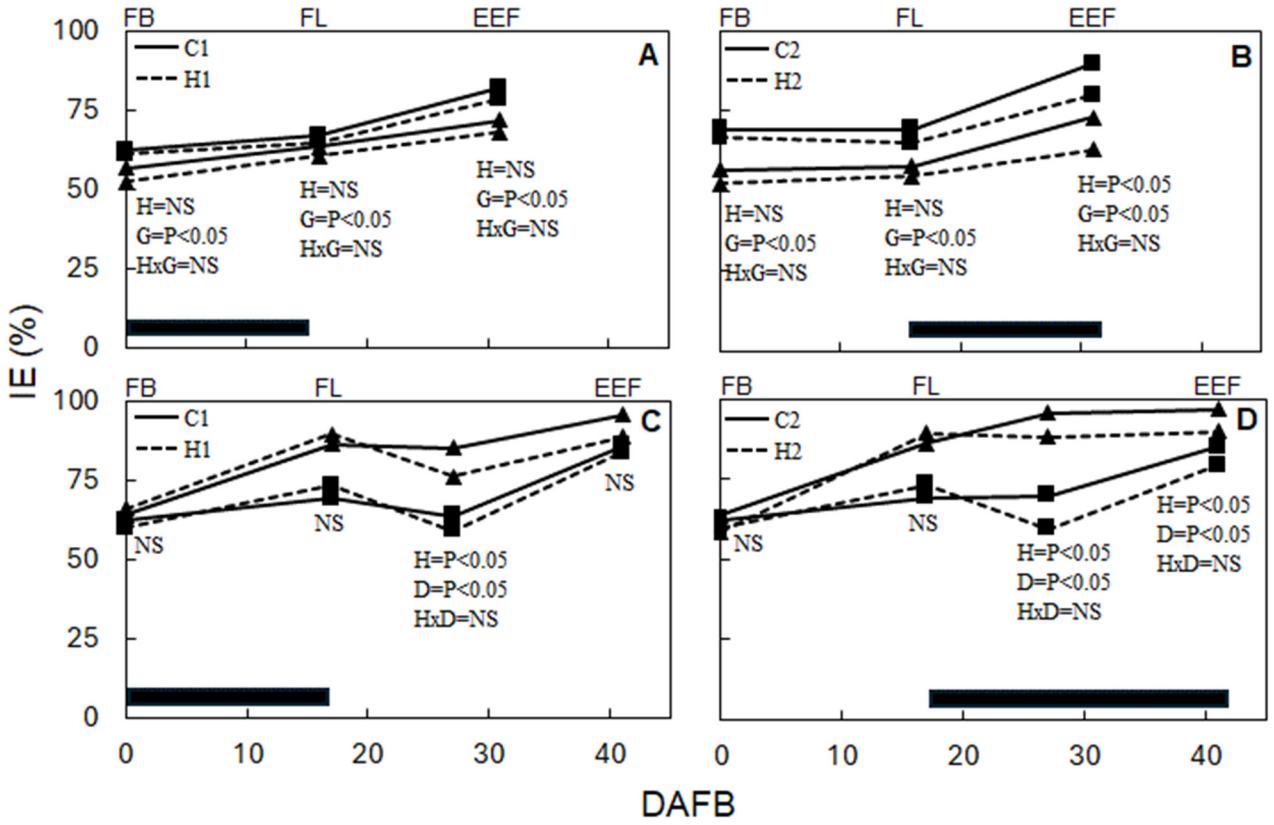

Figure 3. Interception efficiency (IE) as a function of the days after the onset of the flowering-bud stage (DAFB), for the short (Gs, ▲) and long (Gl, ■) genotypes, in Experiment 1 (**A**,**B**) and for the non-defoliated (D_0, ▲) and defoliated (D-, ■) treatments in Experiment 2 (**C**,**D**), subjected to control (entire lines) and heat (dotted lines) temperature treatments. Horizontal black bars indicate the period covered by the treatments between FB and flowering (FL) and between FL and the end of the effective flowering period or cut-out (EEF) for pre-flowering (**A**,**C**) and post-flowering (**B**,**D**) treatments, respectively. Two-way ANOVA analyses were performed for each measuring date and the results are indicate inside the Figure. H = temperature treatments, G = genotype, D = defoliation treatments, NS = non-significant.

Table 1. Radiation use efficiency (RUE) values, Cumulative Intercepted PAR Radiation (IR_{cum})) and Current dry weight (CDW), for the different thermal treatments (control: C and heat H) estimated during FB to EEF phase. Data are presented for the short cycle (Gs) genotype DP402 and for the long cycle (Gl) genotype DP1238 in Exp.1. Statistical analyses were performed for each trait using two-way ANOVA tests (n = 3). RUE values were estimated as the adjusted slopes of the functions fitted in Figure S1. Asterisks indicate significant differences from their respective control treatment.

	Gs			Gl		
Treatments	**RUE (g MJ^{-1})**	**IR_{cum} (MJ m^{-2})**	**CDW (g m^{-2})**	**RUE (g MJ^{-1})**	**IR_{cum} (MJ m^{-2})**	**CDW (g m^{-2})**
C1	4.83 ± 0.62	221.64	834.84	3.81 ± 0.46	241.11	733.03
H1	0.72 ± 0.11 ***	202.51 ns	250.6 ***	2.05 ± 0.14 **	237.98 ns	421.06 ***
C2	2.64 ± 0.13	222.39	527.2	3.39 ± 0.48	250.35	704.29
H2	1.79 ± 0.15 ns	205.30 ns	380.42 ***	1.60 ± 0.15 **	244.83 ns	341.52 ***

** $p < 0.01$; *** $p < 0.001$; ns = non-significant.

Table 2. Radiation use efficiency (RUE) values, Cumulative intercepted PAR radiation (IR_{cum}) and Current dry weight (DW), estimated during FB to EEF period, for the different thermal (control: C and heat H) and defoliation (D-) and without defoliation (D_0) treatments in Exp.2. Statistical analyzes were performed for each variable using two-way ANOVA tests (n = 3). The values were obtained through the adjusted slopes of the functions in Figures S2 and S3. ns = non-significant.

	D_0			D-		
Treatments	**RUE (g MJ^{-1})**	**IR_{cum} (MJ m^{-2})**	**CDW (g m^{-2})**	**RUE (g MJ^{-1})**	**IR_{cum} (MJ m^{-2})**	**CDW (g m^{-2})**
C1	2.76 ± 1.28	221.47	868.86	4.67 ± 0.75	182.83	982.22
H1	3.88 ± 0.86 ns	222.38 ns	1153.33 ns	3.66 ± 1.21 ns	182.95 ns	922.22 ns
C2	2.47 ± 0.60	262.82	1067.78	1.08 ± 0.40	251.99	648.44
H2	1.60 ± 0.45 ns	268.8 ns	760.0 ns	1.07 ± 0.51 ns	257.14 ns	646.66 ns

ns = non-significant.

3.5. Radiation use efficiency (RUE) estimated separately for the pre and post reproductive phases

With the aim of increasing the precision of the RUE analysis, this trait was also separately estimated for the FB to FL (pre-flowering) and FL to EEF (post-flowering) sub-phases. In Experiment 1, the heat treatment clearly decreased RUE in both genotypes. However, significant interactions between heat treatments and genotypes were detected, with RUE values 80% and 20% lower than the control ones in the Gs and Gl genotypes, respectively, when applied during pre-flowering (Figure 4). Thus, the effect was considerably more detrimental in the shorter-cycle genotype than in the longer-cycle one. Interestingly, an opposite trend was observed when heat stress was imposed during the post-reproductive phase, as the negative effect was more pronounced in the longer-cycle genotype. Indeed, reductions of around 30% and 70% were detected in the Gs and Gl genotypes, respectively. Nevertheless, this outcome is a consequence of the extremely high RUE value, close to 6 g MJ^{-1}, estimated for the C2 treatment.

Unlike the RUE estimated for the entire FB to EEF phase (Table 2), significant effects and interactions between temperature and defoliation factors were detected in Exp. 2 (Figure 5). When D0 plants were exposed to high temperatures, a drastic reduction in RUE values of 60% was observed in both the pre- and post-reproductive phases, compared to their respective controls. However, in the defoliation treatment (D-), RUE drastically decreased to values close to 1 g MJ^{-1}, regardless of the heat treatment or phase.

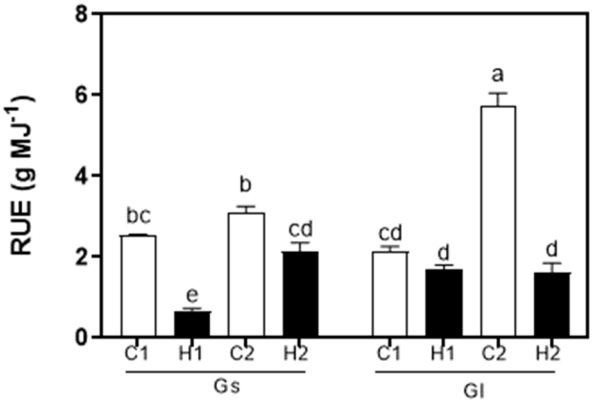

Figure 4. Radiation use efficiency (RUE, g MJ^{-1}) estimated for the period FB-FL (pre-flowering; C1-H1)) and FL-EEF (post-flowering; C2-H2)) for control (C1-C2) and heat (H1, H2) treatments. Data are presented for the short cycle (Gs) genotype DP402 and the long cycle (Gl) genotype DP1238 in Experiment 1. Vertical bars are standard errors for means (n = 9) and different letters indicate significant differences among treatments ($p < 0.05$).

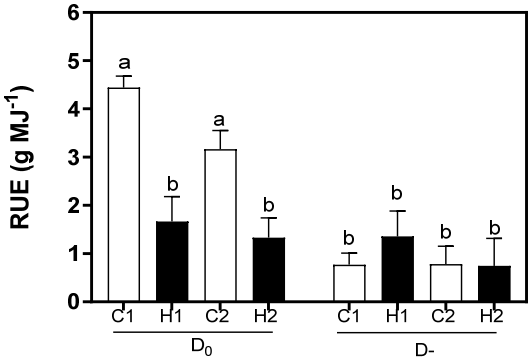

Figure 5. Radiation use efficiency (RUE, g MJ^{-1}) estimated for the period FB-FL (pre-flowering; C1-H1) and FL-EEF (post-flowering; C2-H2)) for controls (C1-C2) and heat (H1, H2) treatments. Data are presented for the undefoliated (D_0) and defoliated (D-) treatments in Experiment 2. Vertical segments are standard errors for means (n = 9) and different letters indicate significant differences among treatments ($p < 0.05$).

3.6. Yield

Control yield was approximately 2700 kg ha^{-1} in Exp. 1 and was differentially reduced by heat stress, decreasing by 66% and 42% in H1 and H2 plants, respectively, compared to their respective controls (Gs genotype, Figure 6A). Surprisingly, in Exp. 2, conducted in Buenos Aires, raw yield reached up to 6000 kg ha^{-1} in the control treatments (Exp. 2, Figure 6B), more than double the yield obtained in Experiment 1. This result is noteworthy, given that Buenos Aires lies outside the traditional cotton cultivation region in Argentina, whereas Experiment 1, conducted in Reconquista, is located within that region. No significant interactions were detected, and the yield reduction due to heat stress was more pronounced when heat was applied during the post-flowering phase (approximately 75%) compared to the 50% reduction observed during the pre-reproductive phase (Figure 6B). Additionally, defoliation had a negative impact, independent of temperature, resulting in average losses of 15%. As expected, fiber yield followed a similar pattern, representing an average of 35% of the raw yield, with the overall pattern remaining consistent (Figure 6C).

3.7. Harvest index (HI) and total dry weight (DW) al harvesting

In general terms, the harvest index (HI) in Exp. 2 was approximately 0.32 in control treatments and significantly decreased under high-temperature exposure during both treatment periods (H1 and H2) when leaf area was not manipulated (D0), with average decreases of 40% and 65% for the pre-flowering (C1 and H1) and post-flowering (C2 and H2) thermal treatments, respectively (Figure 7). Similar trends were observed for both D0 and D- plants, ruling out interactions with the thermal treatments. Reductions in DW also contributed to the yield decrease caused by heat stress (around 23%, averaged across H1 and H2 treatments for both D0 and D- plants, Figure 8). A similar effect was observed for defoliation treatments, regardless of the thermal regime.

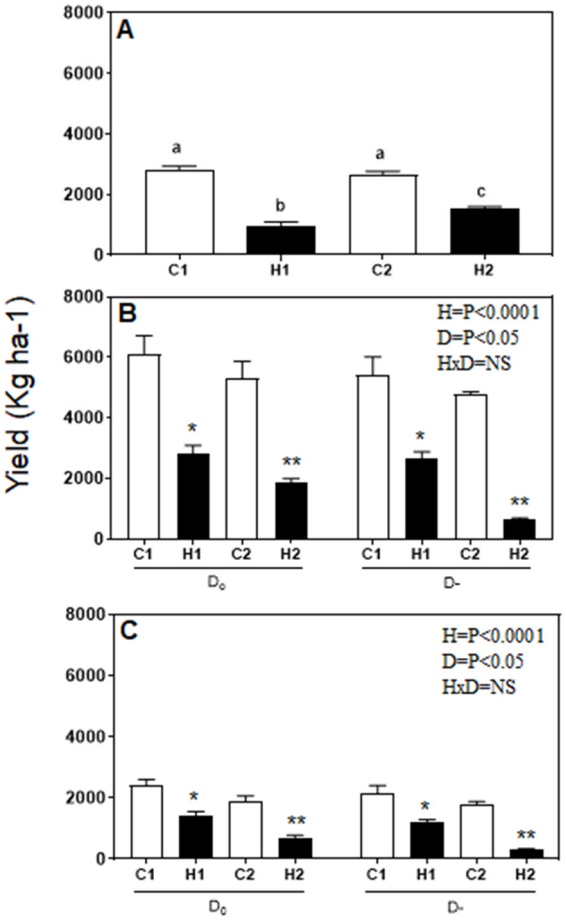

Figure 6. Raw yield (fiber + seeds) in Exp.1 (**A**, short cycle genotype only) and Exp.2 (**B**), and fiber yield for Exp.2 (**C**) for their respective control (C1-C2) and heat (H1, H2) treatments. Data are presented for the undefoliated (D_0) and defoliated (D-) treatments in Experiment 2. Vertical segments are standard errors for means (n = 24). Two-way ANOVA analyses were performed in Exp. 2 for temperature (H) and defoliation (D) treatments, and the results are indicated as insides. In Exp. 1, different letters indicate significant differences among treatments ($p < 0.05$). In Exp. 2, asterisks indicate significant differences for the H factor respect to their respective controls (* = $p < 0.05$; ** = $p < 0.01$).

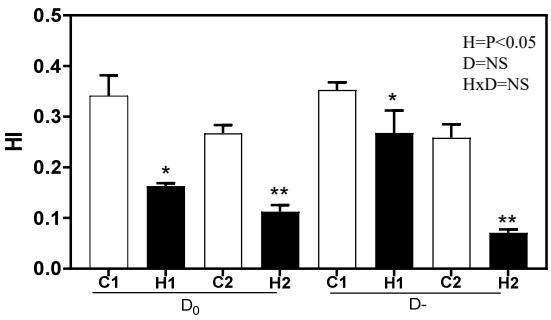

Figure 7. Harvest index in Exp.2 for control (C1-C2) and heat (H1, H2) treatments, for the undefoliated (D0) and defoliated (D-) treatments. Vertical segments are standard errors for means (n = 24). Two-way ANOVA analyses were performed for temperature (H) and defoliation (D) treatments, and the results are indicated as insides. Asterisks indicate significant differences for the H factor respect to their respective controls (* = $p < 0.05$; ** = $p < 0.01$).

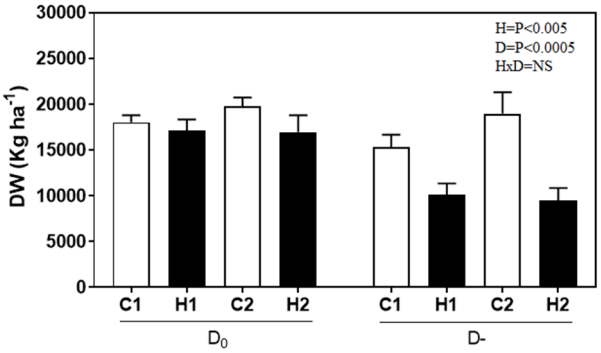

Figure 8. Total aerial dry weight in Exp.2 for control (C1-C2) and heat (H1, H2) treatments, for the undefoliated (D_0) and defoliated (D-) treatments. Vertical segments are standard errors for means (n = 24). Two-way ANOVA analyses were performed in Exp. 2 for temperature (H) and defoliation (D) treatments, and the results are indicated inside.

4. Discussion

Knowledge about the impact of high-temperature episodes on crop variables related to radiation utilization in cotton has been quite limited, with most research focusing on the effects on water use efficiency (Conaty et al., 2015; V. R. Reddy, K. R. Reddy, & Hodges, 1995). Previous studies on temperature stress have primarily concentrated on its impact on harvest index, particularly failures in the pollination process (Abro et al., 2023; Snider et al., 2009; Snider & Oosterhuis, 2012). The reproductive phase encompasses a significant portion of the critical period for yield determination in cotton, spanning from the flower bud (FB) stage to 10 days after the end of the effective flowering period (EEF) (Paytas et al., 2023), Thus, the effect of heat stress on raw and fiber yield (Figure 6) was closely associated with similar reductions observed in the harvest index (Figure 7). The stronger yield and harvest index reductions during the post-reproductive phase support the idea that heat stress exacerbates the detrimental effects on pollen viability and fertilization when applied during this stage, as flowers are fully exposed to pollination and fertilization processes (K. R. Reddy, Hodges, & V. R. Reddy, 1992).

Interestingly, our results clearly reveal an additional detrimental effect of heat stress on yield due to a reduction in plant size (total aboveground dry weight) at harvest, (Figure 8). This finding is particularly relevant, as total dry biomass has been a key trait for improving cotton productivity over the past 65 years of breeding. Modern cultivars require increased biomass accumulation to achieve higher yields (Singh et al., 2023). This contrasts with approaches used in other crops (such as wheat during the Green Revolution), where dwarfing genes were introduced and harvest index was enhanced rather than plant size (Ferrero-Serrano, Cantos, & Assmann, 2019). Although LAI and interception efficiency were significantly affected by heat stress (Figures 2 and 3, respectively), a surprising lack of differences caused by temperature treatments was observed in both experiments regarding cumulative intercepted PAR radiation, measured from floral bud to the end of the effective flowering stage (Tables 1 and 2). Therefore, our results suggest that yield reduction cannot be attributed to diminished radiation capture, at least under the 20 plants m^2 density used in the experiments, higher than the 15 plants m^2 typically employed by farmers in Argentina (Scarpin et al., 2022, 2023).

Fiber yield in the control treatments of Experiment 2 was around 2200 kg ha^{-1}, conducted in Buenos Aires, a site located outside the traditional cotton cultivation region in Argentina (Figure 6C). This productivity aligns with other reports from irrigated cotton in Australia (Grundy, Yeates, & Bell, 2020; Yeates, Constable, & McCumstie, 2010a) and the USA (Hu et al., 2018; Siegfried et al., 2023). Paradoxically, yield was 38% lower in Experiment 1, carried out in Reconquista, a location within the traditional cotton-growing region of Argentina. However, lower lint yields of 1171 and 1135 kg ha^{-1} for the Gs and Gl genotypes, respectively, were also reported by (Scarpin et al., 2022) in that location, supporting the idea that yield may be higher in non-traditional regions compared to traditional ones. Further research is needed to understand the physiological basis underlying these productivity differences.

RUE estimated during the critical period of yield determination emerged as the main determinant of the detrimental effects on yield, produced not only by heat stress but also by the reduction of source availability generated in our work through defoliation (Figures 4 and 5). Certainly, this aligns with findings in cotton subjected to reductions in other resource availabilities such as nitrogen, radiation, or waterlogging (Milroy & Bange, 2013; Pokhrel et al., 2023; Yeates, Constable, & McCumstie, 2010a). However, this contrasts with a report on potato, conducted across a broad range of genotypes, which revealed that radiation interception played a more significant role than radiation use efficiency in determining yields (Sandaña & Kalazich, 2015). In fact, the RUE estimations reported in this work are the first for the species under heat stress. Our results for control treatments ranged between 2.47 and 4.83 g MJ^{-1} in both experiments throughout the reproductive phase (Tables 1 and 2), which is consistent with the approximately 3 g MJ^{-1} values reported by (Yeates, Constable, & McCumstie, 2010b) during the pre-reproductive phase at 27 °C, as well as other studies on the same species (Grundy, Yeates, & Bell, 2020; Pokhrel et al., 2023). Much higher values (up to 6 g MJ^{-1}) were obtained when estimated separately for the pre- and post-reproductive phases in both experiments (Figures 4 and 5). This discrepancy is presumably due to the estimation methodology (calculating the difference in biomass between two consecutive harvests divided by the corresponding amount of intercepted radiation), which can introduce errors associated with the calculated differences. Furthermore, the growing rate could have differed along the crop cycle (Sinclair & Muchow, 1999). Although further research is needed to determine more accurate RUE values for the pre- and post-reproductive phases separately, the methodology used in this work was sufficiently precise to detect differences not only among heat stress treatments but also across different source/sink relationships.

In general terms, no interactions between heat stress and source/sink relationships were found in the study, with the notable exception of RUE. Thus, unlike the much smaller reductions in yield caused by defoliation compared to heat stress (without interactions between factors) (Figures 6–8), RUE was dramatically affected in Exp. 2 by defoliation to the same magnitude in both heated and non-heated plants (Figure 5). Therefore, the causes of RUE depletions could be explained through different mechanisms: while the decrease due to source restriction may be produced by a higher proportion of the canopy subjected to saturating irradiance, as observed in other crops such as sunflower (Lambers, Chapin, & Pons, 2008; Trapani et al., 1992) or wheat (Tao et al., 2022), lower photosynthesis rates likely contribute to RUE reduction under heat stress in cotton, as indicated by recent research (Mercado Álvarez et al., 2022; Yousaf et al., 2023). Thus, the impact of RUE reductions on cotton productivity, during the reproductive phase, strongly depends on the nature of the limiting factor.

The results from Experiment 1 significantly contributed to consolidating the knowledge regarding the role of heat stress on the studied traits. However, the behaviors of both genotypes (with different cycle durations) were not sufficiently contrasting to generate solid trends about how source/sink relationships affect the variables analyzed in our study. Fortunately, this was achieved in Experiment 2 by introducing a manipulative defoliation treatment, which reduced yield by approximately 15%, regardless of temperature (Figure 6). Our results contrast with the increases in cotton fiber yield observed by (Liu et al., 2024), who performed partial leaf removals after the crop reached its maximum LAI. One possible explanation is that, in that study, LAI was adjusted from a maximum level close to 6 to an optimum level near 3, which would enhance crop architecture and, hence, RUE. Such values are significantly higher than the 3 and 0.5 LAI values observed in unheated plants of Experiment 2 for intact and defoliated plants, respectively (Figure 2). Thus, the results obtained in our work contribute to the understanding of the effect of source/sink relations on cotton productivity. Our results clearly demonstrate that when reductions in LAI fall below an optimal level, productivity would be reduced not only associated to a decrease in RUE (Figure 5) but also to interception efficiency (Figure 3).

5. Conclusions

Heat stress drastically reduced raw and fiber yield by 50% and 75% during the pre- and post-reproductive phases, respectively. The defoliation treatment also diminished yield by 20%, without interacting with the thermal regime. The effect of heat stress was closely linked to reductions in harvest index, with lesser total plant dry weight also contributing to yield reduction due to both heat stress (21% reduction in the pre- and post-reproductive phases) and source reduction (25%) compared to unheated and intact plants, respectively. No impact on cumulative intercepted radiation during the pre- and post-flowering periods was observed from temperature or defoliation treatments at the given plant density, despite significant reductions in LAI detected in this study. Interestingly, strong interactions between heat and defoliation treatments were observed for RUE, supporting the notion that the association of this variable with cotton yield greatly depends on the nature of the limiting factor. This work represents a significant advancement in understanding the crop variables involved in yield generation in cotton subjected to heat stress under different source/sink relationships during the reproductive phase. However, further research is needed to determine the impact of these combined treatments on the number and weight of capsules, as well as the spatial distribution of capsules along different reproductive branches.

Supplementary Materials

The additional data and information can be downloaded at: https://www.sciltp.com/journals/PlantEcophys/2025/1/518/s1.

Author Contributions

K.M.A. developed the initial conceptualization and investigation, carried out the methodology and formal analysis, and wrote the original draft. H.D.B. contributed to the conceptualization, supervised the activities, and assisted in manuscript writing. M.J.P. conducted part of the methodology, supervised the activities, and revised the manuscript. E.L.P. managed the project and resources, supervised the conceptualization and activities, and wrote the revised manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

Data are available upon request from E.L.P.

Conflicts of Interest

The authors have no conflict of interest to declare.

Peer Review Statement

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Article

Nitrogen-Driven Changes in Metabolic Profile Modulate Photosynthetic Performance and Antioxidant Defense of *Amaranthus cruentus*

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Abstract: Nitrogen is crucial for plant development and crop production. *Amaranthus cruentus*, a C_4 species, has been pointed out as a high-nutritious and stress resilient crop. Here we studied the effects of sufficient and low nitrogen supplementation on the photosynthetic efficiency and metabolic responses of *A. cruentus*. Photochemical parameters from dark-adapted and transient chlorophyll fluorescence measurements, antioxidant enzymes activity, and metabolomic analysis, were evaluated to depict the impact of nitrogen availability. Photochemical parameters showed a significant decrease compared to those from gas exchange. The antioxidant enzymes activity revealed variations among treatments, being important at low nitrogen availability. At the metabolic level, there is a significant accumulation of L-glutamine, aromatic amino acids and ascorbic acid in *A. cruentus* with sufficient nitrogen. At low nitrogen, the metabolic profile of *A. cruentus* suggests stabilization of membrane structure and efficient use of available nitrogen by accumulating L-glutamic acid. The differential accumulation of L-glutamine and L-glutamic acid reflects an adaptive strategy for maintaining nitrogen. Nitrogen-rich conditions, the plant stores excess nitrogen as L-glutamine, while in deficiency, it utilizes L-glutamic acid for essential metabolic functions. Overall, *A. cruentus* activates a coordinated metabolic strategy under LN to optimize nitrogen use. This includes effective ROS detoxification via both enzymatic and non-enzymatic antioxidants, structural reinforcement through membrane-stabilizing lipids, and efficient nitrogen storage and redistribution to meet metabolic demands during nitrogen limitation.

Keywords: nitrogen supplementation; chlorophyl fluorescence; gas exchange; antioxidant activity; metabolic profiling

1. Introduction

Nitrogen is an essential component for plant growth, development, and productivity. In fact, besides water, among the environmental factors affecting plant productivity, nitrogen is the one that most limits productivity, especially in crops (Plett et al., 2020). Nitrogen influences photosynthetic efficiency as a fundamental component of chlorophyll and essential enzymes, such as RuBisCO. Hence, a deficit of nitrogen or decreased availability has a profound impact on the primary productivity of ecosystems and agricultural systems.

Nitrogen supplementation promotes biochemical adjustments in plants, leading to improved metabolic activity, even under suboptimal environmental conditions, such as drought stress (Tariq et al., 2019). Nitrogenated metabolites not only serve as building blocks for protein synthesis but also contribute to essential physiological processes and signaling pathways, enhancing plant resilience to stress (Sadak &

Ramadan, 2021). Nevertheless, the level of nitrogen supply, i.e., high, moderate, or low, determines the degree of tolerance or sensitivity against abiotic stresses, such as drought (Song et al., 2019a). For example, nitrogen supplementation in drought-stressed soybean plants increased its yield (Purcell & King, 1996), although in excess produced negative effects on water use efficiency and photosynthesis, affecting its drought tolerance response (Sun, Gao, & Lu, 2007). Also, several studies indicate that optimal nitrogen supply has a positive impact on photochemical parameters while limited nitrogen availability significantly reduces photochemical efficiency in crops such as maize, wheat, oat, rice, and tomato (Wu et al., 2019a; Song et al., 2019b; Peng et al., 2021; Li et al., 2023).

Plant responses to nitrogen availability are complex and usually species-specific (Brueck, 2008; Ren et al., 2017). *Amaranthus cruentus* L., a C_4 plant commonly known as grain amaranth, exhibits significant potential as a climate-smart crop owing to its adaptability to diverse climates and soils (Netshimbupfe et al., 2022). *A. cruentus* possesses a high nutritional value, particularly in terms of protein content, essential amino acids, and micronutrients, making it a valuable resource for combating malnutrition and enhancing food security (Cechin et al., 2022). The effect of sources and doses of nitrogen on growth, gas exchange, and some biochemical traits such as pigments, proline, and phenolic content have been reported for *A. cruentus* (Cechin et al., 2022; Zubillaga et al., 2019; Cechin & Valquilha, 2019). Nevertheless, we are still far from understanding the interplay of nitrogen availability and metabolic pathways and their impact on the photosynthetic performance of *A. cruentus.*

Given the key structural and functional role of nitrogen on primary and secondary metabolism and photosynthetic-related processes, we postulate that low nitrogen availability induces metabolic shifts towards an efficient distribution of nitrogen to sustain photosynthesis. The analyses of key physiological parameters and metabolomics provide novel insights into how nitrogen availability impacts the photochemical yield, antioxidant protection, and differential accumulation of metabolites in *A. cruentus*.

2. Materials and Methods

2.1. Plant material and experimental design

The genotype utilized in the experimental setup was *Amaranthus cruentus* Diaguitas (ACR40). Seeds were obtained from the National Seed Bank collection of the Instituto de Investigaciones Agropecuarias (INIA-Intihuasi) located at Vicuña, Chile (19 J 336895.75 m E 6675781.94 S). At a nursery garden located at the University of Concepción, plants of *A. cruentus* were grown from seeds sown directly on 5 kg of dry soil in 11 L pots (22 cm height by 28 cm diameter; two plants per pot). The soil in the pot contained a mixture of 80% washed sand and 20% peat and a basal fertilization with 4 g of 6 M Basacote Plus Compo Expert (16% N, 3.5% P, 10% K, 1.2% Mg, 5% S, and micronutrients) according to Cifuentes et al, (2023). Pots were irrigated at field capacity (FC) by monitoring the soil water content using a time-domain reflectometer soil moisture meter TDR350 (FieldScout Spectrum Technologies, Inc., Aurora, IL, USA), according to Ostria-Gallardo et al, (2020). The frequency of irrigation was every two days.

The experimental design consisted of evaluating two levels of nitrogen availability as follows: sufficient nitrogen (C), low nitrogen (LN). Each treatment contained 24 pots, each pot containing one plant. For nitrogen treatments, plants with the fourth pair of true leaves were supplemented with urea (CH_4N_2O) to reach both N-level treatments: sufficient nitrogen (C; 0.6 g of N per pot) and low nitrogen soils (LN; 0.30 g of N per pot). These concentrations were used to determine the optimal and insufficient N fertilization levels. We opted for urea as nitrogen source due to its lower risk of causing salt injury, compared to ammonium nitrate or ammonium sulfate, and because it is the most widely used nitrogen fertilizer worldwide. Sampling and measurements were carried out 30 days after the application of the nitrogen doses.

2.2. Quantification of total leaf nitrogen, soluble sugars and starch content

The total leaf nitrogen content (%) was determined using the Kjeldahl method, following the procedure described in https://prometheusprotocols.net/. Briefly, 0.5 g of dried and finely ground leaf samples were placed in a digestion tube with 5 mL concentrated H_2SO_4 and a catalyst mixture composed of 10 g K_2SO_4 and 0.5 g $CuSO_4$. The samples were subjected to digestion and further titration with 0.1 N HCl until the endpoint was reached, indicated by a color change from green to pink. The total nitrogen content was calculated as: Total Nitrogen (%) = (V × N × 1.4)/W, where V is the volume (mL) of HCl used in titration, N is the normality of the HCl solution, 1.4 is the conversion factor for nitrogen in the Kjendahl method, and W is the weight (g) of the sample.

Soluble sugars were extracted from 50 mg fresh leaf material using 80% ethanol, according to Chow & Landhäusser (2004). Following extraction, the extracts were centrifuged at 2500 rpm for 5 min at 4 °C. The total soluble sugar content was determined using the anthrone reagent at 490 nm. Starch was extracted from the residue of the extracts using a boiling solution of 3% perchloric acid. Thus, starch is hydrolyzed to glucose. Glucose in the hydrolyzed extract was colorimetrically determined using anthrone reagents at 525 nm.

2.3. Chlorophyll fluorescence and gas exchange

Chlorophyll fluorescence measurements were conducted with an OS30p+ fluorometer (Opti-Science, Inc., Hudson, USA) to determine the dark-adapted parameters informing quantum yield (protocol 1) and transient OJIP curves (protocol 2). For protocol 1, leaves were dark-adapted overnight before measurements. Leaves were clamped with leaf clips and the probe of the fluorometer was inserted in each leaf clip. The actinic light used was 900 µmol quanta m^{-2} s^{-1}. The maximum quantum yield of PSII (F_v/F_m), the maximum primary yield of PSII (F_v/F_o), and the actual quantum yield (ΦPSII) were

calculated as described in Maxwell & Johnson (2000), and Kramer et al, (2004). For transient OJIP tests (protocol 2), the actinic light used was 3500 µmol and 80% modulation of light intensity. Fluorescence parameters and the log last trace were recorded and double normalized by F_0 and F_m values for further analysis of the OJIP parameters (Pollastri et al., 2022).

