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Doctoral Thesis

ANDER YOLDI ACHALANDABASO

2021

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ZTF-FCT
Zientzia eta Teknologia Fakultatea
Facultad de Ciencia y Tecnología

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June of 2021

I would like to thank the Plant Physiology laboratory from the Department of Plant Biology and Ecology of the University of Basque Country (UPV/EHU), especially to my Thesis supervisors, Dra. Usue Pérez López and Dr. Alberto Muñoz Rueda for allowing me to complete my Thesis and to all the members of FisioClimaCO_2 research group.

I would also like to thank:

Dr. Wieland Fricke, from the University College Dublin (UCD), for allowing me to carry out an online research stay, and for helping me to measure the expression of *AQPs*. Especially to his implication and dedication in the measurements layout and analysis of the obtained data, acting as a mentor.

Dr. Luis Valledor and **Dra. Maria Jesus Cañal** from the University of Oviedo, for the opportunity to enjoy one month of research stage at their laboratory to learn about metabolomics. I would also like to thank Cristina López-Hidalgo and Laura Lamelas (PhD Students) for their support during my stage performing the metabolomics measurements and the subsequent data analysis and their teaching with R program.

Dra. Azucena González, technical and human support by Phytotron Service, SGIker (UPV/EHU) is gratefully acknowledged.

The author of the doctoral Thesis has enjoyed a predoctoral fellowship for the formation as PhD student by the Education Department of the Basque Government.

The research work has received funding from the Basque Government (GV-IT1022-16 and GVCV18-05) and the University of the Basque Country (EHUA16/17).

El camino recorrido para poder haber llegado a este punto ha sido largo y tedioso. Pero por fin lo he/hemos conseguido, y eso que el Covid-19 se había empeñado en querer fastidiarlo. ¡La tesis está escrita! ¡Os he ganado Patrick Rothfuss & George R. R. Martin!

Esta ardua tarea no hubiese sido posible sin toda la ayuda y apoyo que he recibido por parte de la gente maravillosa que me rodea.

En primer lugar, querría agradecer a mis directores Usue y Alberto por haberme dado la oportunidad de realizar la Tesis Doctoral y por toda la dedicación e implicación que habéis puesto en este trabajo.

También querría agradecer al resto de miembros del grupo por haberme ayudado a lo largo de este camino. Somos muchos, con lo que no voy a nombraros a tod@s jeje, pero si a ti Jon, por todo lo que me has enseñado. Por supuesto, no podría olvidarme de tod@s l@s TFG y TFM que han colaborado en la consecución de este trabajo. Espero que, al mismo tiempo, yo haya contribuido en vuestra formación y aprendizaje.

Gracias a tod@s l@s integrantes del laboratorio de Fisiología Vegetal. Hemos vivido buenos momentos juntos, sobre todo fuera del laboratorio. En especial al grupo de amigos que he hecho a lo largo de estos años. ¡Espero que podamos seguir manteniendo nuestra amistad!

Y qué decir de ti Marlon, ¡no hay otro como tú! No es lo mismo esto sin ti. Espero que pronto puedan volver a unirse nuestros caminos. Lo deseo de corazón.

No podría olvidarme de tod@s mis compañer@s de la Universidad de Oviedo, especialmente de Cris y Laura. Muchas gracias por haberme cuidado tan bien durante mi estancia y por todo lo que me habéis enseñado. ¡Habéis tenido mucha paciencia conmigo!

Fuera del laboratorio también ha habido mucha gente que me ha apoyado durante estos años. Gracias al talde y a los amigos de tenis. Me siento afortunado de haberos conocido.

Por supuesto, gracias a toda mi familia. Creo que teníais más ganas que yo de que terminase la Tesis. Ya va, ya va, queda poco.

Por muy raro que suene, también quiero agradecer a Rafa Nadal, por haber sido una gran fuente de inspiración. ¡Vamos!

Finalmente, he querido reservar mis últimas palabras en estos agradecimientos a la persona más importante de mi vida: Nerea. Sin ti, todo lo demás no importa. Espero que la vida nos depare grandes momentos juntos. ¡Gracias por haberme apoyado todos estos años!