Leaf gas exchange measurements were made to estimate the effect of nitrogen supply on carbon assimilation (A_{sat}), stomatal conductance (g_s), and the electron transport rate (ETR) and the intrinsic water use efficiency (iWUE) using a gas exchange system (LI-6400, Li-Cor Inc., Lincoln, USA) with a 2 cm^2 leaf chamber of with an LED light source (LI-6400-40). Leaves were carefully placed in the sensor head, ensuring contact with the leaf thermocouple. Light-saturated CO_2 assimilation (A_{sat}), stomatal conductance (g_s), and apparent transpiration rate (E) were measured in 12 individuals per species from 9:00 to 13:00. Gas exchange parameters were recorded 10 min after clamping the leaf. Leaf chamber conditions were set at 400 µmol mol^{-1} of CO_2, 1200 µmol photons m^{-2} s^{-1} (90:10% red: blue light), 60–65% relative humidity, and 25 °C block temperature. The intrinsic water use efficiency (iWUE) was calculated as the ratio of photosynthesis (A_{sat}) over stomatal conductance (g_s). To verify both proper equipment performance and status of control plants, the ratio of electron transport rate to assimilation (ETR/A) was evaluated according to Perera-Castro & Flexas, (2023).

2.4. Antioxidant activity

The antioxidant enzyme assays i.e., ascorbate peroxidase (E.C. 1.11.1.11, APX), glutathione reductase (E.C. 1.6.4.2, GR), superoxide dismutase (E.C. 1.15.1.1, SOD) and Peroxidase (POD), activities were determined by the methods described in Palma et al, (2014). Briefly, 1g fresh leaves was ground with liquid nitrogen and homogenized with 2 mL of 50 mM potassium phosphate buffer (20% *w/v* polyvinylpolypyrrolidone, 0.1 mM EDTA, 10 mM β-mercaptoethanol, pH 7.8). The homogenate was centrifuged at 13,000× *g* 20 min at 4 °C, and the supernatant was used for determinations of enzyme activity and total protein content by Bradford (1976). Ascorbate peroxidase uses ascorbate as an electron donor and was evaluated spectrophotometrically by the decrease in absorbance at 290 nm after 10 min on a reaction mix of 50 mM Tris-HCl pH 7.8, 0.4 mM ascorbate, and 0.3 mM H_2O_2, the last two added moments before starting the measurements. Superoxide dismutase catalyzes the dismutation of superoxide radicals into oxygen and hydrogen peroxide and was determined at 560 nm by the inhibition of the photochemical reduction of nitroblue tetrazolium chloride (NBT). The activity is expressed as unit min^{-1} mg^{-1} protein, where one unit of SOD is equal to the amount of enzyme that inhibits 50% of NBT photoreduction. Peroxidase (POD) was determined at 470 nm by monitoring the oxidation of guaiacol. The reaction mix contained 200 mM sodium phosphate buffer pH 5.8, 7.2 mM guaiacol, and 11.8 mM H_2O_2 as cofactor. Glutathione reductase (GR) activity maintains the reduced state of glutathione and was evaluated at 340 nm by measuring the decrease in absorbance due the NADPH oxidation. For this, the reaction mix contained 0.2 mM NADPH, 0.1 mM HEPES-NaOH, 3 mM $MgCl_2$, 1 mM EDTA, and 0.25 mM oxidized glutathione.

2.5. Metabolomic analysis

A total of 32 leaves from individuals of the different treatments were collected and freeze-dried. Samples were sent to the Sequencing and Omics Technologies Unit at Pontificia Universidad Católica de Chile (Secuenciación y Tecnologías ómicas–Facultad de Ciencias Biológicas (uc.cl)) for samples preparation and chromatography. Briefly, 3 µL of sample was injected on a Compact QTOF MS + Elute UHPLC spectrometer. Separation was done by using a Kinetex C18 column. Raw data was preprocessed with MS-DIAL for *m/z* peak detection, noise reduction, retention time alignment, and peak integration. Further, the m/z and MS/MS spectra were matched to MassBank database for annotation. For statistical analysis, we used those metabolites that fall within confidence level 2 (Summer et al., 2007). Preliminary data analysis was performed in the software Metaboscape v4.0. The relative quantities of metabolites from both positive and negative ionization modes were merged and imported into MetaboAnalyst v6.0 software (Pang et al., 2024). Data was filtered by interquantile ranges, log10 transformed, scaled by mean-center, and normalized by sum for further analyses according to Cifuentes et al, (2023).

2.6. Data analysis

Data were checked for normality assumptions and variance homoscedasticity with the InfoStat software. Accordingly, the parametric two-way ANOVA or non-parametric Kruskal–Wallis analyses were used to compare means between treatments. When significant differences were found, we used post hoc tests with Tukey or pairwise comparisons ($p \leq 0.05$), depending on the parametric or non-parametric nature of the data. For chlorophyll *a* fluorescence and gas exchange data we calculate the percentage change to assess the level of increase or decrease in the parameters of control (C) versus low nitrogen (LN). The percentage change was calculated by the following formula:

$$\Delta\% = ((M2 - M1)/M1) \times 100$$

where M1 is the mean value of a parameter in C, and M2 is the mean value of the parameter in LN.

For untargeted metabolomics, scaled and normalized data were analyzed for differential accumulation differences, chemometric, and cluster analyses. For these analyses, metabolites were considered significantly accumulated at $p < 0.05$. A Sparse Partial Least Square Discriminant Analysis was used to determine specific metabolites associated with the different treatments. For cluster analysis, the interquantile ranges of normalized data were used to apply the Ward's hierarchical cluster analysis and the Euclidean distance to evaluate the metabolite accumulation patterns regarding nitrogen supply and water availability. Finally, we used the Pathway analysis tool of Metaboanalyst 6.0 (Pang et al., 2024) to predict the impact of

differentially accumulated metabolites in specific metabolic pathways.

3. Results

3.1. Total leaf nitrogen, soluble sugars and starch

Leaf nitrogen content varied significantly depending on nitrogen supply. Specifically, the decrease of total nitrogen was 51.52% from C to LN (p = 0.0013; Table 1). The metabolic partitioning of carbohydrates, specifically total soluble sugars (TSS) and starch, showed non-significant changes (Table 1), suggesting a steady carbohydrate metabolism under the tested conditions.

3.2. Nitrogen availability affects significantly the photochemical response of A. cruentus

Deeper insights into the photosynthetic process of *A. cruentus* under varying nitrogen supply were gained from transient and dark adapted chlorophyll fluorescence measurements. The analysis of chlorophyll *a* transient curve (OJIP) provided insights on the effect of nitrogen availability over PSII efficiency and, consequently, plant photosynthetic capacity (Table 2). All fluorescence-derived parameters showed significant differences (Table 2). The sufficient nitrogen treatment (C) significantly outperformed the nitrogen deficit (LN) treatment, exhibiting higher values in the maximum quantum yield of PSII (F_v/F_m), maximum primary yield of PSII (F_v/F_o), Performance Index (PI) and the amplitude of relative variable fluorescence in the I-P rise (ΔVIP). Except for A_{sat}, nitrogen availability has no significant effects over gas exchange parameters (Table 2). Additionally, A_{sat} showed a significant decrease of 23.19% from C to LN, whereas the other gas exchange parameters showed non-significant changes. However, the largest percentage decrease between C to LN was observed in chlorophyll *a* parameters, except for V_J which showed an increase of 36% (Table 2).

Table 1. ANOVA results for mean values of total leaf nitrogen (in percentage of dry mass), total soluble sugars, and starch content of *A. cruentus* leaves (n = 3 leaves per treatment). Acronyms denote treatments as follows: sufficient nitrogen (C), low nitrogen (LN). Asterisk (*) indicates significant differences at $p < 0.05$.

Treatment	Total Leaf Nitrogen (%)	Total Soluble Sugars (mg g^{-1})	Starch (mg g^{-1})
C	**2.95 ***	2.41 ± 0.595	84.06 ± 10.840
LN	**1.43**	2.08 ± 0.301	72.58 ± 20.56

Table 2. ANOVA and percentage change (Δ%) of chlorophyll *a* fluorescence and gas exchange parameters under control (C) and low nitrogen (LN) treatments. Single asterisk (*) denotes significant effects at $p < 0.05$, and double asterisk (**) denotes significant effects at $p < 0.001$. Arrows indicate the direction of percentage change, with a decrease (↓) or increase (↑) in parameters when comparing C to LN.

Chlorophyll *a* Parameters			
	C (Mean ± S.E.)	**LN (Mean ± S.E.)**	**Δ%**
F_v/F_0	2.91 ± 0.14 **	2.17 ± 0.09	25.43 (↓)
F_v/F_m	0.79 ± 0.01 **	0.68 ± 0.01	13.92 (↓)
V_J	0.45 ± 0.03 *	0.61 ± 0.04	35.56 (↑)
PI	2.41 ± 0.49 *	0.65 ± 0.12	73.03 (↓)
ΔVIP	0.27 ± 0.01 **	0.20 ± 0.01	25.93 (↓)
Gas Exchange Parameters			
	C (Mean ± S.E.)	**LN (Mean ± S.E.)**	**Δ%**
A_{sat}	22.03 ± 0.71 *	16.92 ± 1.86	23.19 (↓)
g_s	0.14 ± 0.01	0.11 ± 0.01	21.43 (↓)
E	2.37 ± 0.10	2.52 ± 0.26	6.33 (↑)
$_i$WUE	158.57 ± 9.85	150.74 ± 9.35	4.94 (↓)
ETR	138.42 ± 4.62	127.96 ± 5.03	7.56 (↓)

3.3. Performance of antioxidant enzyme activities in A. cruentus leaves

The activities of key antioxidant enzymes, including APX, GR, and POD exhibited significant modulation in response to variations in nitrogen supply (Figure 1). Activity of APX, crucial for scavenging hydrogen peroxide, was markedly high in the LN treatment (Figure 1A), showing an increase of 77.8% of activity compared to C.

The activity of GR and POD were significantly higher in the LN condition (Figure 1C,D), with an increase of 133% and 50%, respectively.

3.4. Metabolomic profile and differential accumulation of metabolites in A. cruentus under varying nitrogen supply

The metabolomic results provide a comprehensive insight into the metabolic adjustments of *A. cruentus* in response to nitrogen supply. The partial least square–discriminant analysis (PLSDA)showed a distinct clustering pattern of the treatments (Figure 2A), highlighting these metabolites that contribute the most to these patterns of clustering according to the variable importance in projection (VIP) scores (Figure 2B). L-glutamine, phenylalanine and L-norleucine were the metabolites with the highest contribution to the variability observed along Components 1 and 2 (Figure 2B). Further, we analyzed the

differential accumulation of metabolites and metabolic pathways that were modulated in response to the experimental conditions (Figures 3 and 4). In Figure 3A, the heatmap illustrates patterns of metabolite accumulation across the treatments. Notably, L-glutamine, L-norleucine, phenylalanine, L-tryptophan, and the antioxidant ascorbic acid showed elevated levels in the C condition. Furthermore, high accumulation of ferulic and coumaric acids was also detected in C.

By contrast, lower levels of metabolites such as pheophorbide, phosphatidylcholine, and phosphatidylethanolamine were observed in the C treatment. On the other hand, L-glutamic acid was elevated under low nitrogen supply (LN). Also, lipids such as lysophosphatidylcholine and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, as well as flavonoids and phenolic compounds such as rutin, ferulic acid and chlorogenic acid showed high accumulation in response to LN.

Given the differential accumulation L-glutamine and L-glutamic acid depending on nitrogen supply, i.e., high levels of L-glutamine at C, high levels of L-glutamic acid at LN, we explored the correlations with other metabolites to get insight into the metabolic dynamic of *A. cruentus* across the different treatments (Figure 3B). Arginine and phenylalanine positively correlate with L-glutamine ($p < 0.01$, $r > 0.5$). On the other hand, L-glutamic acid correlated positively mostly with lipids, and a negative correlation was observed with aromatic amino acids and coumarins. Finally, the pathway impact analysis further elucidates the metabolic pathways significantly modulated by the treatments (Figure 4). We found that alanine, aspartate and glutamate metabolism, and nitrogen metabolism exhibit the highest impact, noting the crucial role of metabolites involved in these pathways, particularly L-glutamine and L-glutamate, for the response of *A. cruentus* to different nitrogen availability.

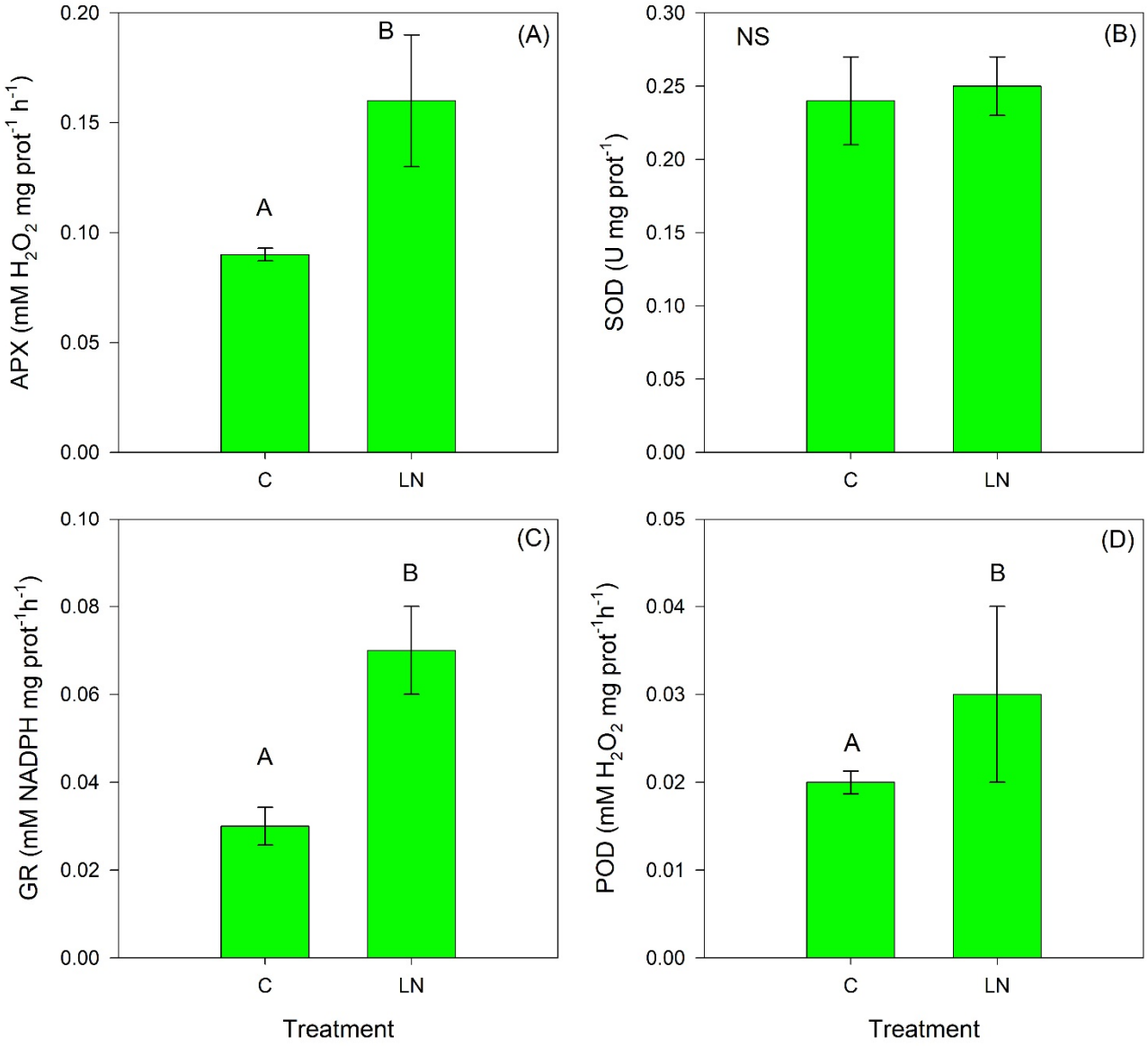

Figure 1. Effects of nitrogen supply and water availability on antioxidant enzymes. (**A**) ascorbate peroxidase (APX), (**B**) superoxide dismutase (SOD), (**C**) glutathione reductase (GR), and (**D**) peroxidase (POD). Enzyme activities were evaluated on healthy and fully expanded leaves of n = 3 individuals per treatment. The bars and vertical lines above the bars indicate the mean and the standard error values, respectively. Uppercase letters indicate significant differences between treatments at $p < 0.05$.

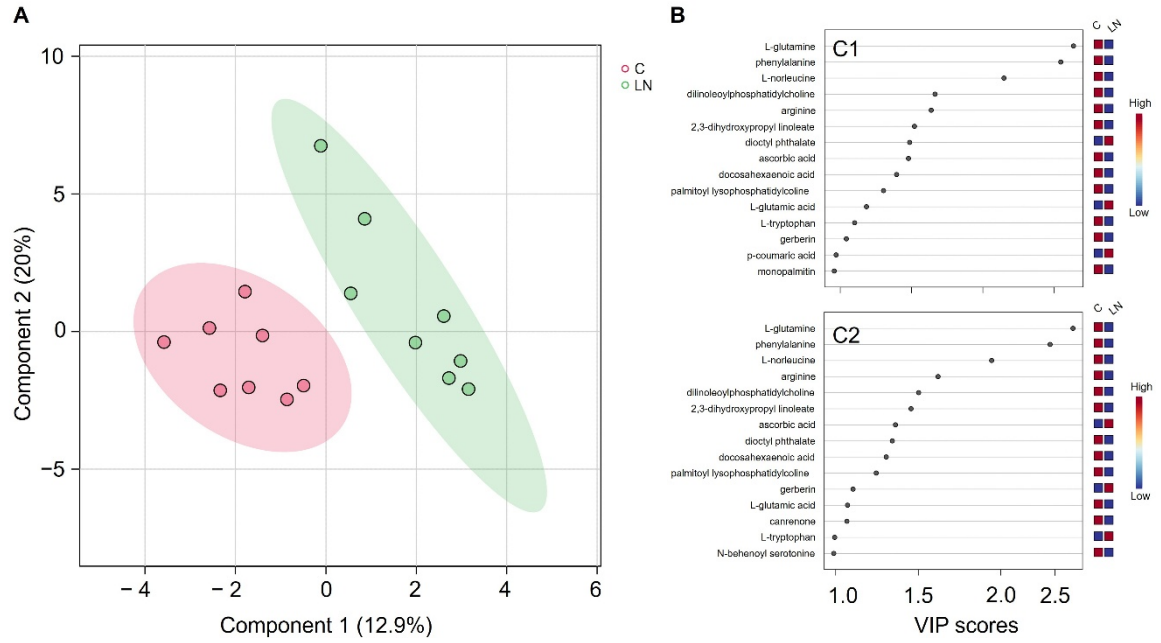

Figure 2. (**A**) Partial Least Square Discriminant Analysis plot (PLS-DA). The two-dimensional space shows the 95% confidence region of samples from control (C, red) and low nitrogen (LN, green) treatments. Components 1 and 2 collectively explain 32.9% of data variability between C and LN. Panel (**B**) shows the Variable Importance in Projection (VIP, X axis) score of metabolites (Y axis) in Components 1 and 2 (**C1**,**C2**), identifying the metabolites that contribute significantly to the variability of the data. The higher the VIP score, the higher the contribution of a metabolite in the variability of the response between C and LN. The color pallet in (**B**) indicates the contribution of specific metabolites that contribute most to the variability among treatments, from high (red) to low (blue) contribution.

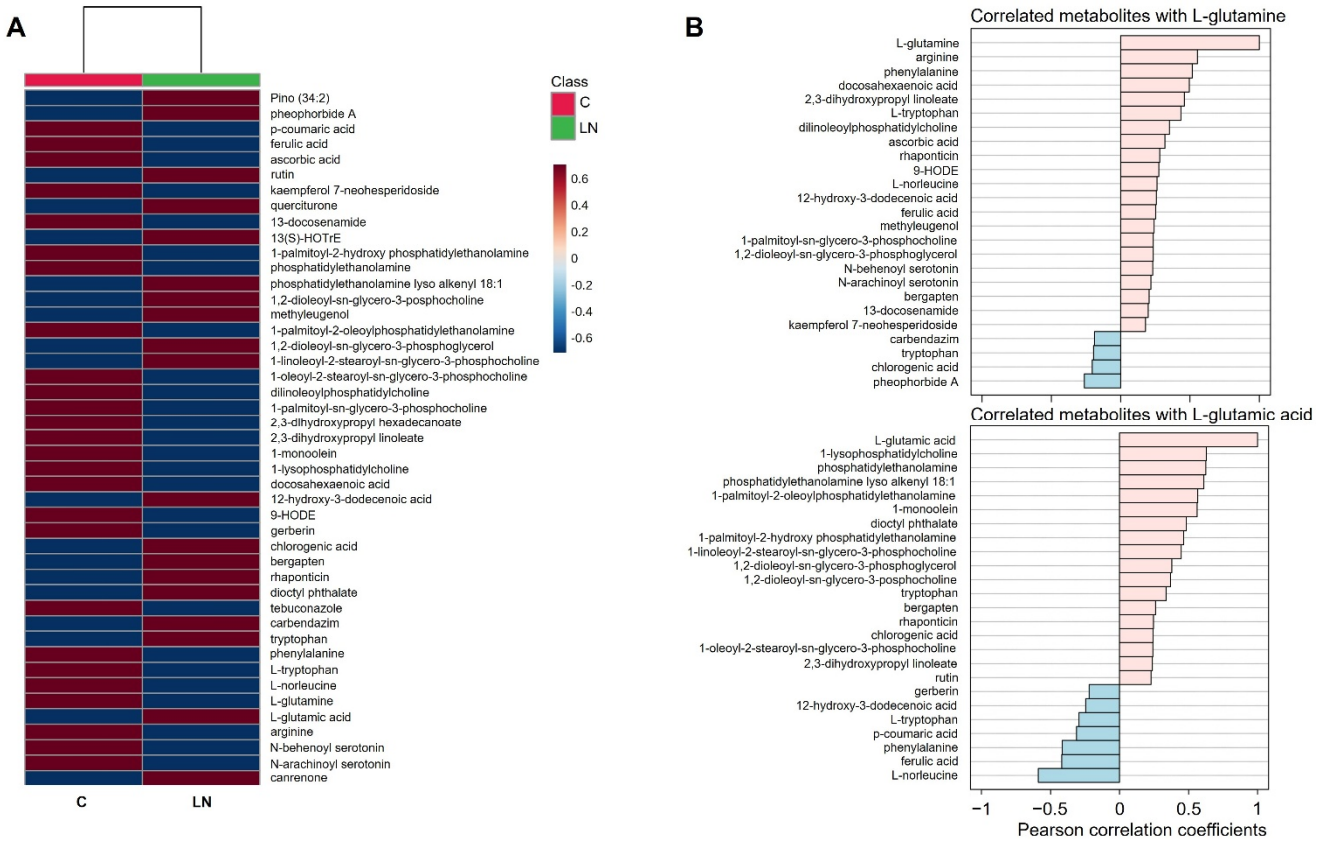

Figure 3. Heatmap for metabolome profiling data (**A**) showing the accumulation patterns of 47 non-repeated identified metabolites under the different treatments. The scale bar shows the normalized value for metabolite quantification from low(blue) to high (red) levels of accumulation. Panel (**B**) shows the Pearson correlation coefficients of L-glutamine and L-glutamic acid with the top 25 highly up-or-down accumulated metabolites in C and LN. Red and blue bars in the Pearson correlation plots denote positive and negative correlations, respectively.

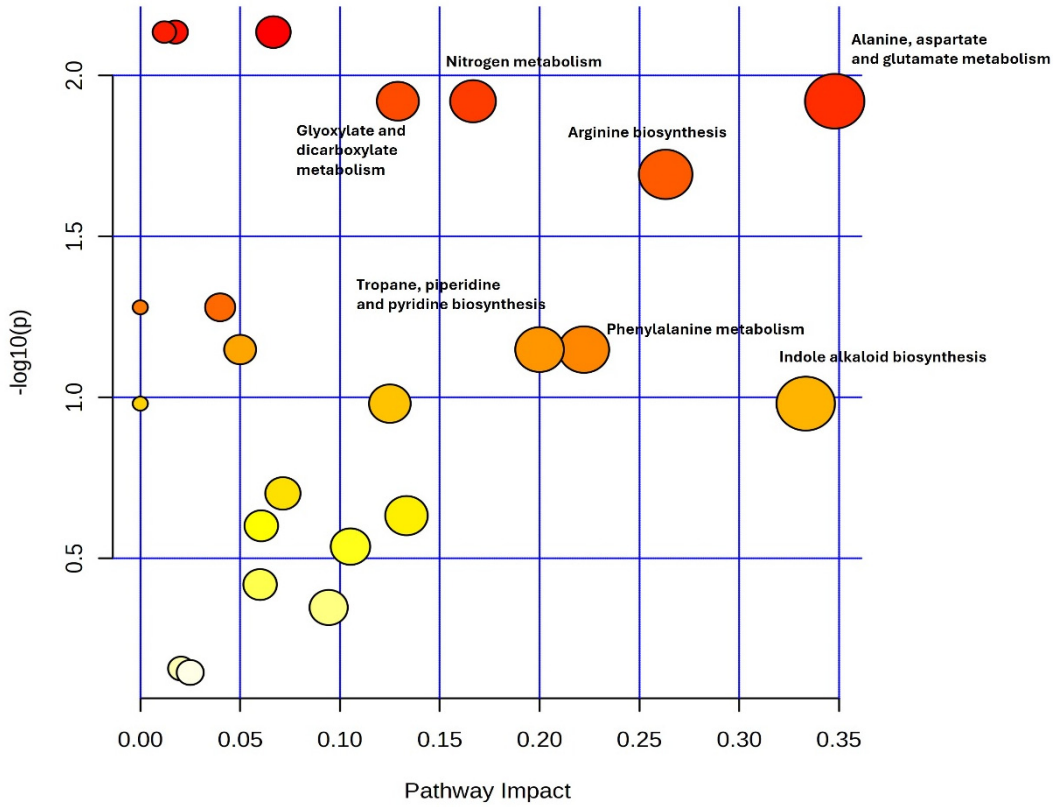

Figure 4. Pathway impact analysis based on the differential accumulation patterns of metabolites between treatments. The analysis predicts the position and roles of each metabolite within a pathway. The plot highlights those pathways that show significant alterations in response to the level of accumulation of metabolites. The size of the circles indicates the level of impact in pathways that are modulated by the treatments. The color inside the circles indicates the significance (log10 of *p* value) of the change of metabolite levels in each metabolic pathway. Accordingly, alanine, aspartate, and glutamate metabolism, arginine biosynthesis, glyoxylate and dicarboxylate metabolism, and nitrogen metabolism pathways exhibit the highest impact of the variation of accumulation of metabolites depending on C or LN conditions.

4. Discussion

Nitrogen availability has significant effects on the total content of nitrogen in leaves (Table 1), highlighting the responsiveness of *A. cruentus* to varying nitrogen supply. Nevertheless, the leaf total soluble sugars and starch showed no significant differences despite observing a decrease under LN conditions. Although the effect of nitrogen over the content of leaf carbohydrates varies widely among species, a common response is either to increase TSS and decrease starch content or vice versa (Wu et al., 2019b; Mariem et al., 2020; Zhao et al., 2020). One possible explanation for our TSS and starch results is a stable carbon metabolism under varying nitrogen content due to the C_4 carbon concentration mechanism of amaranth. Ultimately, this would help the plant to use and invest carbon in root growth to explore soil for nitrogen acquisition while maintaining carbohydrate availability for respiratory demand in response to nitrogen deficiency (Zhao et al., 2020).

We observed a significant decrease in the photochemical efficiency of *A. cruentus* under LN, as evidenced by chlorophyll *a* parameters (Table 2). Further, our analysis showed that the largest percentage decrease changing from C to LN conditions was larger in the photochemical processes than in the gas exchange processes and parameters. The largest decreases were observed in the performance index (PI), while there was a large increase in the V_J parameter. The former is an indicator of the overall photosynthetic performance, while the latter typically reflects the rate at which quinones (QA) transition from the reduced state (QA^-) back to the oxidized state (QA). High values of V_J often suggest optimal electron transference within PSII. Counterintuitively, V_J increases under LN. Under certain stress conditions, such as high light intensity or temperature stress, V_J can increase (Serôdio, Schmidt, & Franenbach, 2017; Marečková, Barták, & Hájek, 2019). The increase under stress conditions has been proposed as a protective mechanism, where PSII maintains a high turnover rate of electrons to minimize damage by excess excitation energy.

Regarding gas exchange, a decrease of nitrogen supply affects mainly to A_{sat} and g_s, yet has negligible impact on transpiration, ETR and iWUE. Sufficient nitrogen supply has been shown to enhance WUE under certain stress conditions (Plett et al., 2020; Sadras & Rodriguez, 2010). A comprehensive meta-analysis highlights that the beneficial impacts of nitrogen on WUE predominantly result from improved physiological processes rather than stomatal dynamics (Brueck, 2008). In our study, physiological adjustments under LN appear to prioritize efficient water management, enabling *A. cruentus* to maintain stable g_s and transpiration rates. Low nitrogen typically decreases photosynthetic enzyme levels, particularly RuBisCO, limiting carbon assimilation. Consequently, C_4 metabolism, as the CO_2-concentrating mechanism, allows *A. cruentus* to maintain internal CO_2 levels in bundle-sheath cells, contributing a stable iWUE.

The interplay between low nitrogen supply and the antioxidant enzyme system resulted in increased activity of key antioxidant enzymes, pivotal for mitigating oxidative stress by scavenging reactive oxygen species (ROS) and maintaining cellular redox homeostasis (Ding et al., 2020). Elevated activity of enzymes such as glutathione reductase (GR), ascorbate peroxidase (APX), and peroxidase (POD) under LN not only highlights the critical role of nitrogen in sustaining redox balance but also suggests mechanisms that support photochemical stability (Hamada et al., 2023). In particular, the enhanced activity of GR under LN contributes to maintaining the redox state of ascorbate and glutathione pools, which are essential for protecting photosystems from oxidative damage. Additionally, there appears to be a feedback mechanism between photochemical efficiency and ROS regulation. Reductions in nitrogen availability impair photochemical efficiency, which may lead to an increase in ROS formation. The elevated antioxidant enzyme activity under LN seems to counterbalance this potential rise in ROS, protecting photosystem II (PSII), particularly at the quinone level. Consequently, these findings suggest that the increase in antioxidant enzyme activity under LN is not only a direct response to redox imbalances but also an integral part of a broader protective mechanism when low nitrogen availability comprises the functioning of photosystems as observed in the photochemical results for *A. cruentus*.

Our metabolomic analysis reveals distinct patterns of metabolite accumulation under varying nitrogen conditions, indicating that nitrogen availability significantly shapes the metabolic responses in *A. cruentus*. Under sufficient nitrogen, metabolites associated with nitrogen storage and cellular protection, such as L-glutamine, ascorbic acid, and aromatic amino acids, were present in elevated levels. Notably, L-glutamine and L-norleucine accumulated abundantly, reflecting their roles in nitrogen assimilation and redistribution, which are essential for maintaining cellular functions and supporting nitrogen storage mechanisms. Under LN conditions, there are metabolic shifts, particularly among nitrogenous compounds, antioxidants, and structural stabilizers, each playing specific roles in cellular stability and stress response. Elevated levels of L-glutamic acid under LN suggest a metabolic adjustment towards efficient nitrogen use, mobilizing scarce nitrogen towards critical processes such as amino acid synthesis, nitrogen transport, and the functioning of citric acid cycle (The, Snyder, & Tegeder, 2021). This shift appears to support sustained metabolic activity under limited nitrogen, with L-glutamic acid also serving as a precursor to glutathione, thereby linking it to the observed increase in GR activity in LN. These nitrogenous metabolites primarily impact the phenylpropanoid and arginine biosynthetic pathways and broader nitrogen metabolism (Figure 4), suggesting that *A. cruentus* activates a coordinated metabolic strategy under LN to optimize nitrogen use. This includes effective ROS detoxification via both enzymatic and non-enzymatic antioxidants, structural reinforcement through membrane-stabilizing lipids, and efficient nitrogen storage and redistribution to meet metabolic demands during nitrogen limitation.

Antioxidant compounds, including rutin, querciturone, and chlorogenic acid, were notably higher under LN. These metabolites likely enhance the oxidative stress defenses, as chlorogenic acid is known to activate antioxidant enzymes such as POD (Rice-Evans, Miller, & Paganga, 1997; Agati et al., 2020). The increase in these flavonoids and phenolic compounds suggests a reinforced non-enzymatic antioxidant response, complementing the enzymatic antioxidant system to provide ROS detoxification under nitrogen deficiency. This dual antioxidant strategy appears vital for maintaining cellular redox balance. Furthermore, the accumulation of L-glutamic acid correlates positively with structural stabilizers, such as lipid compounds like palmitoyl phosphoethanolamine (palmitoyl-PE; Figure 3B). This lipid likely contributes to membrane stabilization under oxidative stress, supporting cell integrity in the face of nitrogen deficiency (Cechin et al., 2022; Colin & Jaillais, 2020). The observed coordination between L-glutamic acid and structural lipids suggests that LN-induced oxidative stress may trigger a reorganization of membrane composition, with glutamic acid playing a key role in fortifying cellular structures through lipid interactions.

Finally, the dynamic regulation of L-glutamine (gln) and L-glutamic acid (glu) levels highlight their central roles in metabolic pathways modulation of *A. cruentus* in response to nitrogen availability. L-glutamine and L-glutamic acid have been widely reported as key for transport and metabolic use in plants, respectively (Watanabe et al., 2013; Lee, Liao, & Hsieh, 2023). Beyond their absolute concentration, the gln/glu ratio can inform about metabolic shifts regarding nitrogen use. Comparing the gln/glu ratio in *A. cruentus*, the control treatment (C) showed a significantly higher value ($p = 0.03$) than in LN (Figure S1). High values of gln/glu indicate that the plant has sufficient nitrogen for storage and transport, whereas a low ratio indicates that the plant is optimizing the use of the available nitrogen to sustain metabolic functions. Given the crucial roles of gln and glu in growth, development, and stress response (Lee, Liao, & Hsieh, 2023), the L-glutamine and L-glutamic acid metabolism and the enzymatic control over their levels would play a crucial physiological role in *A. cruentus* to cope with low nitrogen availability.