Abbreviations list

ABA	Abscisic acid
ADW	Aboveground dry weight
A_{net}	Net photosynthetic rate
ANTH	Anthesis stage
AP	Awn primordium
AQPs	Aquaporins
ASN	Spike number at anthesis
ATN	Tiller number at anthesis
ATP	Adenosine triphosphate
BGF	Beginning of grain filling phase
Ca	Atmospheric CO_2 concentration
Carot	Carotenoids pigments
CATA	Ambient CO_2 concentration and ambient temperature (current conditions)
CATE	Ambient CO_2 concentration and elevated temperature
Cc	Chloroplast CO_2 concentration
cDNA	Complementary deoxyribonucleic acid
CETA	Elevated CO_2 concentration and ambient temperature
CETE	Elevated CO_2 concentration and elevated temperature (future conditions)
Chl-a	Chlorophyll a pigment
Chl-b	Chlorophyll b pigment
CHs	Carbohydrates
CI	Collar initiation
Ci	Intercellular CO_2 concentration
DD	Double drought treatment
DH	Dehydration
DAS	Days after sowing
DMSO	Dimethylsulfoxide
dNTP	Deoxyribonucleotide triphosphate
DW	Dry weight

ε	Cell-wall volumetric elasticity modulus
E	Instantaneous transpiration rate
EAR	Early-vegetative stage
ECO_2	Elevated CO_2 concentration
ET	Elevated temperature
ETR/A_{net}	Electron transport rate to net photosynthetic rate
ETR	Electron transport rate
EU	The European Union
EVC	Early-vegetative stage control treatment
ΦPSII	Actual quantum yield of PSII
FAO	The Food and Agriculture Organization of the United Nations
FSN	Final spike number
FTN	Final tiller number
Fv/Fm	Photochemical efficiency of PSII in dark-adapted leaves
Fv´/Fm´	Photochemical efficiency of PSII in light-adapted leaves
FW	Fresh weight
GADPH	Gliceraldehide-3-phosphate dehydrogenase
GF	Grain filled percentage
GLA	Green Leaf Area
gm	Mesophyll conductance
gs/stomata	Stomata aperture
gs	Stomatal conductance
H^+-ATPase	Plasma membrane H^+-ATPase or proton pump
HNT	High night temperature
Hs	Heading stage
HSP	Heat-shock protein
HSP70	Heat-shock protein of 70 KDa
HT	Heat-shock treatment
Hv-EF1	*Hordeum vulgare* elongation factor
HvPIP	*Hordeum vulgare* plasma membrane intrinsic protein
HvTIP	*Hordeum vulgare* tonoplast intrinsic protein

IGW	Individual grain weight
IPCC	Intergovernmental Panel on Climate Change
IRGA	Infra-red gas analyser
J_{max}	Maximum rate of electron transport
K	Plant hydraulic conductance
Kleaf	Leaf hydraulic conductance
LD	Leaf density
Leaf N	Leaf nitrogen
LHCI	Light harvesting complex I
LHCII	Light harvesting complex II
Lpr	Root hydraulic conductance
LRWC	Leaf Relative Water Content
MAT	Physiological maturity stage
MIPs	Major intrinsic proteins
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NIPs	Nodulin intrinsic proteins
NPQ	Non-photochemical quenching
NSC	non-structural carbohydrates
OA	Osmotic adjustment
PCA	Principal component analysis
PCR	Polymerase chain reaction
PIPs	Plasma membrane intrinsic proteins
PSI	Photosystem I
PSII	Photosystem I
Ψo^{100}	Osmotic potential at full turgor
Ψo_{md}	Midday leaf osmotic potential
Ψo_{pd}	Predawn leaf osmotic potential
Ψt_{md}	Midday leaf turgor potential
Ψt_{pd}	Predawn leaf turgor potential
Ψw_{md}	Midday leaf water potential

Ψw_{pd}	Predawn leaf water potential
qP	Photochemical quenching
qPCR	quantitative real-time polymerase chain reaction
QTL	Quantitative Trait Loci
RC	Anthesis (reproductive) control treatment
RCP	Representative Concentration Pathway
RD	Anthesis (reproductive) drought treatment
Rd	Day respiration rate
Rn/A_{net}	Night respiration rate to net photosynthetic rate
Rn	Night respiration rate
RNA	Ribonucleic acid
ROS	Reactive oxygen species
R_{photo}	Photorespiration rate
Rubisco	Ribulose-1, 5-bisphosphate carboxylase-oxygenase enzyme
RuBP	Ribulose 1, 5-bisphosphate
RWC	Plant Relative Water Content
SD	Stomatal density
SIPs	Small intrinsic proteins
SPAC	Soil-plant-atmosphere continuum
SVWC	Soil Volumetric Water Content
T	Cumulative transpiration per plant
TAE	Tris-acetate-EDTA buffer
TDW	Total dry weight
TGNS	Total grain number per spike
TIPs	Tonoplast intrinsic proteins
TLA	Total leaf area
TPU	Triose phosphate utilization rate
TSS	Total stomata size
TW	Turgid weight
UV	Ultraviolet
VAZ	violaxanthine, anteraxanthine, zeaxanthine cycle