5. Conclusions

Our study elucidates the critical role of nitrogen availability in the physiological and metabolic responses of *A. cruentus*. Under nitrogen deficit, photochemistry is significantly more affected than gas exchange, with enhanced antioxidant defenses indicating elevated oxidative stress that compromises photosystem integrity and reduce photochemical yield. Simultaneously, nitrogen scarcity leads to decreased CO_2 assimilation, although partial stomatal closure occurs without impacting transpiration or iWUE.

The differential accumulation of L-glutamine and L-glutamic acid reflects an adaptive strategy for maintaining and using nitrogen. Under nitrogen-rich conditions, the plant stores excess nitrogen as L-glutamine, while in deficiency, it utilizes

L-glutamic acid to optimize the use of the available nitrogen for essential metabolic functions.

Understanding nitrogen dynamics in *A. cruentus* offers valuable insights for managing nitrogen in related C_4 crops. Future research should focus on breeding strategies to enhance nitrogen use efficiency and resilience in C_4 species, aiming to develop cultivars that can withstand nitrogen limitation. This study underscores the importance of precise nitrogen management for enhancing plant resilience and productivity under varying environmental conditions.

Supplementary Materials

The additional data and information can be downloaded at: https://www.sciltp.com/journals/PlantEcophys/2025/1/448/s1.

Author Contributions

E.O.-G. and L.B.-G. designed the research. E.O.-G., V.C., E.Z.-C., and J.O. performed the research. E.O.-G., J.O., L.B., T.C.d.L.P., J.C., and L.B.-G. discussed and interpreted the data. EO-G drafted the first version, and all authors contributed to the final writing, review, and editing of the manuscript.

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Data Availability Statement

Data are available upon request from E.O-G.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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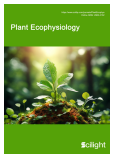

Article

Assessing Nutrient Dynamics in *Vitis vinifera* L. cv. Maturana Blanca: The Role of Training System and Irrigation Strategy

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Abstract: Global climate change presents significant challenges to viticulture, particularly regarding water availability and nutrient management. This study delves into the combined effects of vertical cordon (VC) and gobelet (G) training systems, alongside deficit irrigation (DI) and rainfed (R) regimes, on the physiology, nutrient dynamics, and productivity of *Vitis vinifera* L. cv. Maturana Blanca. The research uncovers that VC training increases vegetative growth and yield through enhanced light exposure and bud load, but careful nutrient management is required to address reduced phosphorus, iron, and zinc levels. DI effectively mitigates water stress, enhances intrinsic and instantaneous water use efficiency, and impacts nutrient uptake, notably increasing calcium and manganese levels while reducing nitrogen. Leaf blade and petiole analyses demonstrated complementary roles in understanding nutrient transport and physiological responses, with petioles reflecting short-term changes and leaf blades capturing long-term trends. The findings underscore the potential of combining VC training and DI to optimize vineyard resilience and productivity under climate stress while maintaining a balanced vegetative and reproductive growth ratio essential for high-quality grape production.

Keywords: grapevine; climate change; vertical cordon; leaf blade; petiole

1. Introduction

The wine sector faces escalating challenges, with climate change exacerbating issues such as increased temperature, altered precipitation patterns, and the pressing need for sustainable practices (Jones et al., 2005). The urgency of adaptation to maintain economically, socially, and environmentally sustainable viticulture is clear (van Leeuwen et al., 2024). Numerous techniques and adaptation strategies have been identified in viticulture (Gutiérrez-Gamboa, Zheng, & Martínez de Toda, 2020, 2021) and oenology (Dequin et al., 2017). It is also worth mentioning that a combination of different adaptation measures can be beneficial (Fraga, 2020). For example, the optimization of training systems and irrigation practices are fundamental, given their profound impact on vine health, yield, and fruit quality (Mirás-Avalos et al., 2017; de Rességuier et al., 2023a).

Researchers have developed numerous training systems to align vine vigor and improve production efficiency more effectively. These systems aim to decrease canopy density, increase its exposure to solar radiation, and enhance sunlight penetration into the interior (Smart & Robinson, 1991). Although little known, the vertical cordon (VC) is a freely directed training system used in some wine regions worldwide (Yuste, 2002). The vertical distribution of this system allows a more significant bud load per vine, resulting in a greater leaf surface area. Additionally, its vertical distribution provides a better canopy microclimate, maintaining a total leaf area similar to the external leaf area (Vanden Heuvel et al., 2004). Given that canopy structure influences water use and evapotranspiration, and considering that water resources for irrigation are expected to become scarcer in the future, understanding how training systems affect crop water needs and water use efficiency becomes essential (Fraga, García de Cortázar Atauri, & Santos, 2018).

Understanding how cultural practices interact with grape varieties and local soil and climatic conditions is crucial to unlocking the oenological potential (Van Leeuwen & Seguin, 2006). Although typical grape varieties are essential, particularly for Protected Designations of Origin (PDO) (Tscholl et al., 2024), the use of minority varieties can provide significant potential for adaptation to climate change (Morales-Castilla et al., 2020; Santos et al., 2020). In the Rioja PDO, the recovered cultivar Maturana Blanca was authorized in 2008 (Martínez De Toda, Balda, & Sancha, 2012) as a result of a project to recover, conserve, and study old genotypes that could represent valuable genetic combinations (Cervera et al., 1998; Martínez De Toda & Sancha, 1997). It is a vigorous and very fertile cultivar but not very productive due to the small size of its clusters. It is also quite rot-susceptible and is sensitive to sun damage (Balda & Martínez de Toda, 2017).

Both irrigation and training systems are practices that aim to enhance both crop production and quality, but they may also impact the plant's nutritional status by affecting nutrient availability, absorption, and distribution (Keller, 2005). Proper nutrient management is essential for maximizing the health and yield of grapevines. Vines need sufficient macro and micro-nutrients to support normal physiological and biochemical functions (Gilda-diana & Maria, 2017). Nutrient deficiencies or excesses can cause physiological disorders that negatively influence vine growth, grape yield, and wine quality. However, researchers have not yet fully established the nutritional requirements for producing high-quality crops. They need large data sets to define desirable nutrient ranges due to the variability in nutrient concentrations across different regions and grape variety-rootstock combinations (García-Escudero et al., 2013). Some researchers have suggested general sufficiency ranges based on adaptable data sets for various grape-growing scenarios (Proffit & Campbell-Clause, 2012). Nevertheless, the precision of these ranges decreases as other sources, such as seasonal weather variations, soil types, and vineyard management practices, introduce additional variability (García-Escudero et al., 2013).

Experts widely recognize plant tissue analysis as the most reliable method for determining grapevine nutritional status, with leaf blade and petiole analysis being the most commonly used practices (Christensen, 1984). Other complementary techniques, such as sap analysis, can help adjust nutrient applications by providing immediate results (Esteves et al., 2021). In European vineyards, growers use leaf blades as the standard tissue for nutrient diagnosis (Gärtel, 1996), while those in the United States and Australia prefer petioles (Robinson, 1992). A combined analysis of both tissues is recommended, as leaf blades offer reliability and petioles provide greater sensitivity (Benito et al., 2013).

This study aimed to evaluate the impact of the vertical cordon training system and deficit irrigation on the physiology, agronomic performance, and nutrient status of *Vitis vinifera* L. cv. Maturana Blanca. Additionally, knowing that researchers have described correlations between nutrient concentrations in plant tissues and plant development for several fruit crops (Mourão Filho, 2004), we sought to identify relationships between nutrient concentrations in leaf blades and petioles with key physiological, vegetative and productive parameters.

2. Materials and Methods

2.1. Location and experimental design

The research was conducted in a commercial vineyard of Maturana Blanca, grafted on R-110 rootstock, located in San Vicente de la Sonsierra, La Rioja, Spain (Latitude: 42°31′25″ N; Longitude: 2°43′23″ W; 466 m.a.s.l.) during the 2023 growing season. The vineyard was planted in 2015 with a row spacing of 2.40 m and a vine spacing of 1.30 m (3205 plants ha^{-1}).

We implemented a 2 × 2 factorial design to investigate the combined effects of two factors. The first factor, training system (TS), involved two free-standing training systems: the traditional gobelet (G), pruned to five spurs with 10 buds, and a vertical cordon (VC) system, with 10 spurs and 20 buds, as shown in Figure 1. The second factor, irrigation treatment (I), compared a rainfed treatment (R), where no additional irrigation was applied during the growing season, with deficit irrigation (DI), which supplied water at 30% of the reference evapotranspiration (ET_0). The ET_0 was calculated using the Penman-Monteith equation (Allen et al., 1998), and irrigation was applied every 10-12 days from July to September. A buffered row was established to ensure clear separation of the irrigated and non-irrigated plots. The R plots only received water from rainfall (a total of 474.2 mm during the season, with 244.4 mm during the growing season), while the DI plots received 76.6 mm of manual irrigation in addition to the rainfall.

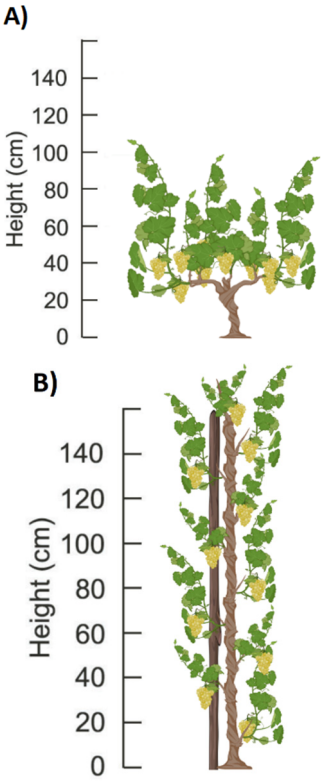

Figure 1. Schematic representation of the two different training systems: (**A**) Gobelet (G), and (**B**) Vertical cordon (VC).

The treatments were organized into the following four combinations: (i) rainfed gobelet (G-R), (ii) deficit irrigation gobelet (G-DI), (iii) rainfed vertical cordon (VC-R), and (iv) deficit irrigation vertical cordon (VC-DI). This study employed a randomized block design with three replicates per treatment, each containing six grapevines, making up a total of 12 replicated plots.

2.2. Environmental conditions

Soil samples were collected from the surface layer (0–30 cm) at sixty points in the study area. The soil samples were homogenized, sieved in the laboratory, and then dried at 40 °C for one week before being sent to the Regional Laboratory of the Government of La Rioja (La Grajera, Logroño, Spain) for analysis. The soil was classified as a Typic Calcixerept (Inceptisols) soil with a sandy loam texture (54% sand, 28% silt, and 18% clay), calcareous ($CaCO_3$ = 12.3%), with 6% of active lime and a pH-H_2O (1:5) of 8.0. The soil had a low organic matter content (1%) and a low salinity (0.14 $dS \cdot m^{-1}$) with a medium cation exchange capacity of 12.65 $cmol(+) \cdot kg^{-1}$. The major limitation of the soil, apart from the low potassium (K) levels (149.5 ppm), was the lack of Magnesium (Mg) (135.0 ppm) and its imbalance with K and Calcium (Ca). The rest of the macro and micronutrient levels were within the normal range, except for phosphorus (P), which was found to be high (41.5 ppm). No type of fertilization was applied during the study.

The climate in the area is Mediterranean continental semiarid, with an average temperature of 12.66 °C over the past 20 years, a reference evapotranspiration (ET_0) of 1108 mm, and annual rainfall of 541 mm, 39% of which occurs during the growing season. During the experimental season (from harvest to harvest), the accumulated rainfall was 474.2 mm, with 244.4 mm falling during the growing season. The seasonal reference evapotranspiration was 1194 mm, and the annual average temperature was 14.4 °C. Climatic data were obtained from the Agroclimatic Information Service of La Rioja (SIAR) near the vineyard.

2.3. Leaf gas exchange measurements and plant water status

Gas exchange and water status measurements were conducted simultaneously on the same days throughout the growing season, specifically on completely clear days between 11:00–12:00 solar hours, when photosynthetic active radiation (PAR) exceeded 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Healthy, fully expanded, and mature leaves exposed to sunlight were selected from the mid-upper region of the primary shoots, positioned both in the middle and outer parts of the canopy. In each replicated plot, measurements were taken from one leaf per plant on two representative vines, totaling six measurements per treatment. Gas exchange measurements were initially performed, followed by water status assessments on the same leaves at three key phenological stages: flowering, veraison, and ripening.

For the leaf gas exchange measurements, a portable infrared gas analyzer (Li-6400, LI-COR, Lincoln, NE, USA) was used to record stomatal conductance (g_s), photosynthesis (A_N), and transpiration rate (E). Intrinsic water use efficiency (WUE_i) was then calculated as the ratio of A_N to g_s, while instantaneous water use efficiency (WUE_{ins}) was calculated as the ratio of A_N to E. All measurements were conducted at ambient air temperature, under natural radiation conditions, and with a CO_2 concentration in the cuvette of 400 $\mu mol \cdot mol^{-1}$.

Vine water status was determined at midday by measuring leaf water potential (Ψ_{leaf}) using a pressure chamber (Soil Moisture Equipment, Corp., Santa Barbara, CA, USA).

2.4. Vegetative growth and yield components

During the veraison period, vegetative parameters, including total leaf area, were assessed on two representative vines per replicated plot, with six vines per treatment. The total leaf area (LA) was estimated using the non-destructive methodology described by (Sanchez-de-Miguel et al., 2010, 2011) and adjusted for the cv. Maturana Blanca (Puelles et al., 2022). Additionally, manual recordings of primary shoot length (PSL), number of primary leaves (PL), number of lateral shoots per primary shoot (LS), and number of leaves on lateral shoots (LL) were also conducted. The average internode length (IL) was calculated by dividing the shoot length by the number of leaves.

At the end of the growth cycle, the pruning weight (PW), number of spurs (NS), and the number of primary shoots (PS) were recorded for each vine, covering all six vines per replicate. The diameter of the second internode (SID) on five shoots from each of the six plants in each replicated plot was measured using an electronic digital caliper (Caliper DIN862, RS PRO, London, UK). For vines trained in the vertical cordon (VC) system, these measurements were extended to include both the top and bottom shoots of the cordon, with five measurements taken on each.

On September 7th, harvest operations were conducted manually, with all six vines per replicate being harvested (18 plants per treatment). During harvest, the number of clusters per vine (CV), the number of clusters per shoot (CS), the weight of each cluster (CW), and the overall yield (Y) (expressed in kg per vine) were meticulously recorded. Additionally, for a detailed assessment of berry weight, a random sample of 500 berries was collected from each replicate. The number of berries per cluster (BC) was calculated by dividing the weight of each cluster by the average berry weight. Moreover, for each of the six vines within a replicate, ratios were calculated for leaf area-to-yield (LA/Y), yield-to-pruning weight (Y/PW), and clusters per shoot, providing valuable insights into the efficiency and productivity of the vineyard management practices.

2.5. Leaf chemical analysis

For the plant mineral analysis, thirty healthy, fully developed leaves (five leaves from each of the six plants per plot) were collected at veraison from each replicated plot and treatment. Leaves were selected from fruiting shoots of medium vigor, positioned opposite to the second cluster (Romero, García-Escudero, & Martín, 2010).

Leaf blades and petioles were separated for independent analysis. They were washed three times with tap water, followed by a risen with distilled water, then dried in an oven (Dry-big, J.P. Selecta, Barcelona, Spain) at 70 °C for 48 h. After drying, the samples were crushed using an ultracentrifugal mill (ZM1, Retsch, Haan, Germany) and sieved with a 0.5 mm mesh.

To assess the total nitrogen content (N-organic + N-NH_4^+), 0.20 g of the ground material was analyzed via dry combustion using a Leco CNS analyzer (St. Joseph, MI, USA), applying the Dumas method (Etheridge, Pesti, & Foster, 1998). For other nutrients—phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), and boron (B)—0.20 g of each sample underwent wet digestion with H_2SO_4 (95%) and H_2O_2 (30%) (Hoenig et al., 1998) and were measured using inductively coupled plasma-optical emission spectrometry (Optima 3000DV, PerkinElmer, Norwalk, CT, USA). All dilutions were prepared with double deionized water (Milli-Q, Millipore, Bedford, MA, USA), and nutrient concentrations were calculated on a dry mass basis.

2.6. Statistical analysis

Data analysis was performed using RStudio software version 4.3.1 (RStudio: Integrated development for R., Boston, MA, USA). Heatmaps were made with R Studio ("ggplot2" package), the others with GraphPad Prism version 8.1.2 (Graph Pad Inc., San Diego, CA, USA). The normality and homoscedasticity were explored using the Shapiro-Wilk test (shapiro-test function) and Levene's test (leveneTest function from "car" package), respectively. A two-way analysis of variance (ANOVA) was performed with a Generalised Linear Model (GLM) (lm function from "lme4" package) to examine statistical differences between training systems and irrigation regimes, as well as the corresponding interaction effects (Tables S1–S5). Physiological parameters were analyzed independently at each phenological stage by two-way ANOVA (lm function) (Table S3). The plot of normalized residuals vs. the fitted values was used to check the model's assumptions. Outliers were initially eliminated before analysis (identify_outliers function from "rstatix" package (Kassambara, 2019)). Any statistical significance was accepted with a p-value < 0.05.

3. Results

3.1. Vine physiology across phenological stages

This study evaluated key physiological parameters, including leaf water potential (Ψ_{leaf}), net photosynthesis (A_N), stomatal conductance (g_s), transpiration rate (E), intrinsic and instantaneous water use efficiency (WUE_i and WUE_{ins}, respectively) across three critical phenological stages: flowering, veraison, and ripening (Table 1). We did not observe statistically significant differences between the treatments at flowering, except for the WUE_i value, which was higher in vines trained in vertical cordon (VC) (Table 1). During the veraison, deficit irrigation (DI) treatments exhibited less water stress, as indicated by a less negative Ψ_{leaf}. Our observations revealed no differences in Ψ_{leaf} between vines trained in gobelet (G) and VC. The application of irrigation significantly increased A_N and g_s, particularly in the G-DI treatment, which displayed the highest values for both variables (19.62 $\mu mol \cdot CO_2 \cdot m^{-2} \cdot s^{-1}$ and 0.295 mol $H_2O \cdot m^{-2} \cdot s^{-1}$, respectively). Likewise, A_N and g_s were significantly lower in vines trained in VC than in G-trained vines. Consequently, WUE_i was statistically lower in the DI treatments, with no differences between training systems. Transpiration rates were also higher in DI treatments, but TS did not affect this parameter. While WUE_{ins} was lower in VC-trained vines than those trained in G, irrigation increased WUE_{ins} in both training systems. A similar trend continued into the ripening phase, accentuating irrigation's cumulative effects and changes in the training system (Table 1). Irrigation treatments consistently yielded more positive Ψ_{leaf} values, with no significant differences between training systems. Similarly, DI led to an increase in A_N and g_s compared to rainfed (R) conditions, although these values were lower than those observed at veraison, reflecting a natural decline as the vines matured. WUE_i values remained lower in DI treatments, but at this stage, G-trained vines had higher values than VC vines. It is worth noting the marked interaction between both factors (TS and I) which informs, for example, that the effect of irrigation on WUE_i is not the same in each training system. At this stage, E values were also higher in the DI treatments, as well as the G-trained vines. Finally, WUE_{ins} followed the same trend observed at veraison, with VC vines and rainfed treatments exhibiting significantly lower values.

3.2. Effects on vegetative parameters and yield components

The analysis of vegetative growth and yield components revealed notable differences between irrigation treatments and training systems (Tables 2 and 3), illustrating how these vineyard strategies impact vine growth and fruit production.

The training system significantly influenced vegetation growth metrics but not irrigation (Table 2). We found the most significant differences between both training systems in the number of shoots per vine (NS) and the number of primary shoots per vine (PS), in which the vines trained in VC practically doubled those G-trained vines. Consequently, the total leaf area (LA) increased by 25% in the VC compared to the G system. However, the rest of the parameters measured were lower in the VC vines. We found that the average length of the primary shoots (PSL) was 29.1% shorter in VC and reduced the internode length (IL). Consequently, we observed a reduction in the thickness of the shoot (measured as the diameter of the second internode (SID)) and the number of leaves per shoot (PL) in VC vines. In addition, the shoots of the VC developed 52.8% fewer secondary shoots (LS) and with a lower number of leaves (LL). Finally, we found no significant differences in the pruning weight (PW) between irrigation regimes or training systems.

Yield and its components varied significantly both with the application of irrigation and with the change of the training system, as can be observed in Table 3. Again, the more significant number of PS per vine in VC caused an increase in yield (Y) of 117.7% compared to the G system. This was mainly due to a 2.5-fold increase in the number of clusters per vine (CV) and a 24.5% increase in the number of clusters per shoot (CS). However, the training system did not significantly influence the parameters cluster weight (CW), berry weight (BW) and the number of berries per cluster (BC). The water regime also had a significant effect on yield components. Specifically, vines under DI exhibited higher Y, CV, CS, and CW values, with increases of 84.1%, 93.0%, 84.7%, and 36.3%, respectively. Although the irrigation factor did not significantly affect BW and BC, slight increases in both parameters under DI caused the aforementioned increase in CW.

Finally, we used two different indices to estimate the vine balance, and both were significantly affected by the two factors studied. On the one hand, the leaf area/yield ratio (LA/Y) significantly reduced its values in the vines trained in VC and the DI treatments, with excessively high values for the G-R treatment (7.35 $m^2 \cdot kg^{-1}$). On the other hand, the yield-to-pruning weight ratio (Y/PW) (known as the Ravaz index) showed the opposite trend, increasing in the VC and with the application of irrigation. Again, the G-R treatment showed values far removed from the rest of the treatments (0.96 $kg \cdot kg^{-1}$).

Table 1. Seasonal values of leaf water potential (Ψ_{leaf}, MPa), net photosynthesis (A_N, µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$), stomatal conductance (g_s, mol $H_2O \cdot m^{-2} \cdot s^{-1}$), intrinsic water use efficiency (WUE_i, µmol $CO_2 \cdot mol^{-1}$ H_2O), transpiration rate (E, mmol $H_2O \cdot m^{-2} \cdot s^{-1}$), and instantaneous water use efficiency (WUE_{ins}, µmol $CO_2 \cdot mmol^{-1}$ H_2O).

Phenology		Ψ_{md}	A_N	g_s	WUE_i	E	WUE_{ins}
Flowering	G-R	−0.99 ± 0.02	12.30 ± 0.76	0.197 ± 0.009	63.41 ± 5.16	4.56 ± 0.12	2.71 ± 0.19
	G-DI	−0.94 ± 0.02	12.69 ± 0.39	0.204 ± 0.011	63.12 ± 3.47	4.72 ± 0.19	2.70 ± 0.11
	VC-R	−0.98 ± 0.03	15.02 ± 0.69	0.201 ± 0.009	74.79 ± 2.31	4.67 ± 0.18	3.22 ± 0.10
	VC-DI	−0.93 ± 0.03	12.20 ± 0.56	0.168 ± 0.012	73.98 ± 5.50	4.24 ± 0.19	2.89 ± 0.15
	TS	n.s.	n.s.	n.s.	*	n.s.	n.s.
	I	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	TS × I	n.s.	*	n.s.	n.s.	n.s.	n.s.
Veraison	G-R	−1.54 ± 0.02	12.18 ± 2.30	0.126 ± 0.015	98.98 ± 4.68	4.00 ± 0.31	3.04 ± 0.07
	G-DI	−1.23 ± 0.02	19.62 ± 0.80	0.295 ± 0.013	66.71 ± 1.68	5.50 ± 0.16	3.57 ± 0.07
	VC-R	−1.58 ± 0.02	10.78 ± 1.01	0.107 ± 0.013	102.37 ± 3.48	3.91 ± 0.36	2.76 ± 0.05
	VC-DI	−1.23 ± 0.03	15.86 ± 0.41	0.216 ± 0.010	73.84 ± 2.13	5.38 ± 0.17	2.95 ± 0.05
	TS	n.s.	**	**	n.s.	n.s.	***
	I	***	***	***	***	***	***
	TS × I	n.s.	n.s.	*	n.s.	n.s.	*
Ripening	G-R	−1.63 ± 0.03	7.23 ± 1.03	0.062 ± 0.009	115.10 ± 3.34	1.83 ± 0.22	3.87 ± 0.15
	G-DI	−1.41 ± 0.04	13.87 ± 0.88	0.171 ± 0.012	81.63 ± 1.67	3.95 ± 0.25	3.52 ± 0.08
	VC-R	−1.63 ± 0.01	2.94 ± 0.82	0.034 ± 0.008	82.19 ± 6.42	1.16 ± 0.24	2.37 ± 0.23
	VC-DI	−1.37 ± 0.02	11.69 ± 0.86	0.136 ± 0.014	88.03 ± 4.61	2.88 ± 0.24	4.07 ± 0.06
	TS	n.s.	**	**	**	**	**
	I	***	***	***	**	***	***
	TS × I	n.s.	n.s.	n.s.	***	n.s.	***

Values are means ± standard error of six measurements per treatment. Significant differences for Training System (TS), Irrigation (I), and its interaction (TS × I) were analyzed with a general linear model (n.s., not significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

Table 2. Mean values of several vegetative growth components of the different treatments. LA (leaf area, $m^2 \cdot vine^{-1}$), PSL (primary shoot length, cm), IL (internode length, cm), PL (primary leaves, $leaves \cdot shoot^{-1}$), LS (lateral shoots per primary shoot, lateral $shoots \cdot shoot^{-1}$), LL (lateral leaves per lateral shoot, $leaves \cdot shoot^{-1}$), PW (pruning weight, $kg \cdot vine^{-1}$), NS (number of spurs per vine, $spurs \cdot vine^{-1}$), PS (primary shoots per vine, $shoots \cdot vine^{-1}$) and SID (second internode diameter, mm).

	LA	PSL	IL	PL	LS	LL	PW	NS	PS	SID
G-R	4.16 ± 0.27	128.28 ± 7.63	4.76 ± 0.21	26.89 ± 0.90	9.72 ± 1.45	27.22 ± 5.30	0.527 ± 0.07	4.93 ± 0.12	9.82 ± 0.10	10.85 ± 0.24
G-DI	4.24 ± 0.33	132.56 ± 7.25	4.67 ± 0.16	28.28 ± 1.03	11.11 ± 1.38	28.56 ± 4.17	0.452 ± 0.03	5.00 ± 0.10	9.69 ± 0.24	10.62 ± 0.26
VC-R	5.23 ± 0.46	91.94 ± 5.06	4.16 ± 0.10	22.06 ± 0.99	4.94 ± 0.86	9.72 ± 2.39	0.495 ± 0.07	9.21 ± 0.33	19.63 ± 0.06	8.97 ± 0.17
VC-DI	5.27 ± 0.19	92.94 ± 6.56	3.94 ± 0.18	23.28 ± 0.84	4.89 ± 0.60	7.89 ± 1.23	0.447 ± 0.02	9.51 ± 0.15	18.83 ± 0.49	9.29 ± 0.16
TS	**	***	***	***	***	***	n.s.	***	***	***
I	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
TS × I	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Values are means ± standard error of six (LA, PSL, IL, PL, LT, and LL) or eighteen (PW, NS, PS, and SID) vines per treatment. Significant differences for Training System (TS), Irrigation (I), and its interaction (TS × I) were analyzed with a general linear model (n.s., not significant; **, $p \leq 0.01$; ***, $p \leq 0.001$).

Table 3. Yield and yield components of the different treatments. Y (yield, kg vine^{-1}), CV (clusters per vine, clusters·vine^{-1}), CS (clusters per shoot, clusters·shoot^{-1}), CW (cluster weight, g), BW (berry weight, g), BC (berries per cluster, berries·cluster^{-1}), LA/Y (leaf area: yield, m^2·kg^{-1}), Y/PW (yield: pruning weight, kg·kg^{-1}).

	Y	CV	CS	CW	BW	BC	LA/Y	Y/PW
G-R	0.57 ± 0.05	8.50 ± 0.50	0.87 ± 0.05	77.00 ± 4.55	1.21 ± 0.05	63.73 ± 2.13	7.35 ± 1.25	0.96 ± 0.04
G-DI	1.47 ± 0.24	14.60 ± 0.61	1.55 ± 0.06	99.95 ± 11.55	1.41 ± 0.04	70.57 ± 6.48	3.04 ± 0.50	3.35 ± 0.77
VC-R	1.78 ± 0.76	19.10 ± 2.50	0.97 ± 0.13	60.70 ± 4.22	1.15 ± 0.16	61.63 ± 9.95	3.78 ± 1.65	3.20 ± 0.78
VC-DI	2.85 ± 0.01	38.67 ± 0.82	1.95 ± 0.04	92.12 ± 2.33	1.26 ± 0.02	73.07 ± 2.51	1.85 ± 0.09	6.40 ± 0.31
TS	**	***	*	n.s.	n.s.	n.s.	*	**
I	*	***	***	**	n.s.	n.s.	**	**
TS × I	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Values are means ± standard error of eighteen (Y, CV, CS, CW, BW, BC, and Y/PW) or six (LA/Y) vines per treatment. Significant differences for Training System (TS), Irrigation (I), and its interaction (TS × I) were analyzed with a general linear model (n.s., not significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

3.3. Vine nutritional status

The mineral composition of leaf blades and petioles varied significantly across training systems and irrigation treatments, highlighting distinct nutrient uptake patterns and translocation, as illustrated in Figure 2. Specifically, the petioles showed more significant variability in response to the two factors studied than the leaf blades. In the leaf blades, the training system had a more pronounced effect than irrigation. Vines trained using the vertical cordon (VC) system exhibited notably lower concentrations of phosphorus (P), potassium (K), iron (Fe), and zinc (Zn), with reductions of 23.7%, 10.1%, 40.4%, and 23.1%, respectively (Figure 2A). DI led to a modest but statistically significant nitrogen (N) content reduction of 3.8%. Conversely, irrigation treatments had a more substantial impact in the petioles (Figure 2B). DI reduced the N content by 12.0% but increased calcium (Ca), P, and manganese (Mn) levels by 10.0%, 41.9%, and 38.9%, respectively. Similar to the leaf blades, the VC-trained vines showed significant reductions in P (18.1%), Fe (42.7%), and Zn (28.9%) concentrations in the petioles.

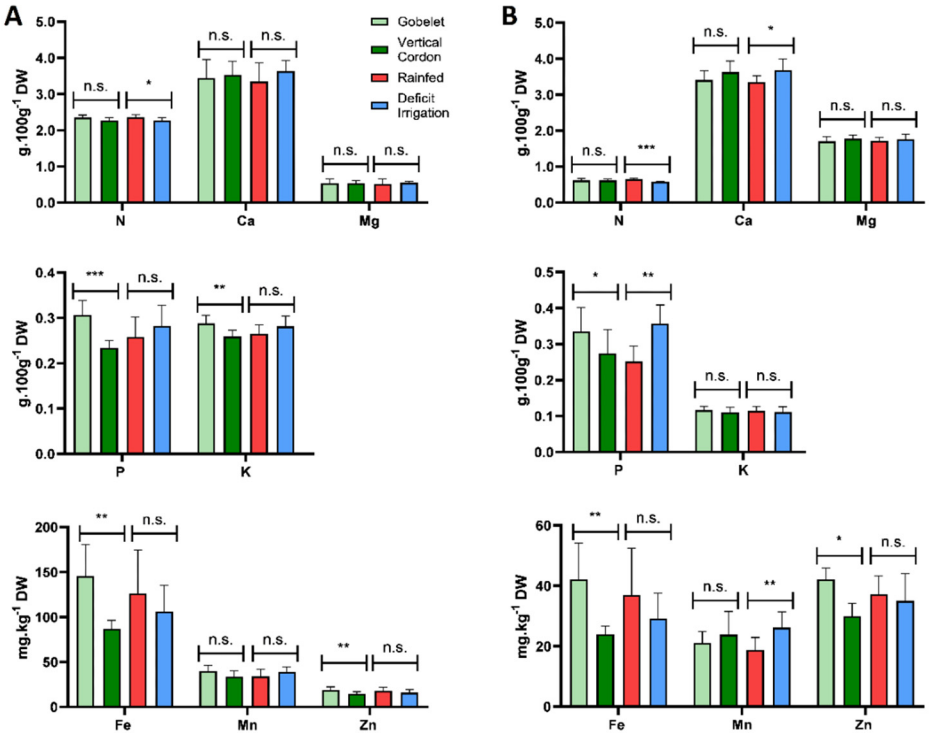

Figure 2. Nutrient concentrations in leaf blades (**A**) and petioles (**B**) at veraison for the factors training system (Gobelet and Vertical Cordon) and irrigation (Rainfed and Deficit Irrigation). Significant differences between training systems and irrigation regimes were analyzed by two-way ANOVA (n.s., not significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

3.4. Relationship between nutritional status, physiological traits and agronomic performance

We could link the observed differences in nutrient concentrations to established correlations with physiological, vegetative, and productive parameters. Notably, the correlations involving nutrient concentrations in leaf blades showed different patterns than those found in petioles (Figure 3). The correlation with vegetative parameters in leaf blades was more substantial, revealing numerous statistically significant associations,

especially for P, K, and Fe (Figure 3A). In addition, several agronomic parameters, including yield and berry weight, showed significant correlations, although to a lesser extent. However, we barely observed statistically significant correlations with physiological parameters.