VC	Vegetative control treatment
$\boldsymbol{Vc_{max}}$	Maximum rate of carboxylation
VD	Vegetative drought treatment
VEG	Vegetative stage
VR	Vegetative recovery treatment
WUEg	Water use efficiency of grains

Resumen

Antecedentes e interés socio-económico

La producción agrícola se ve fuertemente influenciada por el clima, y cualquier cambio en el mismo puede reducir la productividad de los cultivos y poner en riesgo la seguridad alimentaria de la creciente población mundial. Las predicciones sobre el clima plantean un continuo incremento del CO_2 de la atmósfera, asociado con temperaturas más elevadas y con un aumento de la duración e intensidad de las sequías. Ello implica que la productividad de los cultivos se verá afectada por complejas interacciones ambientales entre estos factores. Para hacer frente a este escenario, se estima que la demanda de cereales, tanto para la alimentación humana como animal, se verá incrementada un 70% para el año 2050, siendo necesaria una "Revolución Dorada" en la agricultura.

En este sentido, uno de los mayores desafíos para los fisiólogos, biólogos moleculares y agrónomos será identificar rasgos o atributos para seleccionar variedades de cereales que maximicen su producción en condiciones de cambio climático. Para lograr este objetivo, será necesario, en primer lugar, comprender la respuesta de los principales procesos fisiológicos, bioquímicos y moleculares ante la acción individual y conjunta de los factores asociados al cambio climático (elevada temperatura, elevado CO_2 y sequía). En segundo lugar, se deberá dilucidar cómo tales procesos determinarán el crecimiento y producción de los cultivos.

En términos de producción, la cebada es el cuarto cereal más importante a nivel mundial y el segundo a nivel europeo y estatal, utilizándose tanto en la alimentación animal como humana, así como en la industria cervecera. Además, la cebada presenta una gran riqueza genética heredada de su ancestro: la cebada silvestre (*Hordeum spontaneu*m K. Koch), uno de los primeros cultivos domesticados por el ser humano, confiriéndole una alta potencialidad para adaptarse a diversas condiciones climáticas como las derivadas del cambio climático.

Hasta la fecha, se han publicado más de 80 trabajos en relación a los efectos del cambio climático en las plantas, habiendo sido estudiadas más de 30 especies distintas. Sin embargo, a pesar de su alto valor socio-económico, ninguno de estos trabajos ha incidido en la respuesta de la cebada frente a la acción conjunta de la elevada temperatura, elevado CO_2 y sequía. Además, debido a la disparidad de los resultados encontrados, es difícil establecer la base del mecanismo de interacción entre dichos factores asociados al cambio climático. Por otra parte,

los cambios moleculares y/o fisiológicos específicos observados no suelen integrarse con cambios en los rasgos agronómicos, dificultando así el avance en esta materia. No obstante, se presume que la relación fuente-sumidero que se establece en las diferentes fases del desarrollo del cultivo, y cómo los diferentes factores climáticos incidirán en esta relación, será crucial para comprender el mecanismo de interacción entre los tres factores.

Objetivos y diseño experimental

Bajo esta premisa, el objetivo general de esta tesis ha sido analizar el efecto de la acción combinada de los principales factores asociados al cambio climático –elevada temperatura, elevado CO_2 y sequía–, sobre los procesos fisiológico-bioquímicos y moleculares de la cebada e indagar a través de qué mecanismos afectan el crecimiento, desarrollo y producción.

Para ello, se ha incidido en los dos procesos principales que regulan el crecimiento vegetal: las relaciones hídricas y el metabolismo fotosintético, profundizando a su vez, en la participación de las acuaporinas en la respuesta de ambos procesos. Además, sabiendo que en el futuro no solo se verán intensificados los periodos de sequía, sino que también ocurrirán más frecuentemente, se ha investigado la capacidad de la cebada para recuperarse de un estrés por sequía en el periodo vegetativo, así como para desarrollar un posible mecanismo de endurecimiento al sufrir otra sequía en antesis.