In contrast, we found that nutrient concentrations in petioles were more strongly related to physiological parameters, especially for N, P, and Zn (Figure 3B), with the strongest correlations observed at veraison when collected lead samples for analysis. Although we also observed some correlations with yield parameters, they were less pronounced. Also noteworthy was the high correlation between the concentrations of Fe and Zn in petioles and vegetative parameters, but not for the other elements analyzed.

The correlations varied depending on the nutrient and the part of the leaf analyzed (Figure 3). We observed higher correlations in petioles for N, especially with physiological parameters measured at veraison. P showed strong correlations in both tissues but with different parameters: it correlated with vegetative development in leaf blades and plant physiology in petioles, particularly at veraison. K was highly correlated with vegetative parameters in leaf blades but showed no significant correlations in petioles. Fe and Zn showed fewer significant correlations, though they were primarily associated with vegetative parameters in both tissues, especially in the case of Zn in the petiole. In contrast, Ca, Mg, and Mn hardly showed any significant correlations, neither in the leaf blade nor the petiole.

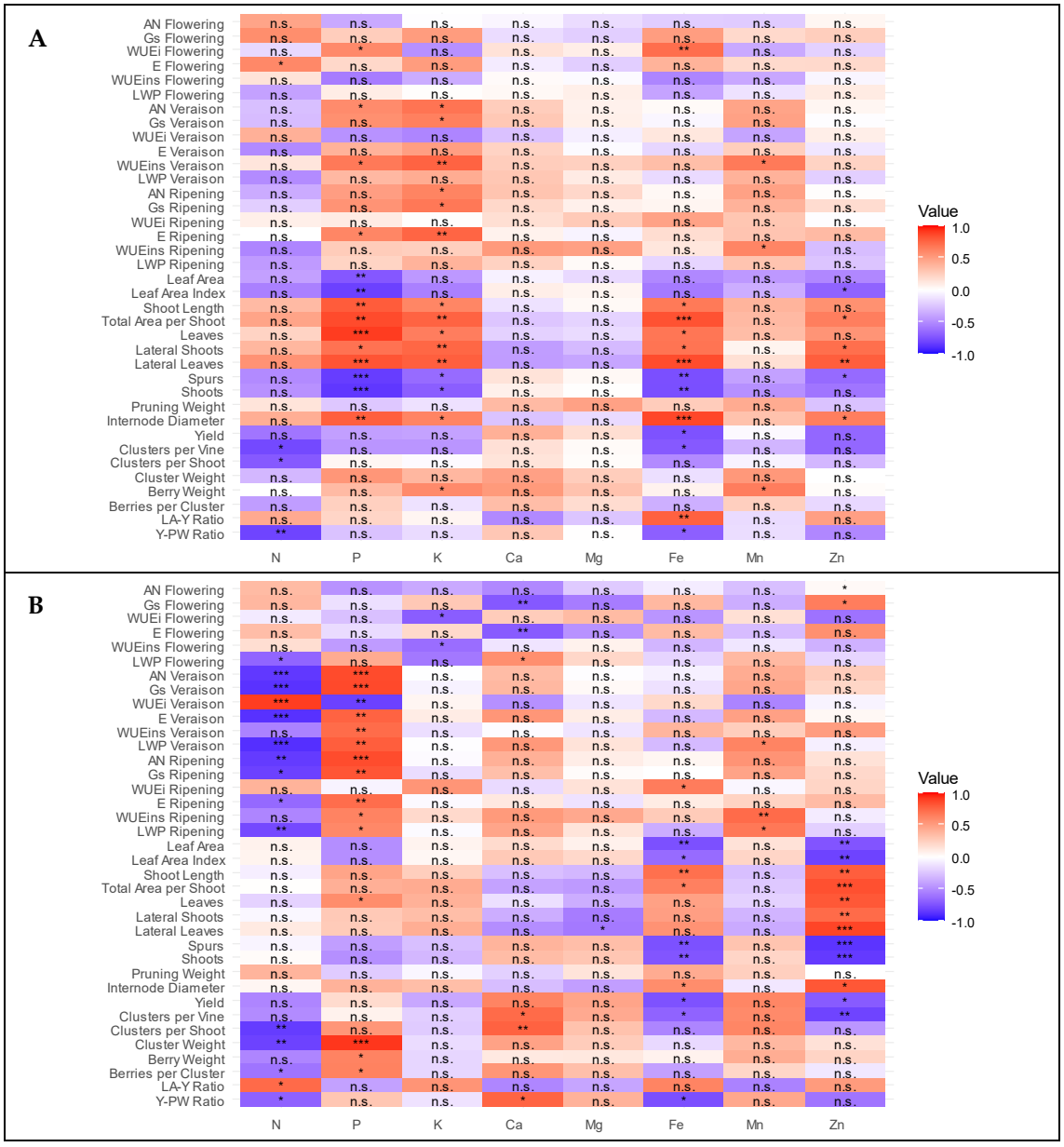

Figure 3. Heatmap of Pearson correlation coefficients between nutrient concentrations in the leaf blade (**A**) and petiole (**B**) with physiological, vegetative, and productive parameters. Figures were produced using R (v4.1.1., https://www.R-project.org (accessed on 15 July 2024)). The color scale shows the values of the Pearson correlation coefficient (positive values in red, negative in blue). The significance of correlations is shown (n.s., not significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$). The abbreviations are as follows: AN, photosynthesis; Gs, stomatal conductance; WUEi, intrinsic water use efficiency; E, transpiration rate; WUEins, instantaneous water use efficiency; LWP, leaf water potential; LA-Y ratio, leaf area-to-yield ratio; Y-PW ratio, yield-to-pruning weight ratio.

4. Discussion

Different training systems represent an effective adaptation strategy to the decreased water availability. Traditional gobelet or bush vines, for instance, are known to mitigate excessive heating of grape clusters, preventing temperatures from rising far above ambient air levels and reducing the risk of sunburn (Gutiérrez-Gamboa, Zheng, & Martínez de Toda, 2020). Additionally, studies have shown that training systems with higher trunks increase minimum temperatures while reducing maximum temperatures in the fruit zone (de Rességuier et al., 2023b). However, there has been little discussion of the differential effects of these two free-standing training systems on water use efficiency and nutrient uptake. It is, therefore, essential to elucidate the effects of these training systems on water use efficiency and nutrient uptake to understand their field requirements better while mitigating the impact of heat waves.

At the flowering stage, the lack of significant differences among treatments suggests uniform environmental conditions and a late application of irrigation, which did not begin until after the fruit set. At this stage, all treatments experienced weak water stress (−0.9 to −1.1 MPa leaf water potential (Ψ_{leaf})) (van Leeuwen et al., 2009). During veraison and ripening, mostly irrigation played a key role in modulating plant responses to stress (Keller, 2005). While rainfed (R) vines showed severe stress (<−1.4 MPa Ψ_{leaf}), deficit irrigated (DI) vines showed moderate water deficit (−1.1 to −1.4 MPa Ψ_{leaf}) (van Leeuwen et al., 2009). These data demonstrate the effectiveness of DI in managing water use without severely stressing the vines (Chaves et al., 2007). Our observation of no differences in Ψ_{leaf} between vines trained using gobelet (G) and vertical cordon (VC) systems suggests that irrigation management may have overshadowed the influence of the training system on water status, which aligns with the findings from some authors (Valentini et al., 2022). However, other studies found higher water stress in those training systems that showed higher vegetative development (Mirás-Avalos et al., 2017; Puelles et al., 2022).

Net photosynthesis (A_N) and stomatal conductance (g_s) were higher in DI-treated vines, demonstrating that appropriate water management can significantly boost physiological activities, critical for fruit development and ripening (Pérez-Álvarez et al., 2021). However, g_s was only partly consistent with Ψ_{leaf}, as additional variables such as light, ambient CO_2, humidity, temperature, and wind influence stomatal closure (Kramer & Boyer, 1995). Nonetheless, g_s could be considered a better indicator of the intensity of water stress (Flexas et al., 2002; Mairata et al., 2024). In general, the g_s analysis indicated the absence of water stress ($g_s > 0.15$ mol $H_2O \cdot m^{-2} \cdot s^{-1}$), except in the rainfed treatments, where we detected moderate water stress at veraison ($0.05 < g_s < 0.15$ mol $H_2O \cdot m^{-2} \cdot s^{-1}$) and severe water stress at ripening ($g_s > 0.05$ mol $H_2O \cdot m^{-2} \cdot s^{-1}$) (Medrano et al., 2002). A_N and g_s were significantly lower in vines trained using the VC system than those trained in the G system. This observation initially suggests a lower physiological performance in VC-trained vines. However, we must carefully consider this interpretation in light of additional agronomic factors, particularly the leaf area. The VC training system potentially increases leaf area by up to 25% compared to the G system (Table 2) and greater sunlight interception (Vanden Heuvel et al., 2004). These increases can significantly affect the interpretation of physiological measurements such as A_N and g_s, which are usually expressed per unit area (μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$ and mol $H_2O \cdot m^{-2} \cdot s^{-1}$, respectively). Despite the inherent complexity in scaling physiological processes from the leaf to the canopy level, Escalona *et al*. (2016) concluded that the outer leaves in the eastern part of the canopy measured at midday were the most representative for estimating the whole-plant gas balance. Therefore, despite reduced g_s and A_N values, VC-trained vines showed increased leaf area (Table 2), which may compensate for lower per-unit-area physiological activity (J. M. Escalona et al., 2016). Transpiration rates (E) followed similar trends as A_N and g_s, with DI and G-trained vines showing higher values, particularly later in the cycle. Adjusting E values to account for increased leaf area in VC-trained vines suggests that overall plant transpiration may be comparable across training systems (L. Escalona, Flexas, & Medrano, 2000; Smart & Coombe, 1983).

Despite increased A_N and g_s, decreased intrinsic water use efficiency (WUE_i) in DI treatments highlights a fundamental trade-off in grapevine physiology. It is well known that WUE_i increases under water stress conditions, mainly due to stomatal behaviour (Schultz & Stoll, 2015). Stomatal closure is pivotal in modifying water use efficiency when vines experience water deficits. During drought, vines regulate stomata opening as a conservation strategy to optimize water usage. This regulatory process is governed by either hydraulic signals or hormonal responses, ensuring that water resources are preserved under stress conditions (Düring, 1988). Furthermore, as the season progressed, G-trained vines showed greater WUE_i, especially under rainfed conditions. The observed interactions between both factors (TS and I) show that each affects the other differently, suggesting, for example, that irrigation can optimize water use efficiency only in VC-trained vines. Although several authors use WUE_i as an indicator of water use efficiency (de Souza et al., 2005), instantaneous water use efficiency (WUE_{ins}) provides a more accurate measure of the carbon and water balance (Pou et al., 2012). Both parameters can frequently go in opposite directions (Schultz & Stoll, 2015). These same authors established a clear relationship between WUE_{ins} and leaf-to-air vapour pressure deficit (LAVPD), showing that WUE_{ins} often declines under water stress due to increased LAVPD, which intensifies stomatal limitations and reduces efficiency (Schultz & Stoll, 2015). Depending on the intensity of stomatal closure in response to water stress and how much LAVPD increases due to increased leaf temperature, these factors may reduce WUE_{inst}.

This study's WUE_{ins} values differed significantly between training systems, particularly under rainfed conditions. At veraison and ripening, vines trained in the G system consistently exhibited higher WUE_{ins} values than those trained in the VC system (Table 1). For example, at ripening,

G-R showed a WUE_{ins} of 3.87 μmol CO_2 ·$mmol^{-1}$ H_2O, significantly higher than VC-R, which had the lowest value at 2.37 μmol CO_2·$mmol^{-1}$ H_2O. These data suggest that the gobelet system, with its compact canopy structure and lower vegetative growth (Table 2), may promote better instantaneous water use efficiency under water-limited conditions by reducing LAVPD and maintaining more favorable microclimatic conditions. Conversely, the VC system, characterized by a larger canopy and increased light penetration, might exacerbate LAVPD due to higher exposure of the leaf area to sunlight, potentially explaining the reduced WUE_{ins} values observed under rainfed conditions. However, under DI, the differences in WUE_{ins} between training systems were less pronounced, possibly indicating that water availability offsets the effects of increased evaporative demand in VC-trained vines. The data from this study are in agreement with other studies on grapevines (Koundouras et al., 2008; Naor & Bravdo, 2000; Schultz & Stoll, 2015), further highlighting the complex interactions between physiological responses, environmental factors, and training systems in determining WUE_{ins}.

Only the training system influenced vegetative growth, and we found no interactions between the training system and irrigation treatments. This finding suggests that the crop load did not affect the growth of the vines under deficit irrigation (Keller, Smithyman, & Mills, 2008). As expected, a more significant crop load resulted in a significantly larger leaf area (Keller et al., 2004). However, although VC vines produced twice as many shoots, their leaf area increased by only 25%. This fact was due to the compensatory effect of more shoots with shorter shoots, close nodes, and fewer leaves (both primary and secondary) (Clingeleffer, 1989; Miller, Howell, & Flore, 1996). Furthermore, the lower number of secondary shoots on VC vines (Table 2), combined with their vertical canopy distribution, made the total leaf area on VC very similar to the total exposed area (Vanden Heuvel et al., 2004). The compensatory effect of fewer shoots per vine in G vines, but with longer and thicker shoots and more lateral shoots, did not result in significant differences in pruning weight between treatments (Martinez De Toda, 2011).

Yield components varied significantly with the training system and the irrigation treatment, confirming that both factors are crucial in determining productivity. On the one hand, the higher yield in the VC was mainly due to a greater number of shoots and, therefore, a more significant number of clusters per vine, which is generally the primary determinant of production (Guilpart, Metay, & Gary, 2014). However, despite doubling the number of buds left per vine in the VC system, the number of clusters per vine increased by 2.5 times. This disparity resulted from a higher number of clusters per shoot, which was not a consequence of greater fertility but of more significant cluster desiccation in the G-trained vines. Shortly before the harvest, a heat wave with temperatures above 42 °C in late August caused sunburn necrosis, leading to shriveling of entire clusters. The damage was more severe in the G than in the VC, with 47.5% of bunches affected, compared to 34.7% in the VC system. The greater exposure of clusters in the VC from the early development stages (Vanden Heuvel et al., 2004) could favour an accumulation of photoprotectants, as observed in several studies with early defoliations (Brandt et al., 2019; J. Gambetta, Holzapfel, & Schmidtke, 2019; Verdenal et al., 2019).

On the other hand, the application of irrigation produced a greater yield per vine through an increase in most of the components analyzed. The heat wave influenced the notable differences in clusters per vine and clusters per shoot. Under water stress conditions, reduced canopy transpiration can cause an increase in fruit zone temperature, increasing the risk of sunburn (Tarara & Spayd, 2005). The higher cluster weight in DI treatments could be due to a lower incidence of berry desiccation (J. M. Gambetta et al., 2021), added to the upward trend in both berry weight and berries per cluster.

Achieving the right balance between vegetative and reproductive growth is one of the most important management issues in quality viticulture (Dry & Loveys, 1998). Our study showed significant differences in the indices used to estimate this balance. The leaf area-to-yield ratio (LA/Y), which ideally ranges between 1.0 and 1.5 $m^2 \cdot kg^{-1}$ (Keller, 2020), was closest to this range in the VC-DI treatment (1.85 $m^2 \cdot kg^{-1}$). The high vigor of cv. Maturana Blanca added to the low yield due to cluster desiccation, also characteristic of the cultivar (Balda & Martínez de Toda, 2017), which led to excessively high values in LA/Y. The VC system reduced this index, increasing yield potential and optimizing the leaf area/fruit ratio. Irrigation also caused a notable decrease, mainly due to production changes. Both factors reduced this ratio, suggesting they could result in better-balanced vines. The other index studied (Ravaz index) also showed values outside those considered optimal (from 5 to 10, Bravdo *et al.*, 1985), although for small-clustered varieties such as Maturana Blanca, the optimal values appear to be between 3 and 6 (Kliewer & Dokoozlian, 2005). The values indicated high vine vigor, especially for the G-R treatment, except for the VC-DI treatment. Again, both the VC and DI systems improved the values of this index.

The nutrient values measured in both leaf blade and petioles at veraison were broadly consistent with previously reported data for different cultivars and growing areas (Benito et al., 2013; Christensen, 1984; García-Escudero et al., 2013; Proffit & Campbell-Clause, 2012). However, manganese (Mn) and potassium (K) were notably lower than typically observed (Figure 2A). Soil alkalinity may have caused the Mn deficiency by affecting the bioavailability of certain nutrients, particularly Mn and iron (Fe) (Longbottom, 2009). Regarding K, several factors might contribute to its deficiency. Firstly, the soil's inherently low K concentrations (less than 150 ppm) can directly limit the amount of K available for vine uptake. Additionally, potential antagonistic interactions with calcium (Ca) and Mg can inhibit K absorption (Stockdale et al., 2013), as supported by a strong and negative correlation between Mg and K in petioles ($r = -0.731$, $p = 0.007$, $N = 12$). Finally, increasing water stress could prompt K early mobilization into the berries, further depleting the available K levels in grapevine foliage (Mpelasoka et al., 2003).

In this study, petioles exhibited more significant nutrient variability compared to the leaf blades, corroborating findings from other research (Fráguas, Miele, & Silva, 2003; Romero, García-Escudero, & Martín, 2013; Wolpert & Anderson, 2007). This sensitivity to environmental changes, such as irrigation, makes petioles reliable indicators for short-term nutrient status alterations (Davis, 1995). For instance, irrigation treatments notably influenced petiole composition (Figure 2B), likely due to its effects on hydraulic conductivity and vessel morphology (Dayer et al., 2017). Conversely, leaf blades maintain more stable nutrient concentrations, particularly for nitrogen (N) and K (Benito et al., 2013; Romero, García-Escudero, & Martín, 2013). This stability renders leaf blades more suitable for assessing the long-term effects of vineyard training systems on nutrient status over time. However, the lack of correlation between physiological parameters and nutrient content at phenological stages other than veraison suggests dynamic shifts in nutrient distribution and physiological responses throughout the season. At flowering, the virtual absence of significant correlations could be because different treatments did not yet clearly affect nutritional content and the physiological variables analyzed. During ripening, nutrient redistribution, environmental influences or mobilization of reserves could have contributed to the lower number of significant correlations observed. To further validate these findings, future research should incorporate multi-stage assessments to better capture nutrient dynamics across different growth stages.

The observed disparities in the nutrient content between leaf blades and petioles under different training systems and irrigation treatments reflect complex physiological mechanisms of nutrient uptake, translocation, and storage within the grapevine. The vertical cordon (VC) training system, which enhances canopy light penetration by reducing leaf layers, significantly impacts nutrient distribution (Vanden Heuvel et al., 2004). While this system promotes greater vegetative and reproductive development, it may also cause nutrient dilution or shifts in allocation favoring reproductive over vegetative growth (Mcgrath & Lobell, 2013; Puelles et al., 2022). The observed decreases in key minerals such as phosphorus (P), K, iron (Fe), and zinc (Zn) in VC-trained vines might reflect these redistribution strategies. Data presented in Tables 2 and 3 support these findings, highlighting the significant impact of training system modifications on vine growth and production. Conversely, the effect of deficit irrigation (DI) on petiole mineral composition underscores the role of water availability in nutrient transport (Plett et al., 2020). The increase in nutrients such as calcium (Ca), P, and manganese (Mn) in petioles under DI could be attributed to their enhanced solubility and absorption, facilitated by changes in soil moisture dynamics and root activity (Ippolito et al., 2019; Liu et al., 2017).

Contrary to previous studies, which reported a decrease in N content with reduced water availability (Spangenberg, Schweizer, & Zufferey, 2020; Torres et al., 2021), our results showed an opposite trend. In our study, N levels in leaf blades and petioles increased under rainfed conditions. This unexpected pattern may be explained by significant changes in source-sink relationships (Table 3), suggesting a possible relocation of N to the fruits. Further research is needed to determine whether this trend is consistent across different vineyard conditions and growing seasons. Finally, the numerous correlations observed between nutrient concentrations in leaf blades and petioles with physiological, vegetative, and productive parameters emphasize the complexity of plants as integrated systems, where multiple components interact with each other and with the environment (Tomkins, 2023). Correlation patterns between leaf blade and petiole analyses highlight their complementary roles in monitoring vine nutritional health (Benito et al., 2013). The stronger correlations between nutrient concentrations in leaf blades and vegetative parameters suggest that leaf blades may serve as reliable indicators of the overall nutrient status and vegetative growth (Christensen, 1984; Schreiner & Scagel, 2017). In contrast, petiole nutrient concentrations exhibited stronger associations with physiological parameters. These associations align with the understanding that petiole nutrient content reflects the plant's dynamic nutrient transport and real-time physiological processes (Shen et al., 2019).

Despite the known variability of N in petioles (Christensen, 1984), we found significant correlations with physiological parameters measured at veraison and ripening. The differential correlation patterns for P, which showed strong associations in both leaf blades and petioles but with different parameters, underline the dual role of P in supporting both growth and metabolic processes (Azeem et al., 2015; Duff, Sarath, & Plaxton, 1994). K is fundamental in stomatal function and water regulation (Zörb, Senbayram, & Peiter, 2014). However, we hardly correlated it with plant water status (Ψ_{leaf} or g_s), possibly due to its remarkably low content in both tissues analyzed. The correlations for micronutrients such as Fe and Zn, primarily with vegetative parameters across leaf blades and petioles, highlight their crucial roles in maintaining chlorophyll content and ensuring efficient photosynthetic activity (Keller, 2020). The pronounced association between Zn in petioles and vegetative growth could be particularly relevant for diagnosing micronutrient deficiencies that affect vine growth before visible symptoms appear. The minimal significant correlations observed for Ca, Mg, and Mn suggest that, despite their essential functions in plants (Rengel, Cakmak, & White, 2023), these elements may not vary distinctly enough with the measured parameters to serve as effective indicators of physiological, vegetative or productive status under the conditions studied.

5. Conclusions

Practices such as vertical cordon training and deficit irrigation have proven to be effective strategies for adapting vineyard management to the challenges posed by climate change (Gutiérrez-Gamboa, Zheng, & Martínez de Toda, 2020). These methods affect nutrient uptake and distribution, significantly impacting vine physiology, vegetative growth, and overall productivity. This study demonstrated that while the training

system significantly influenced vegetative development and yield components, irrigation was key in managing grapevine physiological responses to environmental stress, particularly regarding water availability. The results suggest that integrating adapted training and irrigation strategies can significantly improve vineyard productivity and resilience to climatic variabilities. Furthermore, research on gas exchange responses within grapevine canopy illustrates how grapevines can adapt their physiological processes based on the timing and extent of regulated deficit irrigation. This adaptability is critical for understanding how deficit irrigation practices can optimize water use efficiency without compromising growth and productivity, especially in systems like VC, where increased exposure to light and air might otherwise increase water demand.

Variations in nutrient concentrations found in leaf blades and petioles suggest that the training system and irrigation regime differentially affect nutrient uptake and transport processes, necessitating appropriate management to avoid nutrient deficiencies and imbalances. For example, lower levels of key minerals (P, K, Fe) in CV-trained vineyards may require specific nutrient management practices to counteract potential deficiencies, especially in vineyards aiming for high-quality fruit production under conditions of reduced water scarcity.

Therefore, choosing between petioles or leaf blades for nutrient diagnosis hinges on the specific nutrients, the assessment's intended precision, and the vine's phenological stage. Leaf blade analysis provides a better indication of overall nutritional status and is more closely correlated to vegetative growth, while petiole analysis offers insights into real-time physiological processes, making it valuable for assessing immediate plant responses to environmental conditions. However, it is important to note that we only analyzed nutrient concentrations at veraison. Therefore, we should interpret these conclusions cautiously, as nutrient dynamics may vary at different phenological stages. Future studies should validate these findings by integrating multi-stage nutrient assessments throughout the vine cycle to develop more precise nutrient management strategies.

Supplementary Materials

The additional data and information can be downloaded at: https://www.sciltp.com/journals/PlantEcophys/2025/1/512/s1.

Author Contributions

M.P.: formal analysis, investigation, methodology, resources, writing—original draft; P.B.: conceptualization, resources; I.M.: investigation, methodology, resources; D.L.: investigation, methodology; A.M.: investigation, methodology; F.M.: supervision; A.P: funding acquisition, investigation, methodology, project administration, writing-review & editing. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The data is in the process of being uploaded to the National Digital Repository (CSIC) (https://digital.csic.es/). In the meantime, it is available upon request.

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Conflicts of Interest

The authors declare no competing interests.

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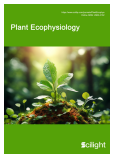

Review

Cold Air Pools (CAPs) as Natural Freezers for the Study of Plant Responses to Low Temperatures

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Abstract: The stratification of cold air is a phenomenon that typically occurs under certain topographic (closed ground depressions) and atmospheric conditions (stability and nocturnal radiative cooling). Under such conditions the drainage of the heavier cold air from the higher elevations causes its accumulation for days or weeks in the bottom of these depressions, leading temperatures to dramatically decrease and to decouple from regional climatic conditions. These particular locations which are frequent in karstic, volcanic and glacial landscapes, have been proposed to act as microrefugia of biodiversity in the context of climate warming. The existence of these cold air pools (CAPs) has been reported worldwide, and their biotic communities differ from equivalent sites out of these locations. However, there is an almost complete absence of ecophysiological studies concerning plant communities inhabiting CAPs. Thus, one of the objectives of this review is to hypothesize the effects of these specific conditions on the biology of the soil and the manner in which these plants should respond to such particular environmental conditions. Furthermore, given that temperature can decrease dramatically over short distances inside CAPs, in the present review we also propose their use as natural freezers for the study of plant responses to low temperatures.

Keywords: Cold Air Pool (CAP); microrefugia; low temperature; plant ecophysiology; freezing-tolerance

1. Introduction

Primary production in terrestrial ecosystems is essentially constrained by the availability of water (liquid or vapor) (Grossiord et al., 2020) and by air temperatures (Whittaker, 1975). In fact, the active metabolism of plants is only possible in a comparatively low range of leaf temperatures from around 70 °C in sun exposed CAM desert plants to a few degrees below the freezing point in cold acclimated species. In contrast, the range of temperatures that allow plant survival is considerably wider than for metabolic activity, ranging from the boiling point in orthodox seeds to the liquid nitrogen (−196 °C) in desiccated vegetative tissues (Larcher, 2003). However, despite this wide range of survival temperatures, plants need to encounter periodically thermal conditions favorable to metabolic activity to achieve a positive carbon balance, otherwise plant life is not possible. This is for example the case of the highest world elevations in the Himalayas or the Antarctic Plateau, that are completely devoid of plant life. Consequently, latitude and elevation are considered to be the main factors determining the thermal boundaries of plant life. Although this general picture is correct at a coarse scale, it ignores the influence of local topographic factors on the fine scale of temperature distribution. This is notably the case of the well characterized orientation effect (Körner, 2021), where microclimatic conditions of sun-exposed *slope*s are uncoupled from those of the regional climate. Another example of a microtopographic effect is the occurrence of cold air pools (CAPs) (Pastore et al., 2022), the subject of present review, where the denser cold air accumulates at the bottom of topographic depressions.

2. Where, When and Why Do CAPs Occur

Air density decreases with temperature, and as a consequence, cold air tends to descend. Thus, during clear nights,

ground radiative cooling causes cold air to accumulate wherever air drainage is topographically restricted and atmospheric conditions are stable enough to prevent air mixing (McCaffrey et al., 2019). When these two conditions are met, air temperature decreases to values much lower than those of the surroundings, leading to the formation of cold air pools through a process that is particularly noticeable when air stability and clear skies last for several consecutive days. This causes a thermal inversion, with colder temperatures at lower elevations. As a consequence, CAPs typically occur at the bottom of fluvial valleys or basins (Vosper et al., 2014). Cold air pooling typically occurs during clear and stable nights, disappearing when sunlight favors air mixing, but it can also be maintained during longer periods, particularly in winter or high latitudes (McCaffrey et al., 2019).

This phenomenon has been theoretically modeled using predictive algorithms (Chung et al., 2006; Lundquist, Pepin, & Rochford, 2008; Daly et al., 2007) and confirmed by numerous theoretical and observational studies (John et al., 2024; Iijima & Shinoda, 2002). CAPs have been reported in a diversity of environments and geographical locations such as the sub-escarpment lowlands of South Africa (Duker et al., 2020), Sierra Nevada in California (Curtis et al., 2014), the mountains of central Japan (Iijima & Shinoda, 2002), Southwest Australia (Matusick et al., 2014) or the mountains of central Europe (Frei et al., 2023). In addition to the above-mentioned factors, cold air pooling and thermal decoupling from the regional climate is enhanced in those sites with a large collecting basin and in those forming close topographic depressions, ranging in depth from a few to hundreds of meters (Pastore et al., 2022). Examples of such topographic sites prone to the formation of CAPs are karstic depressions (dolinas) (Frei et al., 2023), glacio-karstic closed depressions (Giovagnoli & Tasinazzo, 2014), glacial cirques closed by frontal moraines, salt diapirs or volcanic craters and calderas. Figure 1 illustrates four distinct examples of topographic locations in Spain that are susceptible to the formation of CAPs. Thus, CAPs can be considered as a global widespread phenomenon that is observed wherever topography favors it (Pastore et al., 2022). Furthermore, in these particular locations, typically sheltered from the wind, the phenomenon of cold air stratification is further amplified by the reduction of air mixing, finally resulting in surprisingly large thermal gradients in very short distances. Apart from their intrinsic scientific and environmental interest, the specific features of these small topographic locations, particularly their steep thermal gradients, make them natural freezers for the study of plant responses to low temperatures, as will be discussed in Section 7.

Figure 1. Examples of CAPs: (**A**) a glacial cirque with a frontal moraine, notice the long-lasting snow accumulation (Corral de Veleta, Sierra Nevada, 37°03′, −3°22′); (**B**) a volcanic crater (Pico Viejo, Canary Islands, 28°15′, −16°40′); (**C**) a glacio-karstic depression (Hoyo Sin Tierra, Picos de Europa, 43°10′, −4°50′) and (**D**) a karstic dolina (Lubierri, Sierra de Urbasa, 42°51′, −2°05′).

3. Environmental Conditions inside CAPs

The direct effect of radiative cooling and subsequent atmospheric stratification is a significant decrease of air temperature, particularly during nighttime and close to ground. This generates relevant differences between the conditions inside and outside CAPs which are especially relevant for living organisms, which depend on these microclimatic conditions and not on temperatures prevailing in the free atmosphere (Lembrechts et al., 2020). Figure 2 illustrates the atmospheric characteristics occurring on CAPs, and their main impacts on vegetation. Many observational studies have described

temperature gradients between the bottom and upper part of CAPs ranging between 2 and 20 °C (Pastore et al., 2022). For example, microclimatic studies in karstic areas of Hungary show that mean temperatures are between 1.5 °C and 2.4 °C lower at the bottom of CAPs compared to the surrounding plateaus (Frei et al., 2023), while in the mountains of Central Japan the maximum temperature difference measured in the bottom of a hollow was 12.5 °C with respect to the summit (Iijima & Shinoda, 2002). Another biologically significant factor is the occurrence of extreme minimum temperatures that may fall below the survival limits for many organisms, constraining the presence of freezing-sensitive species. In fact, the regional absolute minimum temperatures are sometimes measured in CAPs, as is the case of Vega Liordes in the Cantabrian Mountains where the coldest temperature ever in Spain (−35.8 °C) was recorded in 2021 (Iglesisas, 2021). These conditions of thermal stress can be exacerbated by much larger diurnal and seasonal temperature fluctuations (John et al., 2024). Another physical consequence of the lower temperatures inside CAPs is the higher air relative humidity (on average 15%) (Frei et al., 2023). The primary factor influencing the disparity in air temperature and relative humidity within dolines is the sun's trajectory throughout the day, combined with the bowl- or funnel-like structure of these depressions. For instance, eastward slopes capture more sunlight in the morning, whereas westward slopes absorb greater sunlight during the afternoon and evening (Bátori et al., 2023).

In this review, the focus has been on natural areas where the phenomenon of CAPs occurs. However, it is important to note a particular phenomenon studied in urban areas that can also affect natural areas, even if to a lesser extent. The accumulation of pollutants generated in urban areas has been observed to coincide with the occurrence of CAPs phenomena, due to the strong atmospheric stability. This phenomenon has been studied in different valleys or cities, such as Salt Lake (Utah) using air quality simulations (Sun et al., 2021), Coimbra, using field data loggers (Cordeiro, Orenlas, & Silva, 2023) or Wasatch Mountains (Utah) were they study the ion concentrations in different snowpacks (Hall et al., 2014).

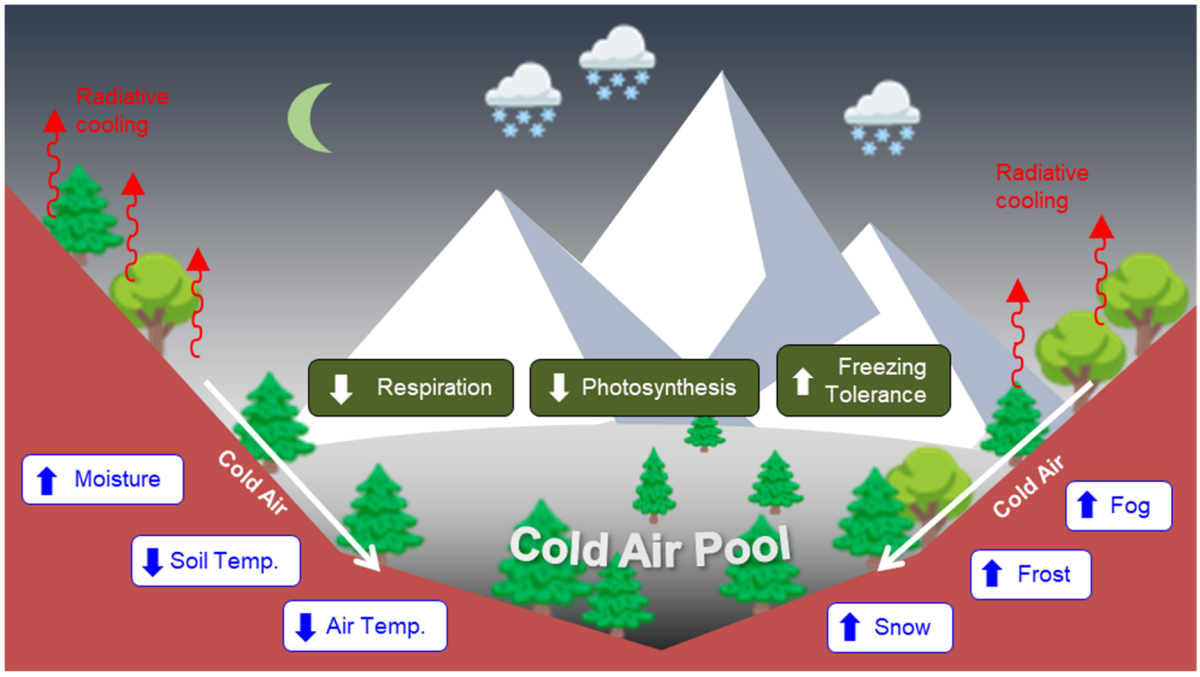

Figure 2. Schematical representation of main environmental conditions in CAPs (blue boxes) and their effects over main physiological responses of plant (green boxes).

4. Effects on the Biology of Soils

4.1. General effects of CAPs on soil properties

As a result of the air temperature decrease in CAPs, soil temperatures reach lower temperatures than those prevailing in the surrounding areas, which has a determinant effect on the biology of the soil (Novick, Oishi, & Miniat, 2016; Soler et al., 2002). One of the principal characteristics of CAPs is the potential for an extended duration of the snowpack (see Figure 1A). This phenomenon can be attributed to two key factors: the lower soil temperatures and the influence of microgeomorphic features of the ground surface and wind action on snowpack distribution, which removes snow from small, exposed ridges to accumulate in shallow depressions (Giovagnoli & Tasinazzo, 2014). Several studies have demonstrated this empirically, for example Curtis et al. (2014), at Sierra Nevada (California), using a water-balance approach for modeling snowpack duration. As a porous medium with high air content snow has high insulation properties. Its thermal insulation capacity mitigates ground temperature fluctuations and, in winter, keeps soil temperatures relatively high as compared to the surroundings above the snow (Vuosku et al., 2022). Therefore, a snow cover protects underlying vegetation and soil against very low temperatures, creating conditions that are favorable to plant survival and plant metabolism (Körner, 2021). However, under snow, plants experience other stresses such as light deprivation (Robson et

al., 2019) and prolonged snow cover, which could weaken plants by impeding metabolic processes and reducing the length of the growing season (Larcher, 2003; Sakai & Larcher, 1987).

Apart from temperature and light, gas diffusion represents another significant factor, which is also conditioned by the snow cover. The presence of snow (especially when ice layers are formed), impedes the flow of gases, such us; soil-atmosphere gas exchange, plant tissues and soil microorganisms gas exchange (Martz et al., 2016). In conditions of low light intensity, the rate of respiration in plants will exceed the rate of net CO_2 assimilation. While oxygen will be consumed by heterotrophic respiration, CO_2 will be released, which can lead to periodic anoxia beneath the snow cover (Körner, 2021; Martz et al., 2016; Edwards, Scalenghe, & Freppaz, 2007). These conditions can be detrimental for plant life by reducing the efficiency of cellular ATP production and lead to the accumulation of toxic metabolites as a result of anaerobic metabolism (Vuosku et al., 2022). Nevertheless, it is important to highlight that snow presence depends on CAPs location and latitude and may not always play a significant role in the biology of the soil. In fact, soils in snow-free CAPs can experience lower temperatures and be subjected to more extreme freeze-thaw cycles compared to those outside CAPs during the winter season.

Water and moisture accumulate in CAP soils because of two main reasons. First, cold air pooling itself can affect dew formation and vapor pressure deficit and thus, indirectly affect soil moisture through effects on plant ecophysiological processes (Pastore et al., 2024). Additionally, cold air pooling events may contribute to the retention of soil moisture due to the lower vapor pressure deficit and reduced wind speed that accompany these events (Frei et al., 2023). Second, due to the concave topography and location (low-lying regions), CAPs can retain more water upon precipitation events, potentially enhancing water availability in soil. Besides, these low- laying areas are frequently covered by deep soils with high water retention capacity (Frei et al., 2023; Bátori et al., 2017). Apart from moisture, higher nutrient and organic matter concentration can be found in these locations. For example, it is known, that soils in CAPs of karst landscapes have high nitrogen and phosphate concentration (Frei et al., 2023).

Cold, wet (or waterlogged) and snow covered (if it is the case) soils protect organic matter from decomposition, preserving soil carbon. Low temperatures reduce gaseous soil carbon losses and wetter soils may favor lower rates of soil respiration when oxygen becomes limiting and as a consequence, soil carbon storage is higher (Pastore et al., 2022). A more persistent snowpack can reduce soil freeze-thaw cycles (Pastore et al., 2022; Wipf et al., 2015). The frequency and duration of freeze-thaw events are critical to nutrient and carbon cycling, so reducing freeze-thaw cycles limits soil carbon loses through respiration and leaching (Edwards, Scalenghe, & Freppaz, 2007). For example, this reduction of soil respiration in CAPs was observed in a southeastern US Appalachian Mountain site (Novick, Oishi, & Miniat, 2016). It should be noted, however, that, in addition to temperature and moisture, the influence of local soil properties on plant community development may outweigh the influence of cold air pooling microclimates.

4.2. Potential CAPs-effects on soil microbiology

To the best of our knowledge the specific microbial communities present in soils where cold air pooling events occur have never been studied. Nevertheless, we hypothesize that their composition and dynamics could be driven by three interacting factors: lower temperatures, longer and thicker snow coverage and higher soil water content. Additionally, it is crucial to acknowledge that these characteristics can exhibit considerable variability depending on the location, topography and soil type.

In regard to low soil temperatures, Schnecker et al. (2023) observed a notable increase in microbial activity during the winter season and postulated a number of potential explanations for this phenomenon. One possibility is that microbial carbon use efficiency (CUE) rises at cooler temperatures (Frei et al., 2023), which can derive from respiration being more temperature-sensitive than growth (Pietikäinen, Pettersson, & Bååth, 2005; Cruz-Paredes, Tájmel, & Rousk, 2021). Alternatively, at lower temperatures, predators may become inactive while their prey microorganisms can continue to increase in biomass. A third potential explanation is that soil microorganisms augment their cellular contents during winter, either as reserves (Manzoni et al., 2021; Mason-Jones et al., 2022) in anticipation of substrate scarcity, or as osmotic agents or cryoprotective substances.

As previously stated, the coverage of snow on a CAP may be longer-lasting than on the surrounding area, contingent on the location and altitude of the CAP. In such instances, microbial activity will manifest in a different way. Under the snowpack, as microorganisms decompose organic matter and release minerals, they accumulate nitrogen, and microbial biomass reaches an annual peak at snowmelt. This safeguards nutrients from being depleted from the soil during this moist period, and as the season advances, these nutrients are released for plant uptake, while microbial biomass diminishes (Körner, 2021; Brooks, Williams, & Schmidt, 1998; Lipson et al., 1999). Microbial activity under snow recycles a large fraction of growing season primary production and thus determines the following season's nutrient availability.

Soil may be subjected to a multitude of physicochemical transformations in response to flooding, a phenomenon that can occur frequently in CAPs. The saturation of soil pores, which typically facilitate gas exchange between the atmosphere, soil, and soil microorganisms, results in a significant reduction in gaseous diffusion. Oxygen levels can rapidly decline due to the activity of aerobic microorganisms, reaching anoxic conditions even in the soil surface layers within the first hours of flooding. This alteration in oxygen availability can then result in a gradual transition in the microbial community from aerobic species to facultative anaerobic organisms, and ultimately to strict anaerobic organisms (Hartman & Tringe, 2019).

5. Plant Communities inside CAPs

5.1. Species present

The exceptional microclimatic conditions typically associated to CAPs can strongly determine the vegetation stablished within them. Thus, the extent and recurrence of low temperature events can play a detrimental effect on non-tolerant species but also a favoring effect on tolerant species, as it has been reported in different geographical locations (Pastore et al., 2022). For example, deleterious effects of low temperatures associated to topographic depressions caused strong seedling mortality on a plantation of spruce and pine in central Sweden (Blennow & Lindkvist, 2000). By contrast, the current distribution of the rare endemic alpine plant *Saxifraga florulenta* in the Maritime Alps matches CAPs microtopography, more than regional macroclimate (Patsiou et al., 2017). This species is favored by cold-air pooling sites where current macroclimate warming effects are buffered and its persistence over the last millennia in that area is explained by topo-climatic cold-microrefugia (Patsiou et al., 2017). Similarly, a recent study in a Hungarian Karst landscape demonstrates higher species cover and richness at the bottom of dolines than on their corresponding plateaus and this correlates with events of lower temperatures (Frei et al., 2023). Additionally, cold-adapted species are more frequent in coldest dolines (Bátori et al., 2017). Similarly, it has been described in the mountains of Japan the existence of a vegetation inversion, with tundra communities occupying the bottom of CAPs and the surrounding higher elevation slopes covered by subalpine conifer forests (Iijima & Shinoda, 2002). Thus, specific microclimate of CAPs can either limit or favor the presence of particular species, likely being low temperature the main selective factor.

5.2. Changes on the biology of present species

The species and/or individuals present in CAPs can also show biological differences when compared to nearby areas out of the CAPs. Although these evidences directly obtained from CAPs are very scarce, information can be inferred from basins recurrently subjected to temperature inversions. Alterations in phenology have already been reported. For example, in a year with frequent anti-cyclonic weather that promoted cold-air pooling at Cascade Range (USA), spring bud break of many species was delayed at low elevation sites (Ward, Schulze, & Roy, 2018). Timing of leaf expansion and of leaf fall is also related with the occurrence of nocturnal temperature inversions at small basin in Central Japan (Kusunoki & Ueno, 2022). Most of the scarce studies available so far, are mainly focused on community assembly and species composition (Pastore et al., 2022; Frei et al., 2023), being physiological studies virtually unavailable. Two further aspects that are misrepresented in bibliography are: climatically warm environments (since most of the literature is centered at mid latitudes) and cryptogamic vegetation (i.e. very little is known on ferns, and nothing on lichens or bryophytes potentially inhabiting CAPs). While temperature is the primary factor influencing species presence in CAPs, other elements such as soil moisture, nutrient availability, and snow cover (see Section 5) frequently interact with it (Frei et al., 2023).

6. Physiological Adaptations. General Effects of Low Temperatures

As previously mentioned, low temperatures can significantly influence vegetation establishment. Despite the absence of direct evidence, the ecophysiological responses of CAP plant communities can be inferred from the better characterized responses to low temperatures. In general, these plants will be subjected to a significantly greater degree of temperature stress than adjacent populations situated outside of CAPs. Therefore, plants in CAPs require activation of signaling pathways that enable processes and structures to minimize climatic impact by either avoiding or tolerating it. Since plant survival under low temperature stress has been reviewed extensively (Körner, 2021), Figure 3 will focus briefly and directly on the most relevant plant strategies in response to low temperatures. Within the lower end of the thermal range, two distinct yet overlapping types of plant stress can be identified: chilling and freezing. Chilling stress occurs at temperatures ranging from 0 to 15 °C, while freezing stress occurs at temperatures below 0 °C. Chilling stress typically interferes with plant metabolism and growth while freezing temperatures lead to ice formation causing structural damage and cell dehydration. Plants respond to such stresses with a series of strategies ranging from stress avoidance to tolerance of ice formation in the apoplast.

Considering these strategies, supercooling is generally effective for brief frost periods, lasting only a few hours. However, if the frost period extends inside CAPs, ice formation may occur, which is often fatal. In such cases, it is more advantageous for the plant to rely on tolerance mechanisms.

As it has been previously mentioned, most of the limited studies available so far primarily focus on community structure and species composition (Pastore et al., 2022; Frei et al., 2023), with physiological research being almost nonexistent. A more specific focus should be placed on the subject of how CAP plants cope with freezing temperatures, with particular attention to the role of antifreeze proteins and solutes, as well as ice nucleation. Additionally, further exploration is warranted into the function of cryoprotectants or ROS-scavenging mechanisms, supercooling, and extracellular freezing tolerance. Further avenue of investigation lies in the comparison of photosynthetic performance between CAP and non-CAP plants. In Section 8, we present preliminary results from our research on this topic, which includes the analysis of osmotic potential, freezing tolerance, and photosynthetic performance of plants both inside and outside of CAPs. Undoubtedly, a more in-depth study in this area would be highly beneficial.

Whether ecophysiological strategies of photosynthetic organisms inhabiting CAPs differ much from those already studied in polar or alpine plants is a key point. Unfortunately, currently available knowledge on plant physiology inside CAPs is so scarce that we are unable to give answer to these and other more

specific questions. On the light of main environmental differences between CAPs and other potentially cold emplacements (such as Alpine and Polar ecosystems), could be reasonable to expect that plants adapted to CAPs may have unique morphophysiological characters. Very likely, these could be strongly related with the possibility of importantly low temperature at vegetatively relevant periods along the year. Complementarily, some of the morphosysiological features could be related to attenuated irradiance and wind. In consequence, it could be reasonable to expect that plants adapted to CAPs may have unique morphophysiological characters, i.e. big leaves (as inferred from plants adapted to shade), but constitutive physiological adaptations to low temperatures, i.e., low osmotic potential.

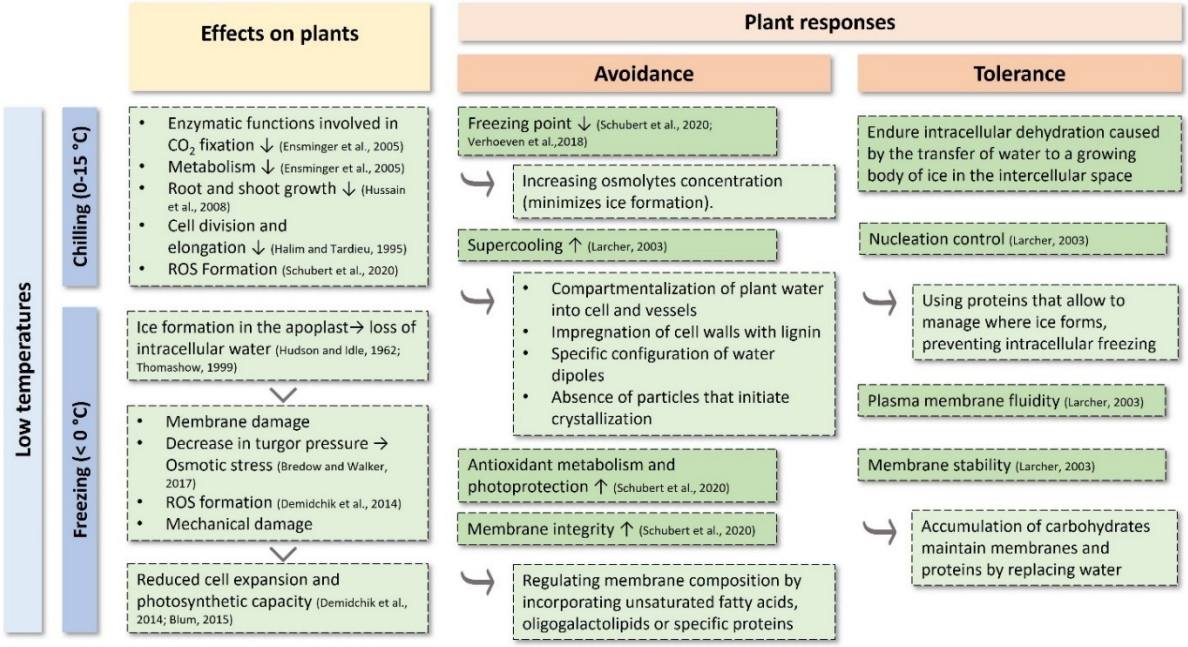

Figure 3. Effects of chilling and freezing temperatures and plant mechanisms in response to low temperatures, avoidance and tolerating strategies (Larcher, 2003; Ensminger, Busch, & Huner, 2005; Hussain et al., 2018; Ben-Haj-Salah & Tardieu, 1995; Schubert et al., 2020; Hudson & Idle, 1962; Thomashow, 1999; Bredow & Walker, 2017; Demidchik et al., 2014; Blum, 2015; Verhoeven, García-Plazaola, & Fernández-Marín, 2018).

7. CAPs as Natural Freezers for the Study of Plant Responses to Low Temperatures

Geodiversity sometimes offers unique opportunities for the study of relevant ecophysiological issues. This is for example the case of the geothermal CO_2 springs in central Italy where a natural plant community grows in a naturally enriched CO_2 environment, providing the opportunity to study long-term responses of vegetation to future atmospheric conditions (Körner & Miglietta, 1994; Miglietta, 2006). In an analogous way, we propose here the use of CAPs as natural freezers to deepen into the study of plant responses in situ to low and freezing temperatures.

Manipulative treatments of temperature stress have been applied in situ to study the responses to both heating stress (Buchner et al., 2015) and freezing tolerance (Buchner, Neuner, & Ball, 2011) thanks to temperature control devices adapted for their use in the field. An alternative approach to study thermal stress is to take advantage of the periodic occurrence of heat (Esteban et al., 2008; Schär et al., 2004) or cold waves (García-Plazaola et al., 2003). However, these extreme meteorological events are essentially unpredictable, which constrains their systematic use in ecophysiological studies. Given that temperature decreases with elevation, another option is to study plant traits across steep altitudinal gradients (Cabrera, Rada, & Cavierers, 1998). However, at high elevations, low temperatures are accompanied by reduced atmospheric pressure, leading to a decrease in oxygen partial pressure. This reduction has been found to decrease frost resistance in some plant species (Larcher, 2003), while enhancing it in others (Halloy & González, 1993). Thus, CAPs offer the opportunity to study variations in low temperatures by comparing nearby positions situated inside and outside the CAPs, avoiding the influence of different oxygen partial pressure for the same species. However, in any study that using CAPs as an experimental model, it must always be taken into consideration that CAPs are natural systems and not experimental chambers, implying that temperature always co-varies with other key environmental factors such as atmospheric humidity, snow cover, or hidden precipitation in the form of dew or fog.

In the context of a climate change scenario, the role of CAPs as microrefugia for cold-adapted species is well-established in this review. However, the future stability of CAPs remains uncertain, and the existing literature on the subject is limited. CAP persistence is contingent on stable nocturnal radiative cooling, a process that may be influenced by factors such as increasing cloud cover and changes in air circulation patterns. It is also plausible that climate change

could lead to a reduction in snowfall, which could in turn affect the thermal insulation properties of CAPs.

8. CAPs at Teide National Park, a Case Study at Subtropical Latitude

8.1. Characteristics of studied CAPs in Teide volcanic caldera

Teide National Park (28°16′N, 16°38′W) features the Teide stratovolcano which has its highest elevation at 3715 m. The peak itself is surrounded by a much wider volcanic caldera, which is delimited by a huge escarpment and consists of a series of natural sedimentary basins locally called "cañadas", where all the eroded material from escarpments accumulates (see Figure 4A). The characteristic configuration of Cañadas del Teide is formed by a huge caldera with a diameter of 17 km in its largest radius (NE-SW) and 7 km in the smallest radius (NW-SE). On days with lower wind speed, the cold air accumulates at lower altitudes resulting in the typical CAP inversion. Figure 5A illustrates the temperature inversion on the nights of 27–28 and 28′29 of December 2018. Temperature at the bottom, where sensor P5 is located (2049 m), was lower than at Montaña Blanca, situated at 2727 m (see Figure 4B), contrary to what would be expected under usual wind speed conditions that prevent air stratification. It should be noted that these sensors are installed at a height of 1.5 m from the ground, measuring air temperature in the free atmosphere and temperatures at ground level might be lower. In Figure 4A, the location of every sensor is displayed.

There are also occasional CAPs of smaller extension, within the large pool of circumstantial cold air of the Caldera, such as Seven Cañadas. This is a local geographic depression that extends for more than 10 km into the base of the great caldera at its southern end and is up to 75 m deep. Here, the stratification events that lead to the formation of CAPs occur at night, when wind speed is at a minimum, and last until dawn, when the sun illuminates this area and generates turbulence that breaks up the air stratification. Figure 5B shows how the process of thermal inversion occurs as the night progresses and the wind speed slows down, so that, if at noon the temperature inside the caldera is higher than at the top of the surrounding area, at midnight and until daylight, the opposite occurs.

This phenomenon is relatively common in the Seven Cañadas and, to a lesser extent, in the whole of the large caldera that makes up the Teide National Park. It affects one third of the days of the year and occurs in every season, even in summer, depending on the characteristics of the local wind regime (López-Díez et al., 2022). It is also known in other summit areas, such as the Ucanca plain (Lazar, 1996). Probably any endorheic basin inside the Cañadas del Teide caldera and surrounding high altitude area could behave as a CAP. This is the case of the basin at 3000 m in the Pico Viejo crater. López-Díez et al. (2021) pointed out several locations of these CAPs in the Teide National Park (López-Díez et al., 2022).

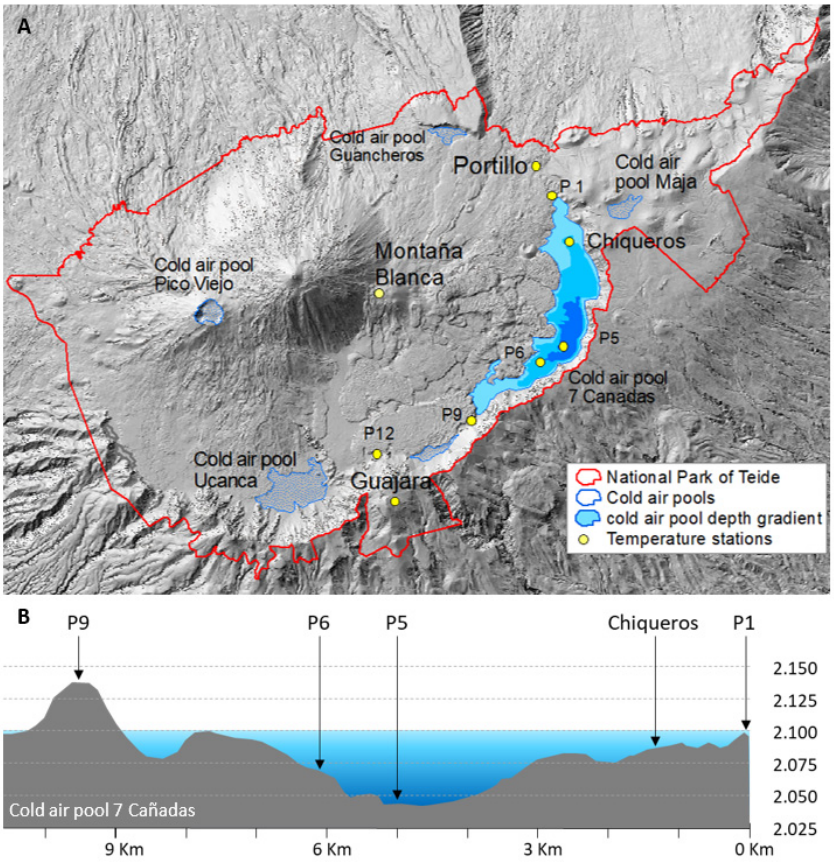

Figure 4. (**A**) Cañadas del Teide map. Yellow points indicate the position of temperature sensors at 1.5 m height from the ground. Red line indicates the limits of the National Park of Teide. In blue CAPs of different depth. (**B**) Elevation profile of the CAP 7 cañadas and the corresponding location of each temperature sensor. Data kindly provided by the National Park "El Teide".

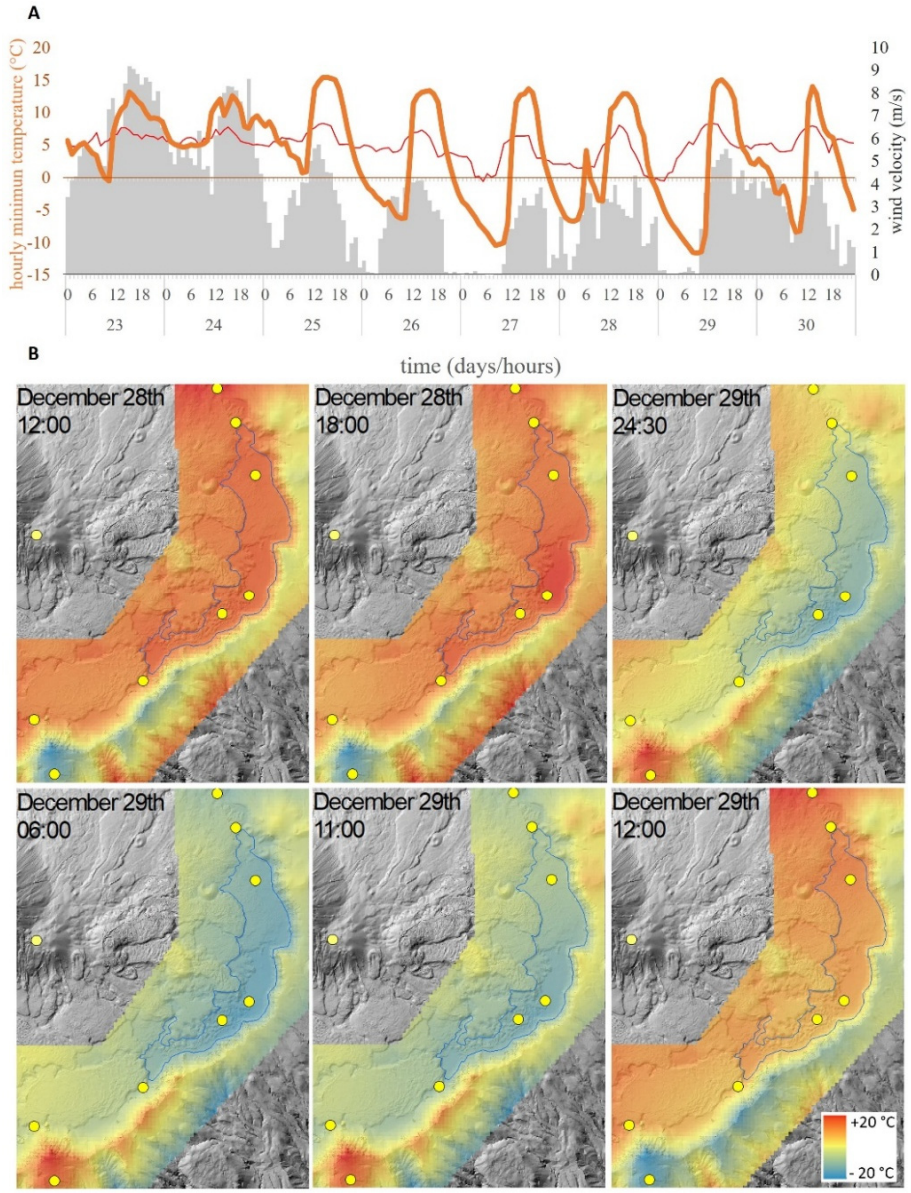

Figure 5. (**A**) Representation of time evolution between 23 and 30 December 2018 of the Caldera de Las Cañadas. The thick orange line belongs to the station P5, in the deepest part of the cold lake of Seven Cañadas (2049 m) and the thin red line belongs to the station Montaña Blanca (2727 m). Note how in the event of the night of 27–28 the phenomenon is repeated twice, as the wind, once activated, slows down again. (**B**) Digital terrain model of a CAP event on the night of 29 December 2018, from weather data from ground stations. Interpolation was performed using multiple linear regression with hourly minimum temperature as the dependent variable and longitude, latitude and elevation from a digital terrain model with 100 m resolution as independent variables. The result was adjusted by adding an inverse distance weighted interpolation of the residuals, according to the methodology proposed by Ninyerola et al. (2000). Data kindly provided by the National Park "El Teide".

8.2. Plant performance on Cañada del Portillo (Teide National Park)

Temperature profiles were followed in a particular location, Cañada del Portillo (see Figure 4A) within the large CAP of Seven Cañadas throughout the growing season (from May to September) of 2024. Figure 6A depicts a photograph of the CAP at Cañada del Portillo, while Figures 6B, 6C and 6D present a schematic representation of the position of the various sensors. Four of them were placed at the bottom of the basin (two on the ground and the other two on rocks), two of them were placed outside and the other four at the southeast-facing slope.

The resulting analysis showed that there was a thermal gradient greater than 2 °C on 96.5% of the days, during the recorded period, while the average gradient was 5.14 °C. As can be seen in Figure 6B, freezing nights (temperatures below zero) occurred 44 % of days at the bottom, while at the plateau never occurred. The absolute minimum recorded by a sensor placed at ground level was −9.36 °C during a cold air pooling event on 6 September 2024 (Figure 6C).

The surroundings of the hollow were occupied by a plant community dominated by two shrubs: *Descurainia bourgeana* (E.Fourn.) and *Pterocephalus lasiospermus* (Link ex Buch), with the first being dominant in the bottom of the hollow and the latter in the upper plateau (Figure 6A). The physiological

responses of both populations (upper and lower) of *Descurainia bourgeana* were characterized in late spring when thermal inversions are large (Figure 6C) and freezing events are still frequent (Figure 6B) at the bottom of the hollow. While no differences were observed between individuals from the bottom and plateau areas in basic photosynthetic or hydraulic parameters (Figure 7A–C,E,F) when examining the species' tolerance to freezing temperatures (Figure 7D), a notable divergence emerges, with individuals from the bottom area exhibiting a considerably higher tolerance to freezing. The physiological basis of this enhanced freezing tolerance remains to be elucidated, but current analysis clearly provides preliminary evidence in support of the hypothesis that CAPs can be understood as natural freezers, thereby facilitating the study of plant responses to low temperatures. In addition, a statistically significant difference was observed in the osmotic potential (Figure 7B), suggesting that a response to freezing temperatures in this species could be the accumulation of osmolytes as it is described in Section 5.

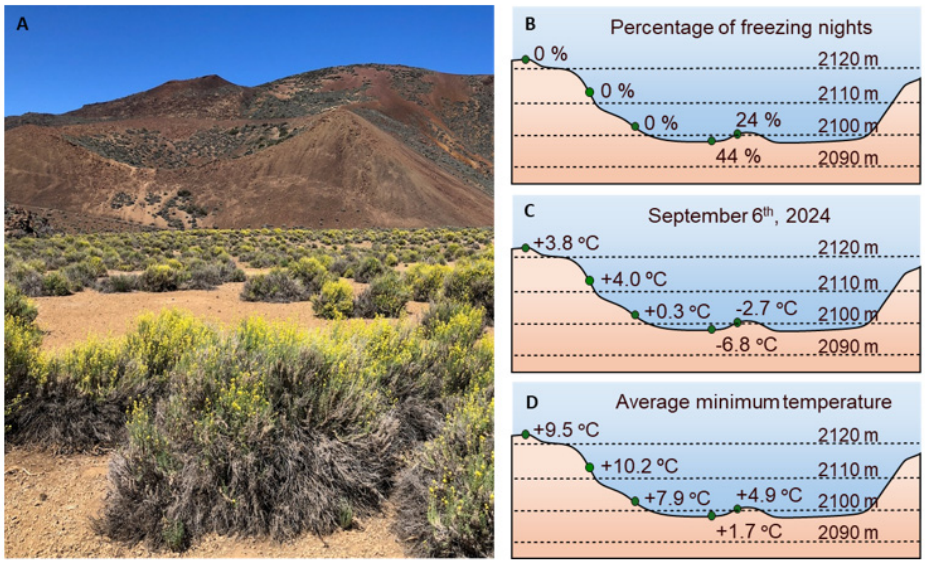

Figure 6. (**A**) Late Spring at the bottom of the "Cañada del Portillo". Vegetation is dominated by flowering bushes of Descurainia bourgeana. (**B**) Percentage of freezing nights at different points at "Cañada del Portillo" during the growing season of 2024. (**C**) Minimum temperatures profile at "Cañada del Portillo" during a cold air pooling event on 6 September 2024. (**D**) Average minimum temperatures at different points at "Cañada del Portillo" during the growing season of 2024.

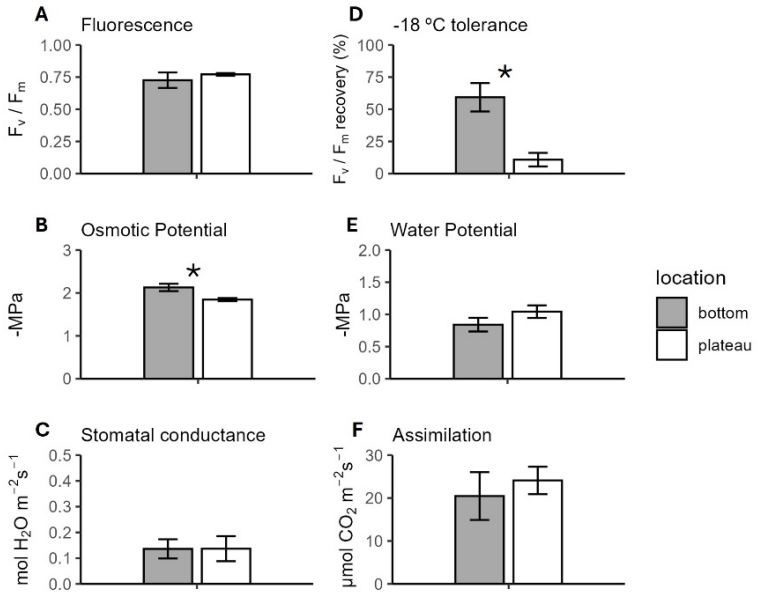

Figure 7. Physiological parameters measured in Descurainia bourgeana, dominant species at "Cañada del Portillo" comparing the response of the individuals growing outside the CAP (plateau, white) and inside the CAP (bottom, grey). Represented as mean ± SE (n = 4) the statistical difference at $p < 0.05$ between bottom and plateau was determined by t-test, indicated by * in the graphs. (**A**) Fluorescence measured *in situ*. (**B**) Osmotic potential measured in sampled leaves. (**C**) Stomatal conductance measured *in situ*. (**D**) Freezing tolerance to −18 °C measured in sampled leaves. (**E**) Water potential measured in sampled leaves. (**F**) CO_2 assimilation measured *in situ*. See Supplementary Materials for Materials and Methods.