La variedad de cebada objeto de estudio fue el cv.Henley, variedad maltera de uso en la Península Ibérica, la cual fue seleccionada por ser una variedad de ciclo corto, habiendo obtenido resultados de producción robustos –especialmente en zonas de sequía–, y valores de calidad de grano adecuados para la elaboración de cerveza, siendo clasificada como “variedad recomendada para la elaboración de malta”.

Para llevar a cabo estos objetivos, las plantas de cebada se cultivaron en una cámara de crecimiento bajo condiciones controladas. El tratamiento de CO_2 se inició desde el momento de la siembra de las plantas bajo dos concentraciones de CO_2: 400 o 700 µmol/mol. El tratamiento de alta temperatura también se inició desde el momento de la siembra y consistió en el incremento de 3 °C tanto de día como de noche (23/17 en el control *vs.* 26/20 °C a elevada temperatura). Por último, el tratamiento de sequía se aplicó en 2 estadios diferentes del desarrollo de las plantas. El primero de ellos comenzó 28 días después de la siembra, coincidiendo con el periodo vegetativo, mientras que el segundo se impuso al comienzo de la antesis.

Resultados más destacados y conclusiones

Bajo condiciones climáticas futuras (700 µmol/mol CO_2 y 26/20 °C día/noche), las plantas de cebada presentaron un mejor estado hídrico al final del periodo de sequía en antesis –el periodo de sequía que más afecto a las plantas de cebada–, gracias a un mejor control estomático y una menor deshidratación celular, respuesta parcialmente modulada por una regulación isoforma específica de las acuaporinas.

A su vez, debido al mejor estado hídrico, las plantas que crecieron bajo condiciones climáticas futuras pudieron mantener unas tasas fotosintéticas más altas. Nuevamente, la participación de una respuesta isoforma-específica de las acuaporinas pareció jugar un papel clave. Esto hecho se pudo deber a un cambio en la función transportadora de las acuaporinas hacia el movimiento de CO_2 de las plantas crecidas bajo condiciones de elevado CO_2.

En relación a lo mencionado hasta ahora, las plantas que crecieron bajo condiciones climáticas futuras y sufrieron un período de sequía en antesis, presentaron una mayor biomasa en comparación a las plantas que sufrieron una sequía bajo condiciones actuales. Sin embargo, esto no se tradujo en una mayor producción de grano final, incluso se obtuvieron valores inferiores en comparación con los valores actuales. Esto, por un lado, se debió al hábito de crecimiento indeterminado que desarrollaron las plantas crecidas bajo condiciones climáticas futuras, viéndose incrementados tanto los órganos fuente como sumidero, pero especialmente el tejido vegetativo. Por otro lado, dichas plantas padecieron los efectos negativos de la elevada temperatura en la formación de los granos y el llenado de los mismos, dando como resultado final un menor número de granos formados por espiga, una reducción en el llenado de grano y una disminución del peso individual del grano. Por lo tanto, la futura producción de cebada –al menos la del cv. Henley– podrá verse comprometida, poniendo en jaque la seguridad alimentaria.

Si bien las plantas que crecieron bajo condiciones climáticas futuras registraron menores niveles de producción, cabe resaltar que presentaron una mayor potencialidad debido a la mayor formación de sumideros. En este sentido, se abre una vía para que los biólogos moleculares, fisiólogos y agrónomos puedan romper las barreras existentes entre los mecanismos de compensación de la producción de los cultivos como la cebada, y así, se puedan obtener variedades con una mejor capacidad para traslocar recursos al grano.

Otro de los objetivos principales de la tesis fue analizar la capacidad de las plantas de cebada para recuperarse de un estrés por sequía en el periodo vegetativo. En este sentido, tanto las plantas que crecieron bajo condiciones climáticas tanto actuales como futuras, y sufrieron una sequía leve en la etapa vegetativa, no pudieron recuperar un correcto funcionamiento del metabolismo fotosintético debido principalmente a limitaciones difusionales desencadenadas por un efecto de memoria negativo. Una menor expresión de la acuaporina foliar *HvPIP2;1* pudo estar involucrada en dicha respuesta negativa.