9. Concluding Remarks

In light of the aforementioned considerations, it becomes evident that numerous aspects of CAPs remain subject to investigation. A shortage of literature exists about the biological differences between the species in and out of CAPs or about the specific microbial communities present in CAP soils. It is also important to highlight that the formation of these events occur in a diversity of environments and geographical locations with markedly disparate characteristics, which can make challenging to identify the common attributes. Taking this into account, here we focused on the potential of CAPs as natural freezers and their utility in elucidating the plant response to cold and freezing temperatures. Cañadas del Teide has been identified as an appropriate location for further research concerning this aspect of the CAPs and the results of the preliminary tests suggest that the proposed hypothesis is valid. The current analysis offers preliminary evidence that CAPs can be conceptualized as natural freezers, thereby facilitating the study of plant responses to low temperatures. These initial findings serve as a foundation for future investigations in diverse CAPs and in various species.

Supplementary Materials

The additional data and information can be downloaded at: https://www.sciltp.com/journals/PlantEcophys/2025/1/558/s1.

Author Contributions

The conceptual framework of the manuscript was devised by the author J.I.G.P. E.A. had a major contribution in the original draft preparation. All authors contributed to the writing and figure preparation and agreed to the final version of the manuscript.

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Data Availability Statement

Original data are available upon request to the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

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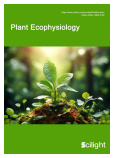

Article

Assessment of Plant Responses to Simulated Combination of Heat Wave and Drought

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Abstract: Background: Among the consequences of global climate change, one of the most significant yet understudied is the increased frequency and intensity of heat waves. This article evaluates the responses to combined heat wave and drought in several crops and a non-crop species using an improved methodology to control temperature using IR lamps. **Results:** The effectiveness and precision of simulated heat waves with the system presented were verified at the experimental field of the University of the Balearic Islands. Using IR lamps, an artificial leaf was used to precisely control environmental temperature, a key aspect in simulating heat wave conditions. Concerning plant physiology, the effects of combined heat wave and drought on leaf relative water content (RWC) and photosynthetic parameters presented different patterns between species. Two remarkable particularities were (1) the observation that photosynthesis was sustained under RWC values well below those previously reported to cause complete photosynthesis cessation in C_3 species and (2) the photosynthetic linear electron transport rate (ETR) was stimulated after retrieval of drought and heat wave far above their own initial values and those for control plants, also in some species. **Conclusions:** The use of an artificial leaf as major temperature sensor is key to provide highly realistic simulated heat waves. Using this technical setup, it was possible to determine that there is a large variability among species and some particularly intriguing observations strongly support the view that systematic experiments should be developed on different species and conditions to grab a significant knowledge on how will heat waves affect crop and vegetation in the near future.

Keywords: climate change; heat waves; drought; affordable commercial infrared heaters; plant ecophysiology; photosynthesis; relative water content

1. Introduction

Global climate change is one of the biggest challenges that humanity faces in the 21st century (Feulner, 2017). Among the numerous effects of climate change, heat waves stand out as extreme phenomena with the potential to devastate ecosystems, agriculture, and human health (Intergovernmental Panel on Climate Change, 2021; Shivanna, 2022; Qu et al., 2024). Heat waves consist of periods (typically days to weeks) with

warmer temperatures than the average for the same site and period over years. The impacts of heat waves on agricultural yields have been reported to induce major negative consequences, leading to numerous direct and indirect social problems in many regions of the world (Hatfield et al., 2011; Lobell, Schlenker and Costa-Roberts, 2011). Although studies on the effects of heat waves on plants follow very diverse methodologies and are difficult to compare, it has been suggested that heat waves have strong negative impacts on plants, with photosynthesis rates and RWC being major targets, as well as inducing alterations in plant development and hormonal levels (Haworth et al., 2018; Xie et al., 2020; Ostria-Gallardo et al., 2023; Rashid et al., 2023; Tokić et al., 2023), germination (Orsenigo et al., 2015), fruit quality (Tomás, Viegas and Silva, 2020), senescence, and others (Qu et al., 2024).

In this context, although it has been predicted that drought would exacerbate the effects of heat waves on photosynthesis and plant water relations—and vice versa—(López, Ramírez-Valiente and Pita, 2022), there is limited and not very conclusive evidence available at present. Thus, Rashid et al. (2018) found, studying two cultivars of wheat, that only in one of them, photosynthesis was more depressed by drought under hot conditions, and only after recovery and not during its imposition. Moreover, Haworth et al. (2018) observed, in olive trees, that a heat wave strongly reduced photosynthesis in irrigated plants, but not under drought, resulting in non-significant differences in photosynthesis between irrigated and drought plants during the heat wave. Conversely, in natural ecosystems, there is evidence that heat waves alone produce only small and transient effects, while when combined with drought they amplify the negative effects of the latter on carbon balance and productivity (De Boeck et al., 2011, 2016). Regarding to RWC, Davies et al. (2018) observed, in native Australian C3 and C4 grasses, that the combined effect of heat wave and drought was a larger decrease of leaf water content. Although RWC was not reported in that study, the observed values of leaf water content in the three C3 species studied point to RWC values well below 65%, i.e., below the value defined by Lawlor and Cornic (2002) as the threshold for complete photosynthesis inhibition in C3 species, which contrasts with the scarce effects of heat waves on photosynthesis observed in the aforementioned works by Rashid et al. (2018) and Haworth et al. (2018). In contrast, Xie et al. (2020) found no differences in RWC between wheat plants subject to drought alone or combined with heat.

As heat waves become more frequent, prolonged, and intense in various parts of the world, understanding and mitigating their effects is becoming increasingly crucial (Marx, Haunschild and Bornmann, 2021). In order to address this issue, scientists have developed several methods, both passive and active, to simulate these extreme conditions in controlled environments, both inside laboratories and out in the field (De Boeck et al., 2010). Despite decades of research, accurate replication of heat waves remains a significant challenge due to the technical and economic limitations of current systems (Schulze et al., 1999; Rich et al., 2015). Passive methods, such as greenhouses, passive nocturnal warming and open-top field chambers are often used in studies with limited infrastructure, such as those performed in remote areas away from the laboratory. These systems present a lower precision and control, resulting in less accuracy in simulating natural heat waves. In contrast, active systems like phytotrons, active warming chambers, heating cables or tubes, and infrared (IR) heaters, although more costly in terms of energy and implementation, are better suited for studies that require precise and constant temperature control (Aronson and McNulty, 2009; De Boeck et al., 2010). These systems can replicate extreme heat wave conditions more accurately, providing reliable simulations of specific climate scenarios (Shen and Harte, 2000). Still, most of these methods have additional limitations, such as, among others, light quality and intensity largely different from natural conditions, or the inability to reproduce outdoors wind conditions.

Among all active methods, heating with IR lamps stands out for its ability to provide heat through infrared radiation, directly warming soil and vegetation similarly to the sun (Kimball, 2005). Further, IR lamps constitute the only active method that can be used in situ, in the field, under the natural dynamic variations of light quantity and quality, wind, precipitation and other environmental factors. Previous studies have shown that IR heaters can have a significant impact on simulating global warming and its effects on ecosystems (De Boeck et al., 2010; Kimball, 2015), while offering significant advantages in terms of thermal precision and speed, thus allowing for specific and consistent temperature increases that accurately mimic heat wave conditions, with minimal disturbance to the ecosystem (Kimball et al., 2008). Nevertheless, the adoption of large-scale IR heaters faces significant challenges, including high installation costs and substantial energy consumption (Kimball, Conley and Lewin, 2012). Moreover, the operational complexity of configurations that require modifying heat intensities and distribution to accurately simulate heat waves in different ecosystems represents another significant implementation hurdle (Kimball, 2015). Harte et al. (1995) presented the first heat wave experiment using infrared heaters, in which the device emitted radiation over a linear space with fixed power throughout the experiment. Later, Nijs et al. (1996) introduced proportional control to maintain a fixed temperature increase in the heated plot compared to a reference plot. Kimball (2005) further improved the methodology by introducing a proportional-integral-derivative (PID) controller. In the present work, some of these and other pioneering implementations of IR-based heatwaves simulations, based on versatile and economical IR heaters, i.e., those often used in bars and terraces, have been used. Overall, one of the crucial issues regarding the use of IR heaters is that they exert a direct effect on the bodies within reach, but not on the surrounding air, while heat waves are defined based on air temperature. This is a crucial issue that constitutes the focus of the present research. A redefinition of heat waves is needed when studying their effects on plants given that leaf temperature is much more dependent on factors beyond air temperature. These factors are

related to (a) leaf or canopy size and morphology with strong influence on the boundary layer, (b) leaf physiology (e.g., transpiration) and (c) additional variable climatic conditions (e.g., wind speed). Consequently, relating the increase in temperature over an artificial leaf which is not influenced by the above-mentioned factors is key to finely controlling temperature changes.

The objectives of the present study are: (1) assessing the suitability of using IR heaters for simulating heat waves on plants; and (2) applying this methodology to study the combined effect of drought and simulated heat waves on leaf RWC and photosynthesis in one native and three crop species.

2. Material and Methods

2.1. Device for simulating heat waves

Commercial, low-cost infra-red based heaters were adapted for simulating natural heat waves to study their effects on plant ecophysiology. A custom control system and dedicated software were specifically implemented for this purpose. A brief description of the main features of the method is provided below with further details in Supplementary Material S1.

Widely distributed and easily accessible commercial IR heaters in Europe (Tristar KA5287, Orbegozo PHF31, Tresko THSL004, Liliana CIPIE2000, and TroniTechnik TT-TH2020) were used. These devices are designed with a circular support to facilitate their placement surrounding those plants under study. Their height is adjustable, ranging from 130 cm to 210 cm, and inclination can be adjusted from 0° to −45°. Heaters are governed by a bus carrier board control system embedded in a Raspberry Pi Compute Module 4 I/O board with a Raspberry Pi Compute Module 4 SBC (Single Board Computer) card that features a quad-core Cortex-A72 processor at 1.5 GHz, also embedded in the I/O board. Temperature was measured using negative temperature coefficient (NTC) thermistors that operate over a range of −30 °C to +105 °C and can function properly in ambient conditions with relative humidity (RH) between 5% and 95%.

Given that IR heaters only affect the temperature of surfaces, temperature sensors were placed in cardboard, simulating a non-exposed artificial leaf without transpiration in two setups (control and simulated heat wave). Two sets of plants were studied, one without IR heaters (control) and another with IR heaters (simulated heat wave). The control system works to increase the temperature reading of the second sensor to a targeted temperature above that registered by the control sensor, using an on/off control with a user-programmable hysteresis within a defined range of ±0.25 °C to ±2 °C (±0.5 °C was selected).

2.2. Assessment of thermal homogeneity across an area surrounded by the heaters

Since the area surrounded by heaters that displays a similar air temperature will depend on the number and spatial disposition of heaters, it is important to assess the limits of what the researchers consider acceptable thermal homogeneity for their experiments before positioning the study plants and subjecting them to a simulated heat wave. Having a given number and array of heaters, this test may allow one to discern the maximum usable area size and, hence, the maximum number of plants that can be assessed in a single run–which would obviously depend on the size of the target plants. Alternatively, if the size and number of plants to be subjected to a simulated heat wave is fixed a priori, a similar assessment may allow one to know how many heaters might be needed, and to adjust their distribution to reach a homogeneous targeted temperature course around all the studied plants.

Thermal homogeneity of the system was evaluated by analysing the sensor temperature data collected when a minimum change of 0.1 °C occurred or when one minute passed without changes greater or equal than 0.1 °C. This data was provided by an array of 15 NTC sensors distributed at three different heights (see Figure 1 for a detailed description of the sensors situation).

Using this sensor array, three basic configurations were studied, all involving the placement of six heaters at the vertices of an imaginary hexagon, either surrounding or being surrounded by the experimental plants. Figure 2A–C illustrate some of the basic configurations that were examined.

More than 150 variants were explored, modifying variables such as the distance of the heaters (IHD) and sensors (TSD) from the centre of the plot, the height (IHH) and inclination angle (IHA) of the heaters, and the power output (Pw).

2.3. Defining and simulating heat waves

Although this is somehow out of the scope of this manuscript, it is worth mentioning that there are various definitions of heat waves at the European and global levels (De Boeck et al., 2010), which should be considered for designing simulating experiments. Given that the present study is focused on Mediterranean environments, heat waves were defined as provided by the Spanish State Meteorological Agency (AEMET). This agency defines a ‘heat wave’ as an episode of at least three consecutive days during which, at least 10% of the considered stations record maximum temperatures above the 95th percentile of their series of daily maximum temperatures for the months of July and August during the period 1971–2000 (Área de Climatología y Aplicaciones Operativas, 2023). It should be noticed that heat waves are defined considering air temperature. On the other hand, IR heaters are designed to heat solid objects—e.g., plants—rather than their surrounding air, which is heated indirectly. Therefore, when simulating the effects of heat waves on plants, it would be legitimate to consider the need for re-defining the heat wave on a leaf or canopy temperature basis. However, this approach has been discarded because the leaf-to-air temperature is a function of (a) leaf or canopy size and morphology (which strongly influences the boundary layer), (b) leaf physiology (e.g., transpiration), (c) additional variable climatic conditions (e.g., wind speed) and, (d) most especially, because empirically-determined leaf temperatures are extremely heterogeneous on an experimental

plot as used here, hence making it difficult to define a 'target' temperature for simulating the heat wave. Thus, given that this experiment compares different species (i.e., different leaf and canopy sizes and morphologies) under naturally variable conditions (e.g., different wind velocities among days and times of the day); and that heat waves are based by definition on air temperature, we developed the solution of placing an 'artificial leaf' in the middle of the two plots (control and heat wave treatment), i.e., a cardboard suspended within the canopies. This artificial leaf was used to register ambient temperature at the control plots as well as on the heat wave plots, the latter serving as the indicator to the heaters for adjusting their intensity as a function of the programmed temperature mismatch between control and heat wave plots. Because using an artificial leaf implies a 'non-transpiring' leaf, hence altering the whole energy balance, theoretical considerations on this and a complete simulation of how leaf temperature can vary at any given air temperature are provided in Supplementary Material S3. It is concluded that for the leaf sizes and environmental conditions used in this experiment, the differential temperatures between leaf and air under the simulated heat wave are sufficiently small as to be considered correct. However, care should be taken when using the present heat wave system with very large leaves, such as tobacco or banana, or under windless environments, such as those in growth chambers.

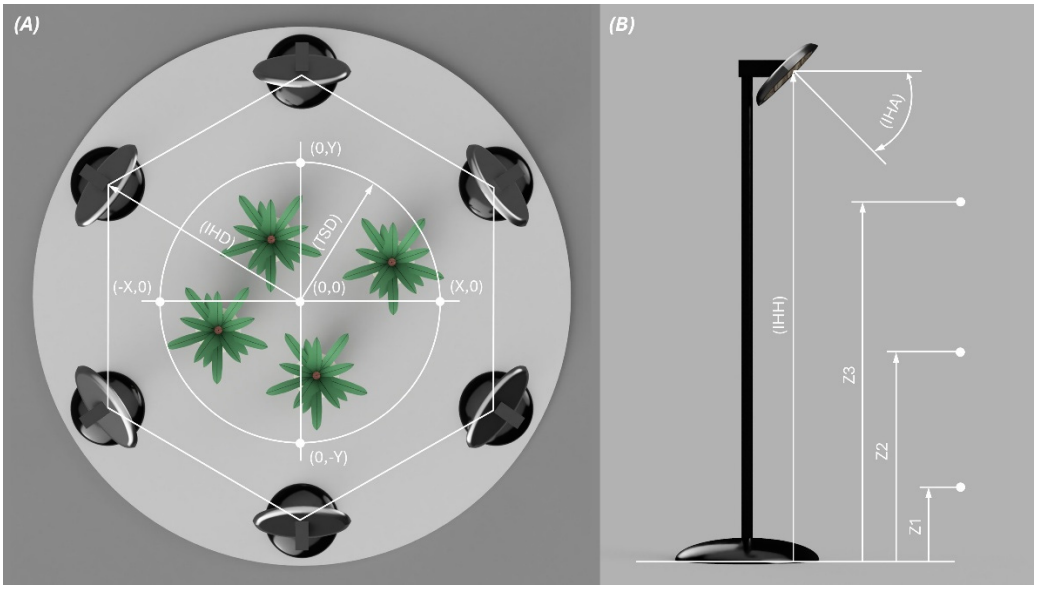

Figure 1. Variable parameters examined for the study of thermal homogeneity in different configurations. Figure 1**A** shows a plan view of the variables located on the X,Y axes: Temperature Sensor Distance (TSD) for homogeneity measurement and Infrared Heater Distance (IHD), both measured from the centre of the plot. Figure 1**B** shows the variables in elevation on the Z axis: Infrared Heater Height (IHH), Infrared Heater Angle (IHA), and height of the temperature sensors for homogeneity measurement, Z1: 25 cm, Z2: 80 cm, and Z3: 135 cm.

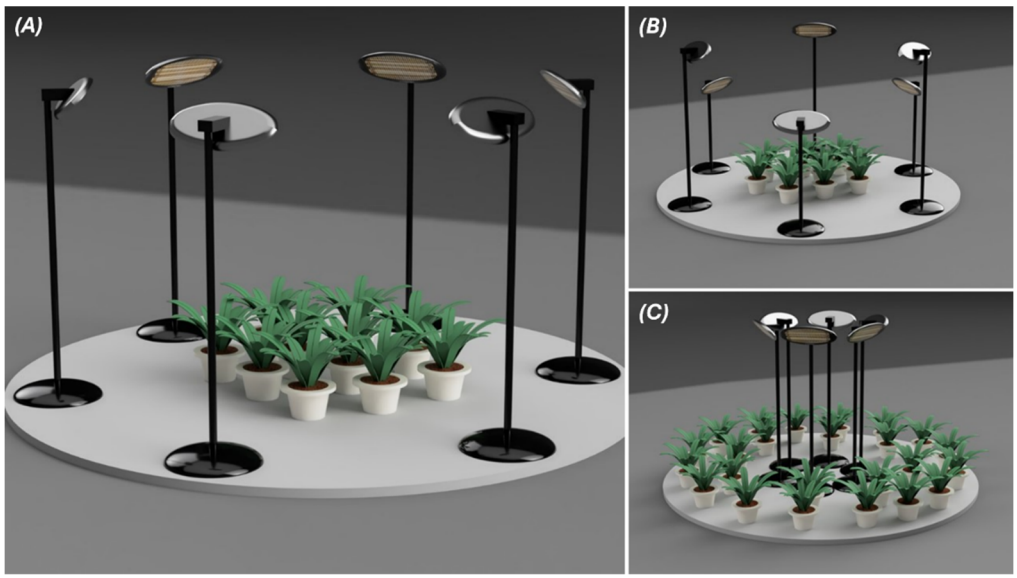

Figure 2. Basic configurations examined to study the combination of parameters that resulted in better thermal homogeneity. Configuration 2**A** shows a plot of plants surrounded by 6 heaters located at the same height over the vertices of a hexagon. Configuration 2**B** shows the same plot surrounded by 6 heaters placed at staggered different heights over the same vertices. Configuration 2**C** shows the heaters in the centre, surrounded by plants forming a circle.

2.4. Plants and treatments

After temperature homogeneity was considered correct (see the Results section), 30 plants in 3.8 L pots were placed in the simulated heat wave area. In both experiments, between 6 and 10 plants per species were used: control, irrigated plants in the absence of heat wave (IC), and plants subjected to a combined drought + heat wave (DHW). Single treatments were skipped as they have been reported to have a much lower effect (De Boeck et al., 2011, 2016; Davies et al., 2018).

In the first experiment, plants of sugar beet (*Beta vulgaris* var. cycla), sunflower (*Helianthus annuus*) and *Limonium gibertii* (an evergreen semi-shrub endemic to the Balearic Islands) were used. Sugar beet and sunflower plants germinated 8th November 2021 and were grown for about two weeks in a growth chamber at 23 °C and 350 µmol m^{-2} s^{-1} PAR, under a 12/12 h photoperiod. Once the first true leaf emerged, plants were transferred to 3.8 L pots filled with a 4:1 mix of commercial horticultural substrate (Prohumin, Projar, Quart de Poblet, Spain) and perlite and placed in a greenhouse at the University of the Balearic Islands (UIB). *Limonium gibertii* seeds were germinated in a growth chamber at 20 °C and grew outdoors under shade in 3 L pots containing a mix of 61.5% coconut fibre, 33% moss peat and 5.5% expanded perlite. They were fertilized with slow release 4.40 mg L^{-1} *Osmocote* NPK 19:10:19 (ICL, The Netherlands) and joined with sugar beet and sunflower plants in the greenhouse two weeks before the onset of the treatments. At this point, all plants were irrigated at field capacity every two days and supplied once with slow release *Multigreen Classic* NPK (Haifa, Israel). A week before the experiment, each plant received 6 g of Poly-feed NPK (Haifa, Israel).

In the second experiment, sugar beet (*Beta vulgaris* var. cycla), sunflower (*Helianthus annuus*) and tobacco (*Nicotiana tabacum* var. Wisconsin) plants were used. Germination and growth conditions were similar to those described for Experiment 1.

Two weeks before simulating heat waves, plants were transferred to a plastic semi-greenhouse (transparent plastic walls covered the top and half the distance between the ceiling and the floor, allowing wind to flow around plants). Irrigation was stopped in plants under DHW treatment until 30% substrate water content was reached. Thereafter, water deficit was sustained by daily supplying the amount of water equivalent to pot weight loss. These plants were kept under these irrigation conditions for a week before they were subjected to a simulated heat wave.

Experiment 1 and 2

Simulated heat waves were slightly different in the two experiments. Thus, in experiment 1, plants were placed on working tables 70 cm above ground, and HOBO temperature sensors (see Simulated heat waves section) were suspended among the leaves forming the canopy. Heat wave was applied for 7 days, using the following differential temperatures: from 10:00 AM, DHW plants would experience a 2 °C increase over control (IC) plants; from 12:00 PM, a 5 °C increase; and from 4:00 PM, a 2 °C increase again. At 6:00 PM, the infrared heaters were turned off until 10:00 AM the next day (Figure 3). In experiment 2, plants were placed directly on the ground with HOBO temperature sensors also suspended within the canopy. Plants were subjected to a 5-days heat wave. The heat wave was stablished as follows: starting at 10:30 AM, the DHW plants should experience an increase of 2.5 °C above IC plants; from 11:00 AM, the increase would be 3 °C; from 12:00 PM, 4 °C; from 1:00 PM, 5 °C; from 4:00 PM, 4 °C; from 5:00 PM, 3.5 °C; from 6:00 PM, 3 °C; and at 7:00 PM, 2 °C, which remained until 10:30 AM the next day, when the cycle started again (Figure 3).

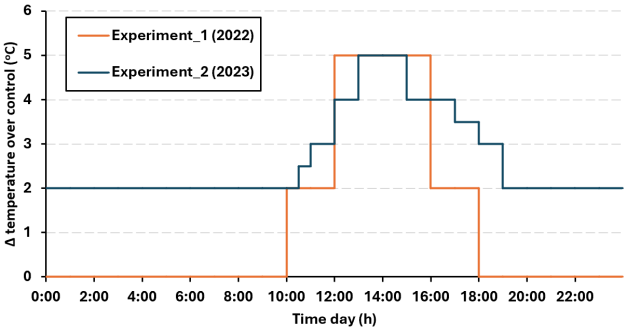

Figure 3. Warm-up regimen based on the time of day. Treated plots were heated by a theoretical air temperature differential (see Supplementary Material S3 for details) with reference to the control plots, depending on the time of day. Temperature regimes varied for each experiment according to the heat wave intended to be simulated.

2.5. Physiological measurements

Gas exchange and chlorophyll fluorescence parameters, and leaf relative water content, were measured between 10:00 AM and 2:00 PM on six different individuals of each species and treatment, on three days: T1 (plants under irrigation or drought the day before the onset of the heat wave), T2 (after four days under combined drought and heat wave), and T3 (three days after re-watering and heat wave simulation removed). All measurements were performed on sun-oriented, young, fully expanded leaves.

Leaf discs were taken and immediately weighed to determine their fresh weight (FW). Then, the discs were rehydrated in distilled water for 24 h under dark conditions at 4 °C to obtain the turgid weight (TW). Finally, leaves were placed in an oven at 70 °C for 72 h to determine dry weight (DW). RWC was calculated as:

$$\text{RWC}\ (\%) = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

Gas exchange and chlorophyll fluorescence parameters were assessed at ambient temperature and vapour pressure deficit (VPD) using an infrared gas analyser (IRGA) LI-6400XTR coupled with a fluorimeter (Li-6400-40; Li-Cor Inc., Lincoln, NE, USA). Photosynthetic photon flux density (PPFD) was fixed at 1500 µmol m^{-2} s^{-1} (90% red, 10% blue light). Net

CO_2 assimilation (A_N), stomatal conductance (g_s), and the rate of linear electron transport rate (ETR) were analysed.

2.6. Statistical analysis

To evaluate the effectiveness of the treatments (DHW vs. IC) on each of the physiological parameters (RWC, A_N and ETR) for each species and experiment, data were analysed independently. First, normality for each dataset was assessed using the Shapiro-Wilk test, and the homogeneity of variances was verified using the Levene test. In cases where both assumptions were met, an independent samples t-test was applied; otherwise, the non-parametric Mann-Whitney U test was used. A significance level of $p < 0.05$ was adopted.

3. Results

3.1. Thermal homogeneity assessment using different configuration variants

Three basic configurations were studied, each involving the placement of six heaters at the vertices of an imaginary hexagon, either surrounding or being surrounded by the experimental plants. Thermal homogeneity of these configurations was assessed using an array of 15 NTC sensors distributed at three different heights. More than 150 variants were explored, modifying variables such as the distance of the heaters (IHD) and sensors (TSD) from the centre of the plot, the height (IHH) and inclination angle (IHA) of the heaters, and their power output (Pw). In general, all measurements obtained from each sensor offered readings close to the target temperature value (Figure 4) with low maximum absolute errors.

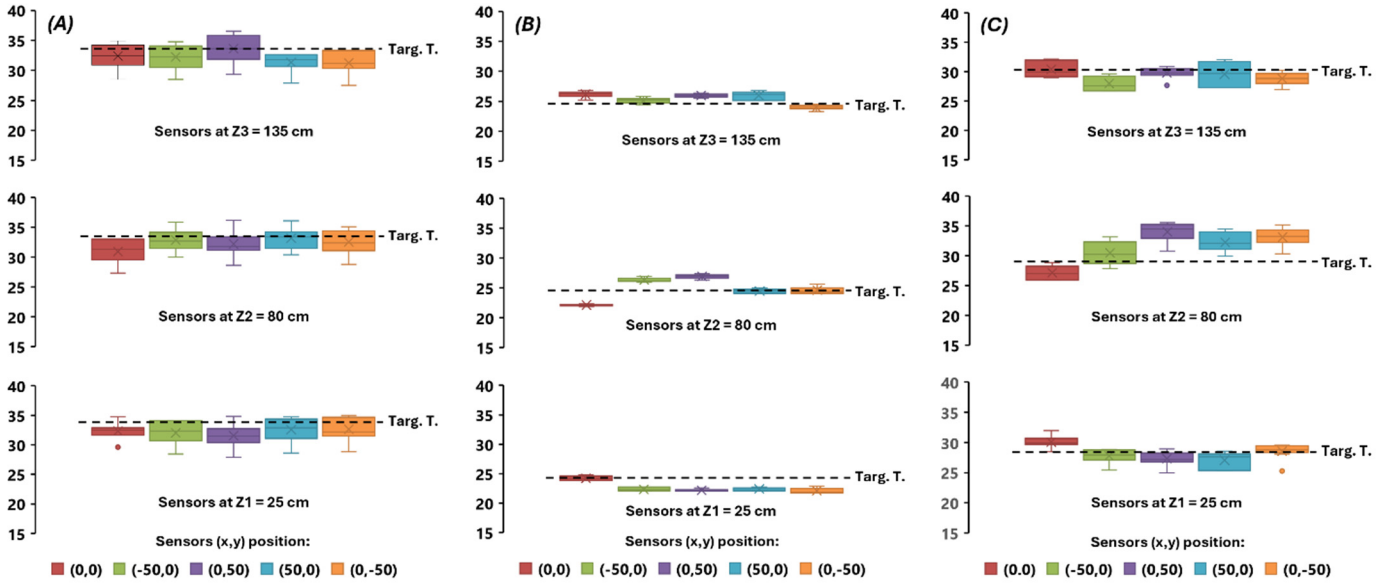

Figure 4. Temperature dispersion across each of the 15 sensors arranged to measure thermal homogeneity. Figure 4**A** corresponds to test 160, which in turn is related to the configuration in Figure 2A. Figure 4**B** corresponds to test 49, which in turn is related to the configuration in Figure 2B. Figure 4**C** corresponds to test 7 which in turn is related to the configuration in Figure 2C. Targ. T, is the target temperature that the system had to reach in the treated plot; refer to Figure 1 for details on the (x,y) and Zn positions.

3.2. Simulated heat waves

The effectiveness and precision of the generated heat waves were measured using temperature and humidity sensors Onset HOBO UX100-003 (470 MacArthur Blvd., Bourne, MA 02532, USA), placed in the centre of the canopy of both control (IC) and treatment (DHW) plots. Real temperatures of control and heat wave plots during several days are represented in Figure 5 showing the viability and fine control of temperature gradients as scheduled.

3.3. Physiological results

The present study combines two primary objectives. The first objective was to describe a heat wave simulating system and prove its accuracy and usefulness, combining this with a second objective which was to depict the response of RWC and photosynthesis in several different species and conditions, owing to the apparently controversial results of previously published studies. Given the duality of goals of the present work, a combination of drought and heat wave stress was applied simultaneously instead of separately in order to limit the number of species and replicates. Beyond this limitation, the obtained results (Figure 6) elucidate the potential impacts of heat waves on plant physiological responses and the expected heterogeneity depending on the plant and conditions during a Mediterranean heat wave.

As soon as plants were randomly assigned to either IC and DHW treatments, and before applying drought and heat waves, all parameters were measured, finding no significant differences for any of them between the two plant groups. Figure 6A shows that the combination of heat wave and drought often results in a severe decrease in leaf relative water content (RWC). This effect is particularly remarkable in sugar beet and sunflower—sugar beet only in Experiment 1, and

sunflower in both experiments—although the decrease in sunflower RWC was not statistically significant in Experiment 1. In both species, RWC dropped well below 65%, a value defined by Lawlor and Cornic (2002) as the threshold for complete photosynthesis inhibition in C3 plants. This agrees with previous findings by Davies et al. (2018) and highlights how extreme the impact of a heat wave can be on plants already experiencing water stress. Net CO_2 assimilation (A_N) and stomatal conductance (g_s) followed almost identical patterns in all cases, for which only the former are shown. Figure 6B shows that, in all cases except sunflower in Experiment 1, A_N was depressed under DHW compared to IC, although this reduction was not statistically significant in sugar beet during Experiment 2. While results in T1 (i.e., plants under drought but before the onset of heat wave) are not shown for simplicity, it is worth saying that A_N in T2 (an additional four days under drought accompanied by heat wave) was significantly decreased compared to T1 except in sunflower in Experiment 1, where it was unchanged, and in Limonium, where it actually increased (data not shown). However, ETR was more stable than photosynthesis in response to DHW, although in some species it was significantly reduced, but to a lesser extent than A_N (Figure 6C). Three days after stress was alleviated (T3), both A_N and ETR of DHW plants increased to values at (Limonium and sunflower in Experiment 2) or even above (sunflower and sugar beet in Experiment 1) control plants (Figure 6D,E). In contrast, in Experiment 2 sugar beet DHW plants kept lower values than control plants upon recovery.

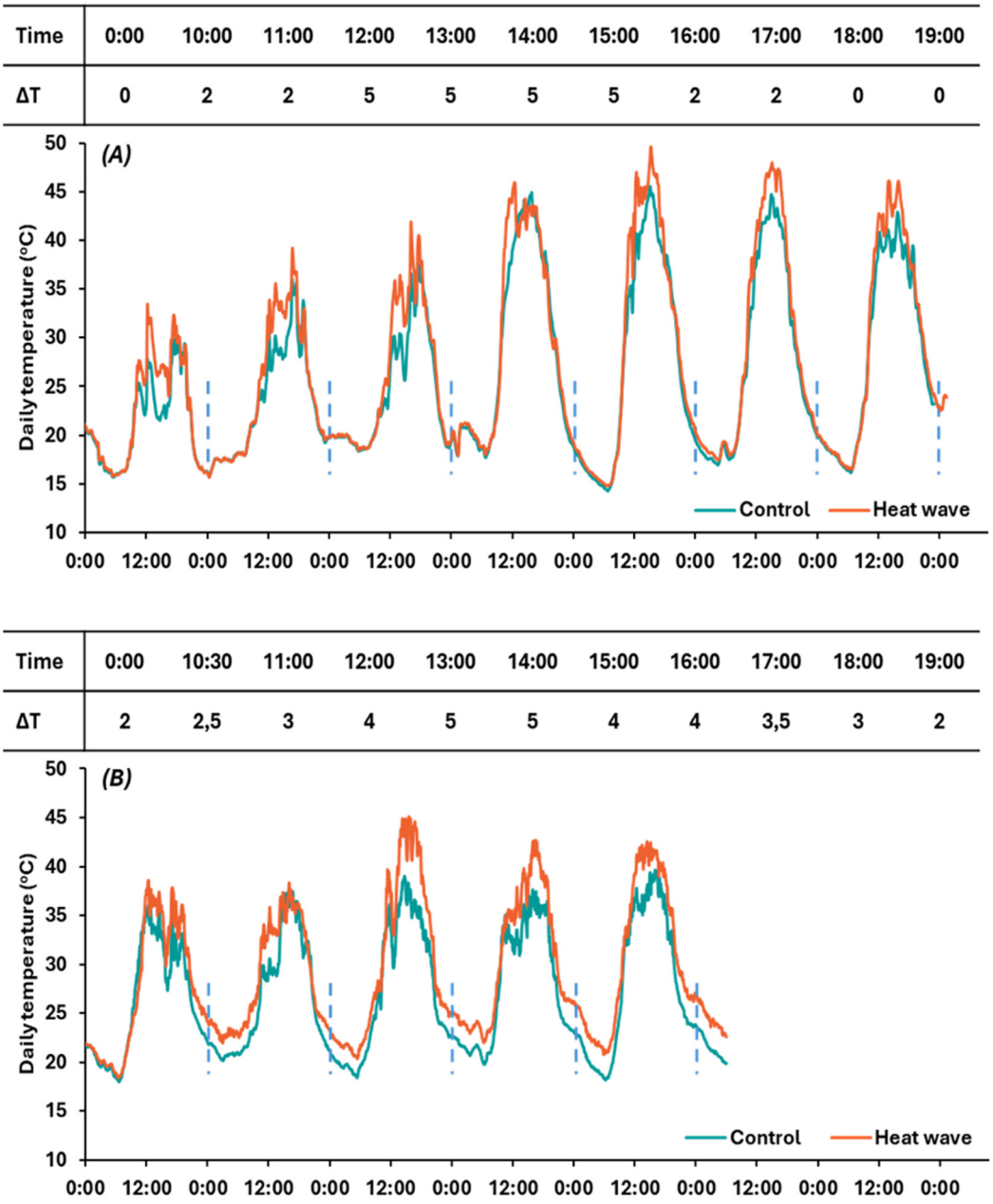

Figure 5. Two experimental heat waves were developed at the University of the Balearic Islands (UIB), simulating typical Mediterranean heat waves. Both Experiment 1 (Figure 5**A**) and Experiment 2 (Figure 5**B**) illustrate the scheduled increases in air temperature over 24-h periods.

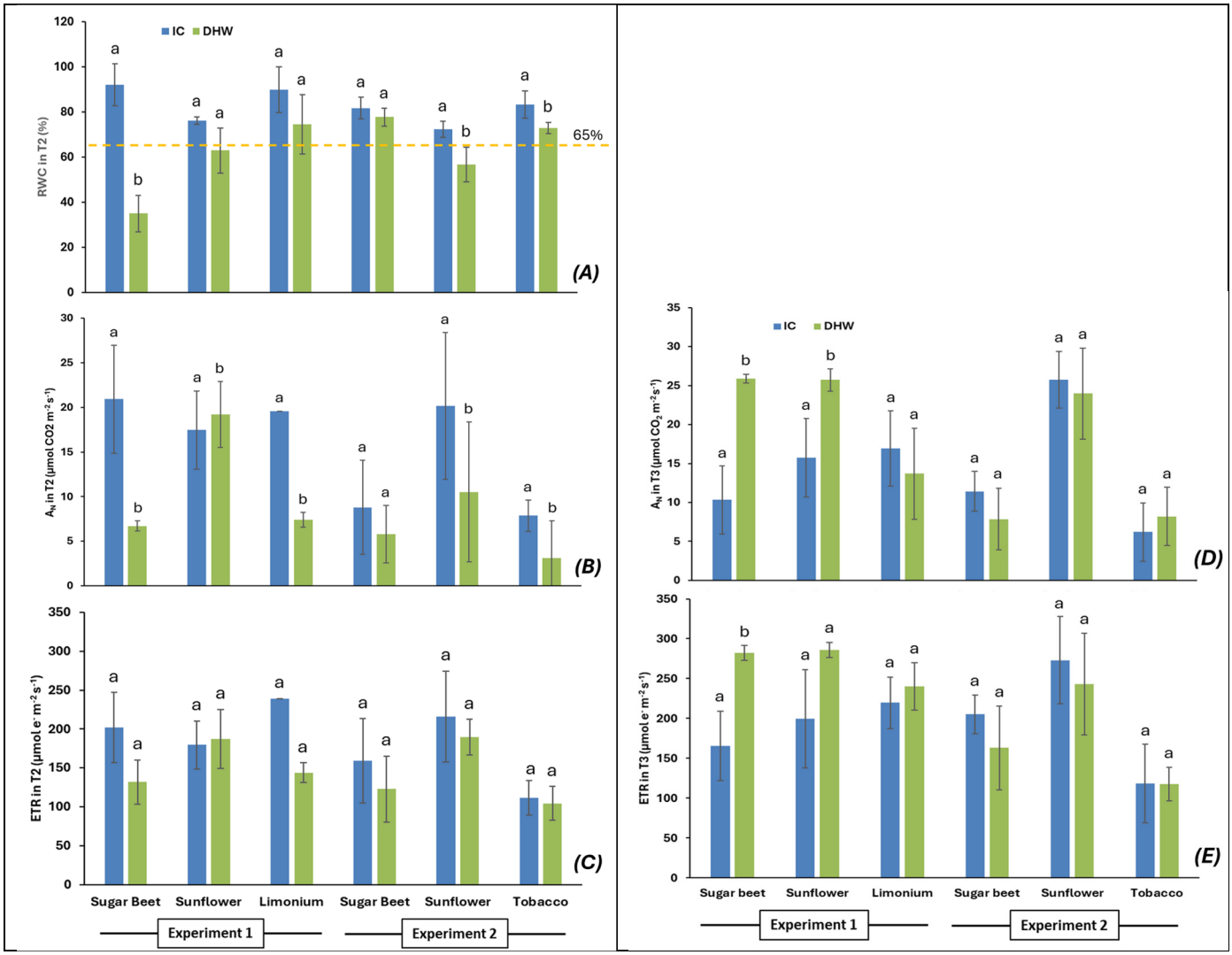

Figure 6. Physiological data indicating the effect of simulated Mediterranean heat waves on experimental plants. Figure 6**A** shows leaf relative water content (RWC) in plants under control conditions (IC) and those subjected to heat wave and drought (DHW), measured immediately after the simulated heat wave (T2). Figure 6**B**,**C** show net photosynthesis (A_N) and the electron transport rate (ETR), respectively, under the same treatments at T2. Figure 6**D**,**E** present A_N and ETR, respectively, for both treatments after a three-day recovery period (T3). The yellow line in Figure 6**A** represents the 65% RCW threshold defined by Lawlor and Cornic (2002) as the point of complete photosynthesis inhibition in C3 plants. Different letters indicate significant differences ($p < 0.05$) between treatments for a given species and/or experiment.

4. Discussion

4.1. An economic commercial device for simulating heat waves

The present paper presents a new system that enables the use of economical commercial infrared heaters, coupled with a microcomputer and dedicated software to accurately simulate natural heat waves under a wide range of conditions, including outdoors. While it is not possible to perfectly simulate past recorded heat waves, especially in the field, due to the inherent differences between current and past weather conditions, our system offers a practical approach.

In experiments simulating heat waves, primarily two types of sensors have been used over the years: thermocouples (contact sensors) and infrared sensors (non-contact sensors) (Bridgham et al., 1999; Nijs et al., 2000; Wan, Luo and Wallace, 2002; Van Peer et al., 2004), each with its own advantages and disadvantages. While IR sensors measure the surface temperature of bodies, contact sensors measure the temperature through direct contact with the body being measured. IR sensors measure temperature by integrating the various temperatures in the coverage area, which can result in unrealistic values on surfaces with low vegetation coverage (Jones et al., 2003; López et al., 2012; Chen, 2015). Additionally, leaf temperature largely depends on transpiration (Nobel, 2020). On the other hand, contact sensors inserted in the canopy are heated by both radiation and convection, which can also influence the measured temperature. Many researchers claim that infrared heaters first heat the surface of plants and then, by convection, the surrounding air (De Boeck and Nijs, 2011; Kimball et al., 2014; LeCain et al., 2015). However, this claim has nuances, as it depends on the efficiency of the heaters, which is linked to the surface working temperature, the emissivity of the material, and consequently, the spectral distribution of the radiation according to Planck's law. The efficiency of IR heaters varies between 40% and 96% depending on these characteristics, indicating how much

energy is used for heating by radiation and how much by convection and conduction (Mor Electric Heating Assoc., 2019). Therefore, using more efficient sources makes heating in windy conditions more effective. In the present study, radiators with 60% efficiency were used, resulting in low efficiency in windy conditions, but justifying the use of contact sensors attached to cardboard or plastic to simulate leaf conditions. In fact, the differential temperatures between leaf and air under the simulated heat wave are sufficiently small to be ignored for the type of leaves and environment analysed here (Supplementary Material S3). Nevertheless, it should be noticed that additional controls or target temperature corrections should be considered if the present heat wave system is to be used with very large leaves, such as tobacco or banana, or under windless environments, such as those in growth chambers.

This is not the first use of IR heaters for simulating heat waves. Harte et al. (1995) presented the first heat wave experiment using infrared heaters, in which the device emitted radiation over a linear space with fixed power throughout the experiment. Later Nijs et al. (1996) introduced a proportional control to maintain a fixed temperature increase in the heated plot compared to the reference plot. Kimball (2005) further improved this by introducing a PID-type controller. He modified the reflector of an infrared heater to expand the coverage area, thus effectively heating a sorghum (*Sorghum vulgare* Pers) cover measuring 1 m wide by 1.5 m long at a height of 60 cm. The researchers concluded that this system offered a radiation angle of 67°. In subsequent research, Kimball et al. (2008) conducted a thorough analysis with the goal of uniformly heating a sorghum plot. Using six heaters positioned at the corners of a hexagon inscribed in a circular plot 3 m in diameter, tilted 45° downward and placed at a height of 1.2 m, it was determined that the optimal ratio between the coverage diameter and height was 0.8. However, this study did not mention the radiation angle. Given that our heaters had a radiation angle of 120° and there was uncertainty about their effectiveness, different configurations were explored. These tests revealed that thermal homogeneity was not a critical factor, as many of the configurations evaluated provided satisfactory results. In the interest of improving future experiments, it is important to note that achieving proper homogeneity involves having identical plots with the same physical and environmental factors: orientation of all elements, shadows and therefore dummy heaters, level of lighting, placement of sensors and heaters, etc. Additionally, it would be beneficial to adopt innovative strategies that ensure uniform heating throughout the plot. A possible improvement could be the use of thermal cameras to visualize and adjust the heat distribution, thereby optimizing the uniformity of the thermal treatment on the plants.

4.2. Physiological effects of combined drought and simulated heat waves

In line with Xie et al. (2020), no significant differences in RWC between IC and DHW treatments for some species were found. However, in agreement with previous observations by Davies et al. (2018), in three cases (sugar beet in Experiment 1 and sunflower in both experiments) RWC values dropped well below 65%, i.e., a value defined by Lawlor and Cornic (2002) as the threshold for complete photosynthesis inhibition in C3 species. Nevertheless, while net CO_2 assimilation generally decreased during heat wave and drought, highlighting the severe impacts of these combined stresses, it did not reach zero, as suggested by Lawlor and Cornic (2002), indicating that plant physiology under a heat wave may differ from that during simple drought. In fact, except for sugar beet during Experiment 1 and Limonium, A_N depression due to combined drought and heat wave was moderate, in line with previous findings in other species by Haworth et al. (2018) and Rashid et al. (2018). In sunflower during Experiment 1, A_N under DHW remained at IC levels despite the very low RWC observed. As previously mentioned, in DHW Limonium, A_N even increased at T2 (drought + heat wave) compared to T1 (drought only).

In the case of sugar beet and sunflower, it is remarkable that the same species grown from the very same seed batch displayed such different results between the two experiments. However, it has to be considered that, while most growing conditions were identical, the heat wave cycles in both experiments differed and plant age at the onset of treatments differed as well (sugar beet plants were ca. 6.5 months-old in Experiment 1 and only 2.5 months-old in Experiment 2; while sunflower was 1.4 months-old in Experiment 1 and ca. 3 months-old in Experiment 2; being tobacco 2.8 months-old and Limonium 1.5 years-old). Additional differences were the natural conditions outdoors or in control plots that the heat wave plots tried to follow adding at each moment the programmed temperature increment, and slight differences in fertilization treatments among years. The different responses observed for each of these two single species despite the relatively small differences in their conditions among the two experiments points to the large complexity of genotype x environment interactions in determining the plant's responses to these stresses.

Interestingly, the electron transport rate (ETR), which is a chlorophyll fluorescence-based indicator used to assess the photochemical and biochemical stability of photosynthesis independently of gas exchange estimates of net CO_2 assimilation, was often not greatly affected by the combined stresses. This suggests that stomatal limitations were the most significant, but again, in a species—and experiment—dependent manner. More intriguingly, in some cases, during recovery (i.e., the simultaneous alleviation of heat wave and drought), ETR of the stressed plants reached values above those of the control plants, most notably in the case of sugar beet in Experiment 1, whose RWC had dropped below 40%. An increase in photosynthetic capacity was shown by Galmés, Medrano and Flexas (2006) in response to long-term plant acclimation to drought and by Galle et al. (2011) in response to repeated irrigation-drought cycles. However, to the best of our knowledge, such an increase upon recovery from combined drought and heat wave has never been reported.

It should be mentioned that an inherent problem of heating experiments is that the relative humidity (RH) and vapour pressure deficit (VPD) surrounding the treatment plants is also

changed. As an example, such changes were observed during experiment 2 (Supplementary Material S4). It can be observed that this problem is much larger during the night than during the day, likely because plants almost do not transpire during the night, so that air temperature is the major driver of RH and, thus, VPD. During the day, although RH was different among treatments, VPD was almost identical in two out of five days and much less increased than RH in heat wave plants than in controls. Since VPD and not RH is the main driver of plant physiological responses, the fact that VPD changes less than RH among treatments suggest that the major observed physiological effects is the heat-wave-related different temperature, but nevertheless it cannot be ruled out that a fraction of them is due to different VPD rather than to different temperature. To the best of our knowledge, this is a limitation of all the systems described to simulate heat waves. Nevertheless, De Boeck et al. (2010), reported that large increases in VPD are a common feature of real natural heat waves.

The insights from the preliminary experiments presented here on the effects of heat waves on plant physiology, using the instrument presented in this study, reflect the potential impact of heat waves on plants, the high heterogeneity in their responses, and even the possibility that investigating heat wave responses could challenge our current perspectives on plant stress responses. The diversity of physiological responses observed within a very limited range of observations, even within the same species under slightly different experimental conditions, highlight the need for intense research in this area on different conditions and species. The affordable device presented here offers an economic and easy opportunity for addressing such research.

Supplementary Materials

The additional data and information can be downloaded at: https://media.sciltp.com/articles/others/2505160945544071/plantecophys-580-Supplementary-final.pdf.

Author Contributions

FC-M directed the development of the device, its implementation in the experiments, and wrote the manuscript with JF. JF conceived the idea and designed experiments 1 and 2. KL conducted the device field testing. MR-O conducted experiment 1 and ÁV conducted experiment 2, with help from MDC, JC and AJF. LC-C developed the control software and assisted in developing the device. MR-C contributed to writing the manuscript and supervised experiments 1 and 2, as well as the overall manuscript preparation. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest

The authors declare that they have no competing interests.

Peer Review Statement

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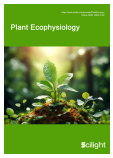

Review

Survival below Zero: Overlooked Aspects of Freezing-Tolerance in Photosynthetic Fern Tissues

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Abstract: The consequences of climate change on the ecophysiology of cryptogams, generally, and in ferns, particularly, are understudied. Phenomena induced by climate change, such as increased frequency of extreme weather events, shifts in precipitation patterns and temperature fluctuations, can significantly impact the physiology and distribution of ferns. The clade of ferns evolved about 400 million years ago and represents the sister group of seed plants. Given their long evolutionary history, ferns offer insights into the resilience and adaptability of plant lineages over geological time scales. Both from an evolutionary and functional perspective, ferns represent a crucial group with intermediate physiological properties between earlier-evolving bryophytes and spermatophytes. Additionally, their life cycle with single-celled reproductive spores and with two independent generations, gametophyte and sporophyte, which have strong anatomical and physiological differences and even different ecological requirements, make ferns a unique case study. While most ferns avoid freezing by living in the tropics or shedding their fronds, wintergreen species deal with subzero temperatures in temperate and cold ecosystems. Additionally, the chlorophyll-containing spores and/or gametophytes of many species also face subzero temperatures. Despite all this, our current knowledge of low temperature- and freezing-tolerance mechanisms in ferns is minimal. In this review we make a comprehensive compilation and re-evaluation of the available knowledge in this topic with a focus on photosynthetic cells/organs of ferns (class Polypodiopsida). We include some recent and relevant findings, identify major gaps and provide baseline for future lines of research.

Keywords: cold stress; frost; gametophyte; pteridophyte; chlorophyllous spore; sporophyte

1. Introduction: Background and Key Concepts on "Below-Zero Plant-Physiology"

Despite the ongoing warming of our planet, coping with freezing conditions is becoming more frequent for many plant species. Due to the presence of warmer temperatures, spring is arriving earlier across much of the globe, which causes plants to emerge from dormancy earlier and increases their exposure to late frosts (Augspurger, 2013). Extreme cold, including cold waves, has become less frequent and less severe at global scale (Lee et al., 2023). However, the frequency of severe winters and late frosts has increased in several regions of both hemispheres as a result of disruptions in atmospheric circulation patterns, which allow cold air from polar regions to reach lower latitudes unexpectedly (Cohen, Pfeiffer, & Francis, 2018; Crimp et al., 2016). Similarly, unseasonal warming episodes during winter or early spring can trigger premature loss of acclimation, leaving non-acclimated tissues susceptible to subsequent cold events. In addition, the reduction of snow cover resulting from warmer winters can impose a double risk on evergreen species, exposing them to both cold and excessive light stress. A comprehensive understanding of the cellular sites and mechanisms underlying injury related to freeze-thaw events in plants is essential to develop breeding programs or genetic modifications aimed at enhancing cold hardiness, as well as for devising effective frost-protection measures in the management of either crops or

at natural environments. While processes and cellular effects of freezing (e.g., intratissular ice formation) on plants were initially studied as early as in the late 1800s (Muller-Thurgau, 1886; Sachs, 1873), many aspects of freeze-thaw effects on plants are still understudied (Arora, 2018). Moreover, while an extensive bibliography is now available concerning crops and conifers, very few works have comparatively focused on ferns (Sutinen et al., 2001; Bannister & Neuner, 2001).

Low temperatures impact plant performance through two main processes (1) a reduction in enzymatic activity and the disruption of membrane function, and (2) the formation of ice and mechanical injury within tissues (freezing stress). The former can induce the overexcitation of the photosynthetic apparatus (photooxidative stress) in chlorophyll-containing tissues, as a result of the unbalance between light absorption by chlorophylls and energy use by photosynthesis, and is regarded as one of the most challenging stress factors in plants and terrestrial algae (Míguez et al., 2017; Van Hasselt & Van Berlo, 1980). Thus, freeze-thaw events predominantly induce two main injuries: oxidative damage induced by reactive-oxygen-species and structural and/or functional perturbations in cell integrity. In vascular plants, freeze-thaw cycles can additionally induce cavitation within the xylem increasing the risk of embolism and producing a third type of damage: loss of hydraulic conductivity (Choat et al., 2011). To investigate this phenomenon, the centrifuge method, which is used to measure xylem resistance to drought-induced cavitation, has been modified to account for additional cavitation caused by freeze-thaw cycles (Davis, Sperry, & Hacke, 1999). The results of these studies indicate a strong correlation between freeze-induced cavitation and average conduit diameter. According to these findings, plants other than ferns with tracheids or smaller xylem conduits (mean diameter <30 μm) do not exhibit freeze-induced cavitation under moderate water stress (xylem pressure = −0.5 MPa). In contrast, species with larger conduits (mean diameter >40 μm) experience almost complete cavitation under the same conditions. Species with intermediate conduit diameters (30–40 μm) show partial freeze-induced cavitation. These results align with a critical conduit diameter of 44 μm, at which or above which cavitation occurs during freeze-thaw cycles at −0.5 MPa. As expected, vulnerability to freeze-induced cavitation is also correlated with hydraulic conductivity relative to the stem cross-sectional area. These findings confirm and expand previous studies, particularly regarding the greater resistance of small-diameter conduits to freeze-induced cavitation. Moreover, the modified centrifuge method, which incorporates freeze-thaw cycles, may be useful in distinguishing the interactive effects of xylem pressure and freezing on cavitation in plants in general (Davis, Sperry, & Hacke, 1999). The loss of hydraulic conductivity can threaten the water transport to photosynthetic cells and thus reduce the photosynthetic capacity of the plant (Choat et al., 2011). Very likely, all these effects, although mostly known from the study of spermatophytes (gymnosperms and angiosperms) also affect to ferns (Figure 1).

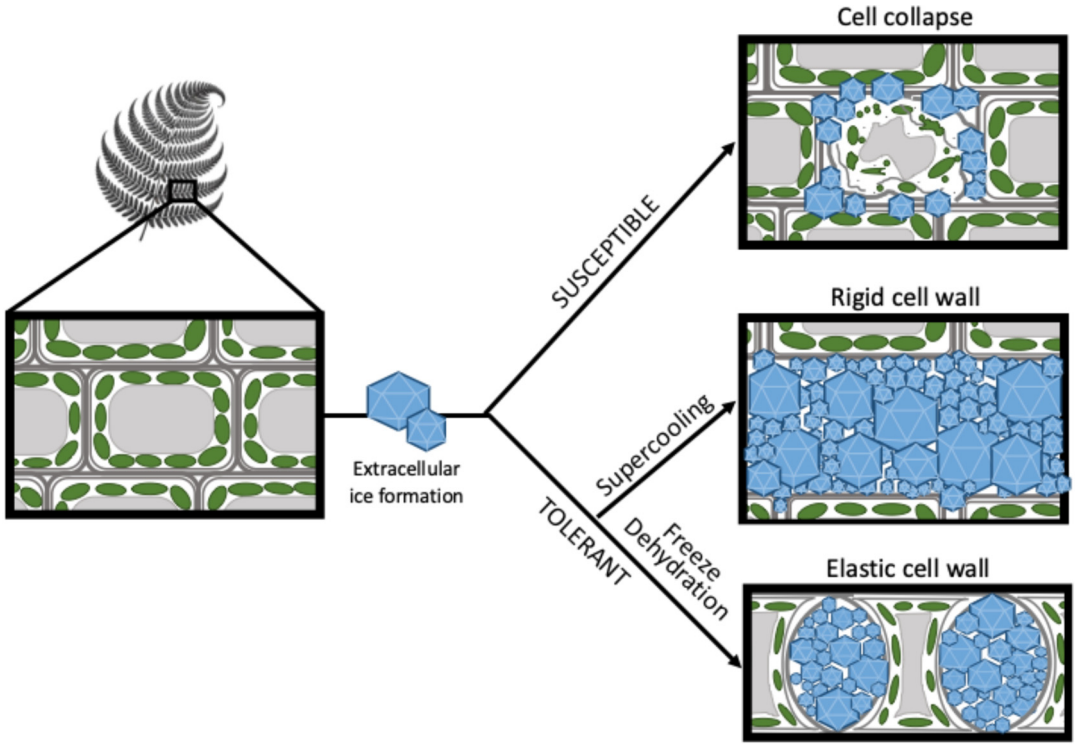

Figure 1. Potential main effects of intra-tissular ice formation on photosynthetic cells, as inferred from studies on seed plants. Essentially, either ice tolerant or ice intolerant cells can be found. Ice intolerant cells will suffer irreversible cell collapse induced by freeze-dehydration and membrane disruption during intratissular ice formation that is initiated in the apoplast. Ice tolerant cells, on the other hand, can show two different strategies. Cells with rigid cell wall will prevent freeze-dehydration and experience supercooling, while ice will be usually accommodated extracellularly in intercellular spaces. Cells with flexible cell wall will reversibly freeze-dehydrate maintaining membrane and organelle functionality upon thawing.

During a natural frost event, plant tissues will typically experience extracellular ice formation, since extracellular fluid has a higher freezing point than the intracellular content as a result of a higher solute concentration. Upon extracellular ice formation, plants can have either ice-tolerant or ice-intolerant cells. Once extracellular ice has been formed, ice-intolerant cells typically collapse (inwards) and suffer irreversible damage to the cell wall (Ashworth & Pearce, 2002), while ice-tolerant cells can either freeze-dehydrate or resist the dehydrating forces and supercool. Some ice-tolerant cells will then freeze-dehydrated and experience "freezing plasmolysis", which means shrinkage of protoplast together with the cell wall, and which will be reversible during thawing (Zhu & Beck, 1991). Other ice-tolerant cells can prevent the plasmolysis and resist the dehydrating forces thanks to relatively rigid cell walls. Finally, after thawing, provided no irreparable damage of the membrane has occurred, melted water will re-enter the cells (in freezing tolerant tissues), which will recover normal functioning. In nature, freezing-tolerant plants typically freeze at relatively slow cooling rates of up to 3 °C/h (Neuner et al., 2013) and show extracellular ice nucleation at relatively high temperatures of −0.5 to −3 °C (Fernández-Marín et al., 2018). During this cooling process, a sequence of processes/structures are affected. With few exceptions, most tissues will first experience a block on photosynthesis (carbon assimilation), while respiration will be maintained until much lower temperatures (Arora, 2018). At cellular level, trans-membrane transporters will be affected (due to alterations in transport proteins), while chloroplast and mitochondria will keep their ultrastructure (Palta & Li, 1978a, 1978b). This makes ultrastructural disorganization a typical symptom of irreversible (lethal) damage after freeze-thaw. Thus, chloroplast, mitochondrial and cell membrane function differ in their sensitivity to a realistic, freeze-thaw stress (Steffen, Arora, & Palta, 1989). While most of these evidences have been obtained in crops, model species, alpine angiosperms and/or conifers, very few studies have focused on fern responses to freezing/thawing events.

2. Biogeography and Phylogeny of Frost-Tolerance in Ferns

The clade of ferns evolved approximately 400 million years ago and is the sister group to seed plants (spermatophytes) (Pryer et al., 2001; PPG, 2016; Shen et al., 2018; Nitta et al., 2022). Specifically, ferns represent one (Polypodiopsida) of the two classes traditionally recognized within pteridophytes, being Lycopodiopsida (lycophytes) the other one. This review is focused on Polypodiopsida. Currently, ferns, with 11,916 species in 337 genera and 51 families, are the second most diverse group of vascular plants, after angiosperms. Ferns comprise four subclasses: Equisetidae (horsetails) with a single genus; Ophioglossidae with 12 genera; Marattiidae with 6 genera; and Polypodiidae (leptosporangiates) which contains the vast majority of extant fern species (PPG, 2016).

The life cycle of ferns is characterised by an alternation of generations: a diploid sporophyte, which produces spores by meiosis, and the haploid gametophyte, which forms the egg and sperm (Figure 2). Most ferns are homosporous, i.e., they produce only one type of spore that gives rise to potentially bisexual gametophytes. However, these gametophytes have several mechanisms that promote outcrossing, such as the asynchronous formation of male and female gametangia (Haufler et al., 2016). Fertilization results in a new sporophyte, which has traditionally been considered the 'dominant' generation based on its significantly larger individual size compared to the gametophyte. The fern gametophyte (prothallus) is generally a photosynthetic heart-shaped individual, although some species have other shapes or even lack chlorophyll (Raghavan, 1989). The gametophytes typically have a surface area of less than 1 cm^2, mostly consisting of a single cell layer, and may have a rudimentary cuticle or lack it entirely. Gametophytes can also be much larger and several cells thick under the notch meristem (Watkins & Cardelús, 2012; Dong et al., 2015) and is thus highly dependent on availability of liquid water for metabolic activity and for the movement of sperms during fertilization (see Section 4 of this review). The alternating generations gametophyte and sporophyte are able to live independently in ferns, in contrast with seed plants.

Fern sporophytes are typically larger and more conspicuous than fern gametophytes. Not to mention that several species (including *Vittaria appalachiana*) are only known to exist as a gametophyte. Like other vascular plants, the sporophytes of ferns consist of three types of vegetative organs: roots, stems and leaves. Fern root systems show high variation comparable to that observed in seed plants (Dong et al., 2015). Stems of ferns are traditionally called rhizomes, most of which are horizontal and located at or just below the soil surface. However, some ferns, particularly tree ferns, may grow their rhizomes vertically, eventually forming a "trunk.". The stems of some ferns with reduced leaves (*Equisetum*) or absent leaves (i.e., *Psilotum* and *Tmesipteris*) are photosynthetic, but this function is normally performed only by the leaves. Fern leaves, called fronds, do have stomata and a well-developed cuticle. Leaf size and shape also vary greatly, and the blade is usually divided into smaller leaflets called pinnae. Spore-producing organs (sporangia) are typically formed on the underside of the blade, and group together into clusters called sori. The spore is the first cell of the gametophytic generation and allows long-distance dispersal by wind. Spores of some species contain chlorophylls and functional chloroplasts (chlorophyllous spores) at maturity (Sundue, Vasco, & Moran, 2011) (see Section 5 of this review). Overall, this alternation of biologically and structurally different photosynthetic generations makes ferns a unique group to study ecophysiological aspects in vascular plants. Despite it, biogeographical and systematic aspects of ferns have been much more extensively addressed than their physiological ecology, as recently reviewed (Anderson, 2021).

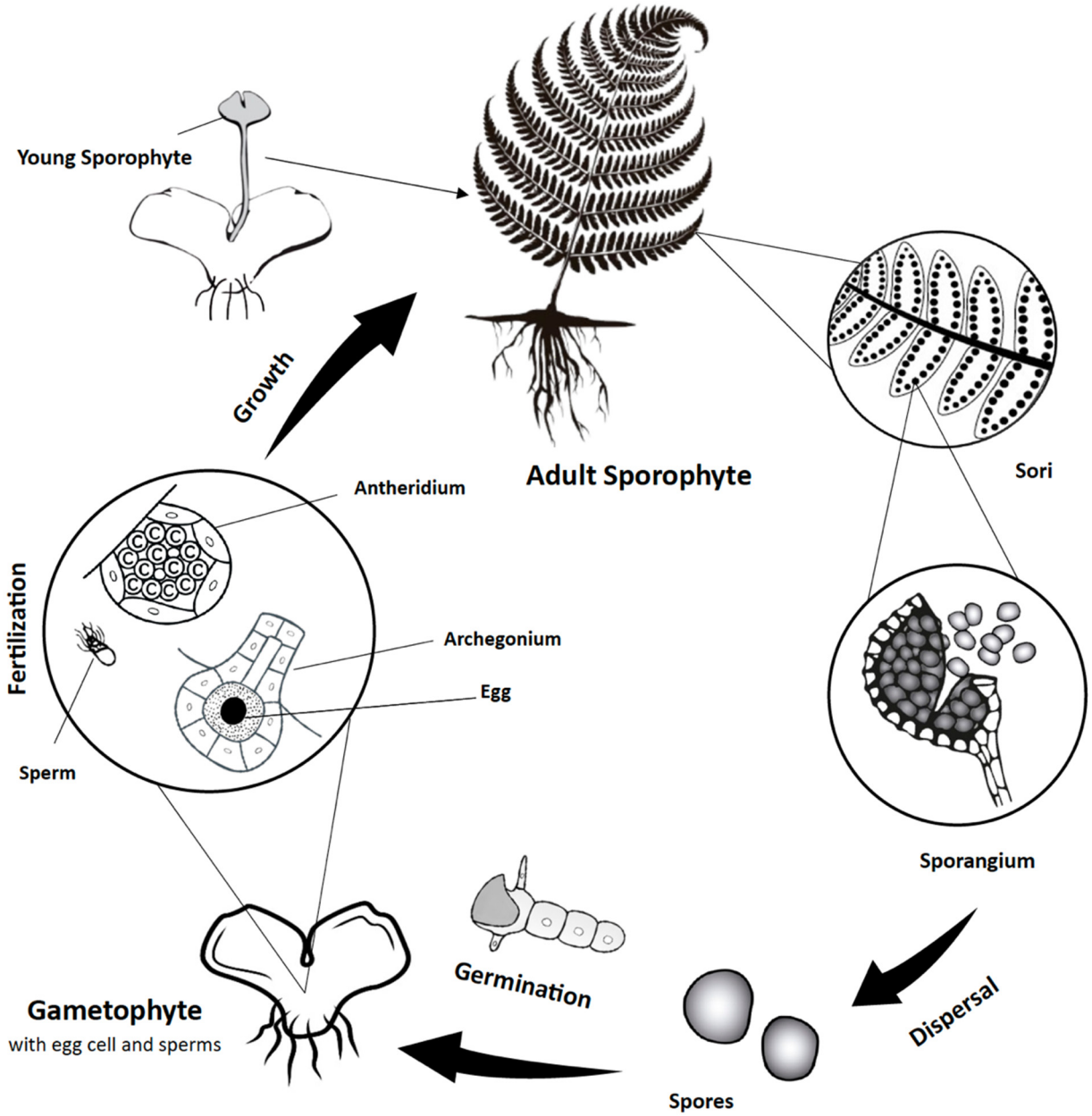

Figure 2. Simplified view of fern life cycle, illustrating the two generations (sporophyte and gametophyte) and the spores, which represent the main focus of this review. Adult sporophyte leaves produce sori with sporangia full of spores. At maturity, the sporangia shed the spores. Spores germinate and produce the prothalli of the gametophyte. Within the gametophyte, fertilization takes place and a new young sporophyte emerges.

In terms of evolutionary diversification, the clade of ferns experienced the most flourishing event during a relatively warm period on the history of the planet (Nitta et al., 2022). Currently, highest species richness is found in tropical moist forests, especially at high elevations (Weigand et al., 2020). Due to these two facts, ferns are often recognized as plants of warm, humid, and shady environments, but many species also thrive in sunny or dry conditions. However, ferns are present in all terrestrial biomes of the world, including the taiga and tundra, and grow well above the timberline in mountains. Therefore, many ferns inhabit areas that experience freezing events, whose intensity, frequency, and temporal distribution vary depending on latitude and altitude. For instance, arctic species are largely exposed to freezing temperatures (Gureyeva & Timoshok, 2016), ferns of temperate habitats (mid to moderately high latitudes) experience subzero temperatures seasonally (wintertime), and ferns at high elevations, even in tropical regions, may face subzero temperatures on a daily basis (Kessler & Kluge, 2022; Sato & Sakai, 1981a; Hill, 1976). Although the sporophyte individual is highly tolerant to overcome hard winters in many species, its annual fronds can be sensitive to freezing. As an example, the underground rhizome of the boreo-alpine fern *Botrychium lunaria* is freeze-tolerant, but its fronds are sensitive to spring frost (Watt, 1981). In this review we focused specially on the freezing tolerance of photosynthetic tissues, including leaves, chlorophyllous stems, as well as chlorophyllous gametophytes and spores. We standardized species names using the Taxonomic

Name Resolution web application, version 5.1 (Boyle et al., 2013) and adopted the families recognized by PPG I (2016).

The highest latitudinal record for fern species has been documented in the Arctic, where a few species exceed 80° N in Canada and Greenland (GBIF Secretariat, 2022), while no pteridophytes are currently inhabiting Antarctica (Peat, Clarke, & Convey, 2007; Colesie et al., 2023). Early works on Russian Arctic flora enumerated up to 22 species of ferns (Gureyeva & Timoshok, 2016). Six species are included in the flora of Svalbard (latitude 74–81° N): *Botrychium lunaria*, *Cystopteris fragilis, Equisetum arvense, E. scirpoides*, *E. variegatum* and *Woodsia glabella* (Stavdal, 2020; Rønning, 1996). Nevertheless, most of these species lose their leaves or, in the case of *E. arvense*, their green shoots at the end of summer, and their sporophyte photosynthetic tissues do not necessarily face freezing temperatures. By contrast, *Equisetum scirpoides* and *E. variegatum* have evergreen shoots adapted to the extreme cold of the Artic winter. An early work compiling the temperature-resistance of Alaskan plants revealed interesting data about *E. scirpoides*. It has a very strong resistance to low temperature (−80 °C) plus the capacity to modulate it across the time, presenting a higher resistance in winter and even higher upon an unusual strong winter occurred in 1968/1969 (Riedmüller-Schölm, 1974).

Fern biodiversity across elevational gradients has been evaluated in the highest mountain ranges on Earth. For instance, Salazar et al. (2015) evaluated diversity patterns in Andean tropical forests up to 4000 m in elevation. None of the locations considered, however, presented average temperatures below zero (provided the data shown in the original articles). Also in America, another study conducted in the Trans-Mexican Volcanic Belt deeply evaluated fern biodiversity, and highlighted three species for the highest elevational upper limit: *Asplenium castaneum* (4,569 m), *Polystichum speciosissimum* (4490 m), and *Cystopteris fragilis* (4377 m) (Hernández-Cárdenas et al., 2019). More recently, in Asia, Umair (2023) and coworkers have evaluated climatic factors affecting fern species richness along an elevational gradient (100–5300 m a.s.l.) at the Tibetan Plateau. In this study, among the 441 fern species found, the family Dryopteridaceae was the richest, with 97 species, 32 of which exceeded 4000 m in elevation (Umair et al., 2023). Some ferns that grow even at an elevation of 5300 m represent a remarkable milestone in this work by Umair and co-workers. In Europe, Marini et al. (2011) studied fern richness along a 0–3000 m elevational gradient in the Alps and suggested that lethal effects of frost are among the factors explaining the decline in richness at high elevations. However, none of these studies addressed ecophysiological questions. Accordingly, Kessler and Kluge (2022) recently brought attention to the lack of ecophysiological knowledge on this aspect after a thorough review of available data. They also concluded that numerous physiological processes determine the elevational ranges of ferns, including not only cold and drought at high elevations but also drought, high temperatures and, importantly, limited frost tolerance at low elevations (Kessler & Kluge, 2022).

3. Freezing-Tolerance in Fern Sporophytes

Among the three types of photosynthetic cells considered in this review (sporophyte, gametophyte, chlorophyllous spores), sporophytes have been more widely studied regarding freezing-tolerance in terms of number of genera and families evaluated (Figure 3, see also Supplementary Table S1). Despite of it, only 14 families have been studied so far. Among them, Dryopteridaceae, Aspleniaceae, Blechnaceae and Polypodiaceae comprise most of the available data (Figure 3). Table 1 presents a summary of the findings on freezing-tolerance in ferns, categorized by tissue type (sporophyte, gametophyte, spore). In the next subsections, several aspects related to either the vascular system, the evolution of research on this topic, the ice formation and propagation and the photoprotection mechanism will be summarised.

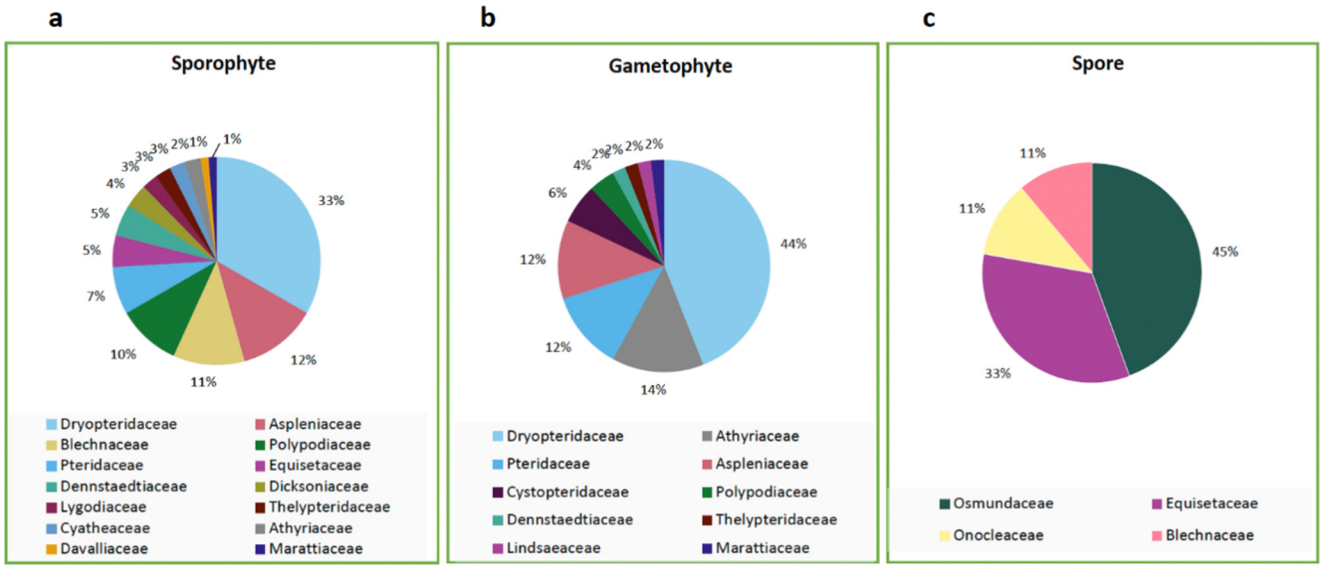

Figure 3. Data available per fern family in the bibliography regarding freezing tolerance of photosynthetic tissues: (a) sporophyte fronds; (b) gametophytes, and (c) photosynthetic spores. Charts are based on data compiled in Table 1 and in Supplementary Table S1.

Table 1. Compilation of already published works in which freezing tolerance was tested in ferns. Data are organized by tissue type. The temperature tested and the key physiological/biological parameters used to test for tolerance in the original references is also shown.

Tissue Type	Freezing Tolerance Range (°C)	Total Number of Species	Key Parameters	Reference
Sporophyte	(−2 to −47)	90	Ice nucleation, leaf damage, regreening, Survival, chlorophyll fluorescence (Fv/Fm), Plasma Resistance, Fatty acid composition of fronds	Sato & Sakai, 1981a; Kappen, 1964, 1966; Bannister, 1973, 1984, 2003; Sato, 1982; Bannister & Fagan, 1989; Noodén & Wagner, 1997; Tessier, 2018; Fernández-Marín et al., 2021a; Voronkov & Ivanova, 2022; Farrar, 1978; Warrington & Stanley, 1987; Bremer & Jongejans, 2010; Klinghardt & Zotz, 2021; Nokhsorov et al., 2021
Gametophyte	(−3 to −196)	57	Survival, regreening, habitat analysis	Sato & Sakai, 1981a; Kappen, 1965; Sato, 1982; Farrar, 1978; Pickett, 1914
Spore	(−18 to −196)	6	Germination rate, survival	Kato, 1976; Whittier, 1996; Ballesteros et al., 2011; Magrini & Scoppola, 2012; Li & Shi, 2014

3.1. Subzero temperatures and vascular system

The sporophyte is typically the vascular generation of ferns. Tracheids have been reported in fern gametophytes, albeit rarely (Whittier, 1976; Goswami & Sharma, 1996). As such, photosynthetic organs, i.e., leaves and, in some species stems, contain a specific tissue for water and nutrient transport, the vascular system, which consists of bundles of specialised cells including tracheids (Pittermann et al., 2011; McElwain, 2011). This is a relevant feature when facing freezing temperatures. Specifically, in addition to photoprotection and maintaining suitable osmolyte and antioxidant metabolism for preserving cell integrity, fern fronds and stems must also be prepared for rapid ice propagation through the xylem and the potential embolization of its conduits during freeze-thaw cycles (Figure 1). Drought-induced xylem embolism has already been quantified in many fern species, although the peculiarities of their stem anatomy result in frequent discrepancies when compared with seed-plants (Pittermann, Baer, & Sang, 2021; Prats & Brodersen, 2020). On the contrary, freeze-thaw induced embolism has not been evaluated on ferns so far.

3.2. Subzero temperatures tolerance in sporophytes: A chronological revision of early literature

Early works evidencing freezing tolerance in fern sporophytes were conducted by Kappen (1964, 1965, 1966) in the 60s (see also Supplementary Table S1). He observed that freezing-tolerance varies both within species along the year, being higher (fronds withstand lower temperatures) in winter than in spring, and among species. After testing 7 different species (*Cystopteris fragilis, Phegopteris connectilis, Gymnocarpium dryopteris, Dryopteris carthusiana, Athyrium filix-femina, Thelypteris limbosperma,* and *Pteridium aquilinum*), tolerance ranged between −2.5 to −12 °C (Kappen, 1964) (Supplementary Table S1). He also brought attention to the relevance of water content of the frond in the survival to low temperature. Specifically, he evidenced that hydrated leaves of *Polypodium vulgare* withstand −17 °C, and that the same leaves are able to survive even immersion in liquid nitrogen when in the dry state (Kappen, 1965, 1966; Sakai & Larcher, 1987).

In the 70s, a couple of studies complemented these results. Bannister (1973) reported the effects of an early frost event (of around −4 °C) at the end of summer over a couple of species. Among other interesting observations, he highlighted the vegetative fronds of *Blechnum spicant* and *Dryopteris filix-mas* as tolerant, while pinpointed some damage in the fertile fronds of *Blechnum spicant*. Few years later, Riedmüller-Schölm (1974) compiled an extensive set of data under natural conditions in Alaska for more than one year. He evidenced (i) very tough tolerance to low temperature in *Equisetum scirpoides* in winter (down to −80 °C, from November to January), and (ii) a strong seasonal variation (acclimation) in this tolerance, which was only around −10 °C in the warmer months (May to August) (Riedmüller-Schölm, 1974).

An increasing number of works was published along the 80s. Sato and co-workers did an extensive work testing

freezing tolerance of a wide number of species (>60) in the north of Japan and comparing, in many cases, sporophytes with other tissues (Sato & Sakai, 1981a, 1981b; Sato, 1982). Frost resistance of the leaves was related to the phenology and ecology of the species, with the leaves of wintergreen and evergreen ferns generally being the most tolerant in the range of −20 to −40 °C (Supplementary Table S1). Almost in parallel, Bannister and co-workers evaluated tolerance to subzero temperatures in other species in New Zeland (Bannister & Fagan, 1989) They concluded that (i) for a similar latitude, fern species original from the Northern Hemisphere had higher frost tolerance than Southern Hemisphere species (Bannister, 1984), (ii) frost tolerance varies within a species temporally and spatially (with season and elevation) (Bannister & Fagan, 1989) and (iii) fertile fronds are less tolerant than vegetative fronds, i.e., in *Blechnum penna-marina* (Bannister, 1984). At the end of this decade, Niklas (1989) contributed to the understanding of possible tolerance mechanisms and highlighted that *Equisetum hyemale* tolerance to subzero temperatures was based on the extracellular freezing of water in the pith cavities of its aerial shoots. Later studies by Schott, Voigt, and Roth-Nebelsick, (2017) further confirmed this by demonstrating that, in addition to the pith cavity, ice formation also occurs in the vallecular canals, suggesting a crucial role of pre-existing lacunae in cold resistance. Thus, main findings until the 90s on fern sporophyte tolerance to freezing indicated that (1) relatively diverse wintergreen taxa can withstand subzero temperatures; (2) interspecific differences are remarkable; (3) most species show seasonal acclimation with stronger tolerance in winter; (4) when present, fertile fronds can show different tolerance than the vegetative fronds within the same species, (5) frond age can be a relevant factor for freezing tolerance and (6) water content of the frond greatly influence the tolerance extent at least in desiccation tolerant species. From the 90s onwards, different ecophysiological aspects have been addressed mostly related to (i) the role of snow protection, (ii) the physiology of ice formation and propagation and (iii) the relevance of photoprotection as is summarised in the following subsections.

3.3. Snow protection

The buffering effect of snow cover over extreme low temperature during wintertime as well as its shading effect (i.e., providing photoprotection) are well known for flowering plants (Briceno et al., 2014; Neuner, Ambach, & Aichner, 1999; Bannister et al., 2005; Wipf et al., 2015). Although largely understudied, similar effects have been observed in ferns. Nooden and Wagner (1997) already evidenced in the 90s that snow cover protection can be so relevant for wintergreen species that some have evolved a specific anatomical mechanism to facilitate snow covering. *Polystichum acrostichoides* and *Dryopteris intermedia* possess a controlled-senescence mechanism at the base of the stipe, which causes it to soften and bend, resulting in the prostration of the fronds. This adaptation helps maintain xylem flow and provides cold protection beneath the snow. Throughout winter, the fronds retain chlorophyll content and PSII photochemical efficiency, remaining capable of CO_2 assimilation at a rate of approximately 2 μmol m^{-2} s^{-1} (Noodén & Wagner, 1997). Additionally, decreasing temperatures lead to the formation of a necrotic zone at the stipe base, allowing fronds to lie flat on the ground and maintain optimal leaf temperatures without disrupting hydraulic function. This mechanism, along with the preservation of xylem conductivity, supports the survival of *P. acrostichoides* in northeastern forests, enabling it to sustain photosynthesis throughout winter (Prats & Brodersen, 2020). Deepening on the consequences of this ecophysiological strategy, the effect of unusual diminished snowfall in the overwintering survival of *Dryopteris intermedia, Dryopteris marginalis* and *Polystichum acrostichoides* was evaluated more recently (Tessier, 2014). The results added new evidences on the protective effect over overwintering leaves, so more damage was found on those not covered by snow during winter. The study also revealed higher damage on the leaves with lower Leaf Mass per Area (LMA) value, suggesting higher susceptibility to frost damage for thin leaves (Tessier, 2014). More recently, two manipulative experiments performed in the field with *Polystichum acrostichoides*, by artificially changing the prostrate into erect position of the frond during winter, shed more light into this aspect (Forget, Parker, & Hughes, 2018; Tessier, 2018). Prostration seems to additionally benefit the leaves by reducing leaf-to-air vapor pressure, enhancing stomatal conductance and photosynthesis during winter. Additionally, prostration enabled slight warming of the fronds on sunny days becoming leaf temperature closer to its optimal for photosynthesis (Forget, Parker, & Hughes, 2018).

3.4. Ice propagation and location within the photosynthetic tissue

While physical aspects of ice formation and propagation are crucial to understand species strategies to deal with freezing temperatures, these parameters have been rarely evaluated in fern sporophytes. Only a couple of works have evaluated this phenomenon in ferns. In *Equisetum hyemale*, extracellular ice formation occurs in the pith cavities and in the vallecular canals within the aerial shoots (hollow stems), inducing tissue dehydration (Niklas, 1989; Schott, Voigt, & Roth-Nebelsick, 2017; Konrad, Schott, & Roth-Nebelsick, 2019). In another recent study, ice propagation was evaluated in 5 temperate European species by using Infrared Differential Thermal Analysis (IDTA): *Adiantum capillus-veneris, Asplenium ceterach, A. trichomanes, A. scolopendrium, and Polypodium vulgare* (Fernández-Marín et al., 2021a). Ice propagation pattern within the frond was fast and disorganized in the freezing-sensitive species, compared to a slower and segmented pattern in the freezing-tolerant species. These observations suggest anatomical controls and barriers to ice propagation probably related to xylem anatomy. Ice nucleation temperature was also evaluated in this work by differential

scanning calorimetry in well hydrated fronds. The ice nucleation temperature occurred around −3 °C in the evaluated species in the field and ranged between −3 and −9 °C in ex situ assays in the lab (Fernández-Marín et al., 2021a).

3.5. Cell wall properties and osmotic adjustment

Very few works have dealt with physiological aspects at cell level that may be involved on freezing-tolerance in fern fronds. By combining pressure-volume curves with gas-exchange measurements, Lösch and co-workers compared the physiological performance of three temperate wintergreen species belonging to the same genus (*Asplenium*) and co-habiting in a limestone rock: *A. trichomanes*, *A. ruta-muraria* and *A. scolopendrium* (Loesch et al., 2007). They found that *A. trichomanes* and *A. ruta-muraria* had fully poikilohydric fronds in winter (that is, tolerant to desiccation), whereas *A. scolopendrium* utilized osmotic adaptation as strategies to deal with winter stress (Loesch et al., 2007). This last species changed its leaf osmotic potential and increased the rigidity of its cell walls (higher modulus of elasticity) during autumn, so the leaves did not dehydrate during wintertime. Interestingly, *A. trichomanes* additionally shifted its optimal temperature for photosynthesis from 20–25 °C in summer to 8–10 °C in winter (Loesch et al., 2007).

3.6. Photoprotection

The deleterious effects of subzero temperatures plus high irradiance for fern leaves can be inferred from evidences obtained with evergreen angiosperms. Thus, most of the studies considering snow-cover demonstrated exacerbated damage to the exposed leaves (uncovered by snow) when additionally exposed to high light for flowering plants (Briceno et al., 2014; Bannister et al., 2005). Although not much research has been conducted with ferns in these directions, at least two studies evidence the need for photoprotection in wintergreen fern fronds (Fernández-Marín et al., 2021a; Putzier, Polich, & Verhoeven, 2022). Both works demonstrate the activation of the photoprotective xanthophyll cycle induced by freezing even in the absence of light, in five different species: *A. trichomanes*, *A. scolopendrium*, *Polypodium vulgare*, *P. virginianum* and *Adiantum capillus-veneris.* Interestingly, this enzymatic response in darkness was first described during the dehydration of the desiccation tolerant fern *Asplenium ceterach* (Fernández-Marín et al., 2009). The dark-induction of zeaxanthin formation has also been observed in vascular plants tolerant to low temperatures in response to freezing (Fernández-Marín et al., 2018). Apparently, this carotenoid can help in the maintenance of integrity of the chloroplast photosynthetic membranes in addition to its direct implication in the dissipation as heat of excessive light energy (Fernández-Marín et al., 2021b). In other species, such is the case of *Dryopteris filix-mas*, the plant optimizes its photosynthetic period through phenological changes, such as differences in the timing of frond development and longevity between sterile and fertile sporophytes. While the fertile sporophytes are summer green, the sterile sporophytes, produce new fronds in mid-summer and remain green throughout winter, extending their photosynthetic period and increase their productivity. This suggests that plants use various mechanisms, including structural and physiological adjustments such as changes in timing of leaf growth or protection of photosynthetic structures, to maintain their photosynthetic efficiency under varying environmental conditions (Bauer, Gallmetzer, & Sato, 1991).

4. Ecophysiological Aspects of Fern Gametophytes and Below-Zero Temperatures

Since gametophytes lack specialized epidermis, stomata, and vascular tissue, they are poikilohydric (López-Pozo et al., 2018), meaning they do not have mechanisms to maintain their water content. As a consequence, gametophytes are exposed to different selective pressures than their conspecific sporophytes, resulting in niche specialization that ultimately determines the establishment of fern populations (Krieg & Chambers, 2022). One of the first studies directly addressing freezing tolerance in ferns was conducted in gametophytes of *Phlebodium aureum* in the 30s (Stuckey & Curtis, 1938). The authors found cellular sensitivity to freezing, and described the freezing sequence of cellular compartments: cytosol, followed by mitochondria and chloroplast (Stuckey & Curtis, 1938).

Given that ferns are present in climates with cold winters, and that the lifespan of fern gametophytes can be longer than one year, as has been recently documented by photographic analysis in Switzerland (Farrar et al., 2008), it is obvious that they have to face recurrent episodes of freezing temperatures. In fact, gametophytes have been found in extreme environments such as the periglacial areas of the Altai mountains (Gureyeva & Timoshok, 2016) or the mountains of Hokkaido (Sato, 1982). Gametophytes are considered to be more stress tolerant than sporophytes (Kessler & Kluge, 2022; Gureyeva & Timoshok, 2016; Farrar et al., 2008). This is, for example, the case of the greater desiccation tolerance of gametophytes in some tropical ferns (Watkins et al., 2007). Furthermore, gametophytes are well adapted to surviving winter in temperate/cold climates. This was described in a study performed in Hokkaido (Japan) showing that gametophytes are in general more tolerant to freezing than their respective sporophytes (Sato, 1982; Sato & Sakai, 1980) and in fact most species were able to survive to experimental treatments at −40 °C (Sato & Sakai, 1981a). Furthermore, in another study, these authors described that the marginal cells of *Polystichum retroso-paleaceum* were able to recover even after exposure to −196 °C (Sato & Sakai, 1981b). Survival to −23 °C under field conditions in Switzerland has been documented by photographic analysis in gametophytes of *Athyrium filix-femina* and *Dryopteris dilatata* (Schneller & Farrar, 2022). In contrast, in a field study in Florida, gametophytes of subtropical *Lygodium microphyllum* and the invasive *L. japonicum* were not able to survive to 6-h exposure to −2.2 °C (Hutchinson & Langeland, 2014), limiting the expansion

of the latter northwards. These results indicate that freezing tolerance is not a universal trait among fern gametophytes.

The so-called 'independent gametophytes' are a well-known example of increased abiotic stress tolerance in the gametophytic generation. These gametophytes are capable of reproducing and dispersing through asexual propagules, which allow them to form populations without the involvement of sporophytes (Figure 2). Independent gametophytes have been found in several epiphytic and epilithic species of five fern families (Yoneoka et al., 2024). Interestingly, in some species, the sporophytes have an exclusively tropical distribution, but the gametophytes reach temperate latitudes due to a greater tolerance to low temperatures and desiccation (Farrar, 1990). Independent gametophytes of exclusively temperate species also withstand harsher conditions at higher latitudes compared to their conspecific sporophytes (e.g., Ben-Menni et al., 2022). The different ecophysiological tolerances of gametophytes and sporophytes can partially explain the observed niche segregation between them, but other factors, such as dispersal and competition may also be relevant. Asexual propagules are larger and, as a result, have less effective wind dispersal compared to spores (Farrar, 1990). Thus, it has been hypothesized that the current populations of independent gametophytes in temperate latitudes could have originated from nearby sporophyte populations that were eliminated by glaciations due to their lower tolerance to extreme cold (Farrar, 1990; PPG, 2016).

The physiological responses of gametophytes to low and freezing temperatures have been rarely studied. It is known that the response of carbon assimilation to low temperature is similar to that of sporophytes, but their respiration is less sensitive to temperature (Johnson et al., 2000). One of the consequences of low temperatures is the metabolic impairment between light absorption and energy use, which is compensated by the so-called photoprotection mechanisms. In this sense it has been described that low temperatures induce a light avoidance chloroplast relocation mechanism in gametophytes of *Adiantum capillus-veneris* (Kodama et al., 2008). This mechanism, which is activated by blue light, also contributes to photoprotection by reducing the absorption of photosynthetic radiation.

A high proportion of fern gametophytes can be considered as desiccation tolerant (López-Pozo et al., 2018; Blake-Mahmud et al., 2024). Since desiccation tolerance and freezing tolerance share many common mechanisms (Verhoeven, García-Plazaola, & Fernández-Marín, 2018), and in fact one of the main effects of freezing is cellular dehydration (Figure 1), it could be expected a constitutive protection against freezing. Moreover, studies of cryopreservation of gametophytes show that a pre-treatment with abscisic acid (ABA) is required to enhance survival (Mikuła, Jata, & Rybczyński, 2009), suggesting a role for ABA in the activation of freezing tolerance mechanisms.

5. Ecophysiological Aspects of Chlorophyllous Fern Spores and Below-Zero Temperatures

Chlorophyllous fern spores occur in around 14% of fern species, mainly included in the families Equisetaceae, Hymenophyllaceae, Onocleaceae, Osmundaceae, and Polypodiaceae (Mellado-Mansilla et al., 2021, 2022). The presence or absence of chlorophyll in spores reflects the diverse strategies that ferns have evolved to optimize their chances of survival and reproduction in varying ecological niches (Mellado-Mansilla et al., 2021; López-Pozo et al., 2019). In particular, the fern species with chlorophyllous spores belong to two ecologically distinct functional groups: epiphytes abundant in humid tropical forests at middle or high elevation; and hydrophytes inhabiting waterlogged soils, particularly in temperate regions, belonging to Onocleaceae (e.g., *Onoclea struthiopteris*), Osmundaceae (e.g., *Osmunda regalis*), and Equisetaceae (*Equisetum* spp.). The presence of chlorophyll on spores has been associated with a lack of dormancy, shorter longevity, and rapid germination (Lloyd & Klekowski, 1970). Moreover, chlorophyllous spores have also been associated with a lower tolerance to stressful environmental conditions, including low or below-zero temperatures, compared to achlorophyllous spores (Lloyd & Klekowski, 1970; Ballesteros, Hill, & Walters, 2017).

Most of the studies regarding chlorophyllous fern spores and below-zero temperatures are focused on effective preservation methods for germplasm banks research or educational purposes (Kato, 1976; Whittier, 1996; Ballesteros et al., 2011). Overall, preservation studies performed in achlorophyllous spores under below zero-temperatures (−10 to −30 °C) indicate a negative effect on their viability (Lindsay, Williams, & Dyer, 1992; Quintanilla et al., 2002; Aragon & Pangua, 2004). In the case of chlorophyllous spores, negative effects of freezing temperatures have also been observed in *Osmunda japonica* (Li & Shi, 2014). However, some chlorophyllous spores present less sensitivity to low temperatures, as observed in *Equisetum ramosissimum* and *Osmunda regalis* (Ballesteros et al., 2011; Magrini & Scoppola, 2012), which show a decrease in spore viability but do not loss it completely at −20 °C for a period of at least 28 months. As for low non-freezing temperatures, green spores of *Osmunda regalis*, *O. claytoniana*, and *Osmundastrum cinnamomeum* also retained viability after long periods of dry storage at fridge temperatures between +2 and +6 °C (Stokey, 1951). Beyond natural freezing temperatures, studies focusing on preservation or storage have also proved the capacity of chlorophyllous spores to survive very low freezing temperatures. This is the case of the green spores of *Equisetum hyemale*, which are able to maintain their viability after been frozen at −70 °C for over a year (Whittier, 1996), and the particular the case of the green spores of *Osmunda regalis*, which can survive up to 18 months stored in liquid nitrogen (Pence, 2000).

The microscopic size of spores (a few tens of micrometres in diameter) is a major challenge for field studies. Although the phenology of spore release has been studied in many species, especially in temperate regions of the Northern Hemisphere (Lee, Huang, & Chiou, 2018), the tolerance of spores to low temperatures after dispersal has not been studied under natural conditions. Spore of many species of northern temperate regions are dispersed from June to September and

germinate before winter begins, giving rise to gametophytes that survive the winter (Sato, 1982). However, there is also indirect evidence of the presence and survival of spores during winter. Specifically, in *Onoclea sensibilis*, a species that reaches high latitudes in North America, the sporangia retain mature green spores during the winter and release them in early spring (DeMaggio & Stetler, 1980; Suissa, 2022). Moreover, in some *Equisetum* species, such as *E. arvense,* spore formation and dispersal take place during early spring, a time in which the spores are exposed to cold temperatures below 5 °C in many areas of the species' range (Cody & Wagner, 1981; Zhao et al., 2015).

The capacity to withstand freezing is closely related to desiccation tolerance. Indeed, studies on spore conservation in germplasm banks highlight the drought tolerance of chlorophyll-containing spores, which is crucial for their survival at subzero temperatures (Ballesteros et al., 2011; Magrini & Scoppola, 2012; Li & Shi, 2014). This is the case for green spores of the fern *Onoclea struthiopteris*, which exhibit significant physiological adaptations that allow them to survive below-zero temperatures during winter, when spore dispersal occurs (López-Pozo et al., 2019). Mature spores of *O. struthiopteris* demonstrated good physiological recovery after desiccation and freezing, showing resilience under harsh conditions. This tolerance is associated with the antioxidant capacity of the spores, as seen in the high levels of α-tocopherol and proline that protect them from oxidative stress. Another example are the green spores of *Osmunda regalis*, which exhibit desiccation tolerance (López-Pozo et al., 2019) and are able to maintain their viability after stored at −20 °C without any pre-treatment (freshly collected with a water content of approximately 17%) (Magrini & Scoppola, 2012). Nonetheless, very few studies have focused on the effects of freezing on green spores and more data are required to understand this interaction between freezing and desiccation tolerance on chlorophyllous spores.

6. Conclusions

Ferns are able to live in cold ecosystems, but very few is known on the physiological mechanisms that ferns have developed to face winter stress. Most of the scarce knowledge currently available on freezing tolerance of photosynthetic tissues in ferns has been obtained either under natural, garden or laboratory conditions mainly in temperate regions with cold winters (Supplementary Table S1). Thus, studies on species covering a much wider biogeographical range would be needed. There are also significant taxonomic biases and gaps that need to be addressed (Figure 3). Overall, although interspecific differences are evident, the gametophyte generally shows higher tolerance than the sporophyte within a single species (Sato, 1982). Fertile vs. vegetative leaves may have different frost-tolerance (being vegetative frond more tolerant), although very scarce literature is available in that sense. There are also interspecific differences in the lethal temperature, being some species able to tolerate temperatures ≤−40 °C in their photosynthetic tissues (Riedmüller-Schölm, 1974; Sato, 1982). Another overall observation is the importance of acclimation and hardening, so a same population can show enhanced tolerance during winter time when compared to summer or after a frost event (Riedmüller-Schölm, 1974; Bannister, 1984; Voronkov & Ivanova, 2022). Water content of the tissue determines the tolerance to subzero temperatures, with higher tolerance reached at lower water contents (Kappen, 1966). In fact, as in the case of flowering plants, a certain relationship between tolerance to desiccation and freezing has also been observed in spores, gametophytes and leaves of ferns. In agreement with this, and although the biochemical/anatomical mechanism behind needs deeper study, ABA could have a role on the enhancement of freezing-tolerance mechanisms in ferns. Also, the preservation of chloroplast functionality by enhancing antioxidant, excess energy dissipation and ultrastructural stabilization, seems to be relevant for freezing tolerance in photosynthetic tissues of ferns. Freeze-thaw induced embolism has not been directly evaluated in ferns but can be inferred from data obtained in *Polystichum acrostichoides* under subzero temperatures. Prats and Brodersen (2020) measured a 25% loss of conductivity in the stipes suggesting the occurrence of embolism. Almost no information is available at cellular level regarding freezing-tolerance mechanisms.

Supplementary Materials

The additional data and information can be downloaded at: https://media.sciltp.com/articles/others/2505201437432693/plantecophys-577-Supplementary-final.pdf. Supplementary Table S1. Compilation of fern species, from which the tolerance to subzero temperatures has already been tested in the literature in green tissues (either sporophyte, gametophyte or chlorophyllous spore). The parameter/approach used to estimate tolerance to subzero temperatures, as well as the country and the original reference are specified.

Author Contributions

BF-M conceived the idea and drafted the work. JIG-P and MIA led the gametophyte- and spore-related information, respectively. LGQ led the taxonomy- and ecology-related information. SF and MIA prepared the figures. SF and BF-M wrote the main body of the manuscript and built the supplementary table. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

Original data are obtained from the literature. Literature sources are detailed in Supplementary Table S1.

Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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