Al mismo tiempo, dichas plantas presentaron una nueva etapa de desarrollo fenológico en comparación a las plantas control, provocada por un retraso en el crecimiento. Una formación de ahijados tardío les permitió alcanzar mismos niveles de biomasa, construyendo principalmente nuevo tejido vegetativo. Sin embargo, esto no fue suficiente para alcanzar mismos niveles de producción que las plantas control, especialmente bajo condiciones climáticas futuras, donde al efecto deletéreo de la elevada temperatura jugo un papel clave.

Por lo tanto, la elevada temperatura se presenta como el principal factor asociado al cambio climático que comprometerá la producción futura de grano de cebada, al que biólogos moleculares, fisiólogos y agrónomos deberían prestarle aún más atención para garantizar la futura seguridad alimentaria mundial.

Una de las posibles estrategias de manejo que podría considerarse para aliviar los efectos negativos de la elevada temperatura en la producción es el efecto de endurecimiento cruzado que podrían desarrollar las plantas después de haber pasado un período de sequía leve. Este efecto podría permitir que las plantas desarrollasen una mayor disipación del exceso de calor, lo que llevaría a un mayor rendimiento fotosintético, producción de carbohidratos, crecimiento de las plantas y producción final. Sin embargo, aún queda un largo camino por recorrer.

Por otra parte, teniendo en cuenta que las predicciones futuras estiman un aumento de los periodos de sequía previos a la época estival, coincidiendo con el periodo vegetativo de los cultivos, se quiso analizar también la posible capacidad de la cebada para desarrollar un efecto de endurecimiento al hacer frente a una sequía en antesis, habiendo previamente pasado una sequía en el periodo vegetativo. En este sentido, los resultados obtenidos vendrían a indicar que dichas plantas no desarrollaron un efecto de endurecimiento cuando sufrieron un estrés por sequía posterior en antesis. Es más, las plantas que sufrieron tanto una sequía en el periodo vegetativo como en la antesis vieron mermada la capacidad fotosintética en comparación a las

plantas que sufrieron una sequía en antesis por primera vez. Dicha reducción se debió principalmente a limitaciones difusionales, coincidiendo con el alcance de la sequía en antesis.

Debido al ahijamiento tardío desarrollado por las plantas que sufrieron una doble sequía, bajo condiciones climáticas actuales, las plantas de cebada fueron capaces de presentar mismos niveles de producción que las plantas que sufrieron solo una sequía en antesis. Sin embargo, en el caso de las plantas que sufrieron una doble sequía y crecieron bajo condiciones climáticas futuras, se observó una menor producción de grano. Este hecho, probablemente, se debió a una falta de capacidad de estas plantas para impulsar el desarrollo radicular, desencadenando un peor estado hídrico y, por lo tanto, un mayor detrimento fotosintético, junto al efecto deletéreo de la elevada temperatura en el propio desarrollo de las espigas y los granos.

Finalmente me gustaría concluir que los datos obtenidos en esta Tesis Doctoral y la discusión realizada de los mismos son relevantes ya que aportan información desconocida hasta ahora sobre los efectos que la triple interacción (elevado CO_2, elevada temperatura y sequía impuesta en distintos estadios fenológicos) causa a lo largo del ciclo de vida de una especie tan importante como la cebada. Además, este estudio combina análisis a distintas escalas (molecular, fisiológica y agronómica), lo que permite relacionar distintos mecanismos fisiológicos con la producción final y así poder hacer un análisis integrado de los distintos resultados.

Abstract

Agricultural production is strongly influenced by climate, and any change in it can reduce crop productivity and, therefore, threaten the world's growing population food security. Climate predictions forecast a continuous increase in the atmospheric CO_2 concentration, associated with higher temperatures and an increase in the duration and intensity of droughts. This implies that crop productivity will be affected by complex environmental interactions between these factors.

In terms of production, barley is the fourth most important cereal worldwide and the second in European Union and Spain, being used both in animal and human nutrition, as well as in the malting industry. In addition, barley has a great genetic wealth inherited from its ancestor: wild barley (*Hordeum spontaneum* K. Koch), one of the first crops domesticated by humans, giving it a high potentiality to adapt to various climatic conditions such as those derived from climate change.

Within this context, we have tried to study the molecular, physiological and agronomical response of a variety of malting barley (cv. Henley) to the interactions of elevated temperature, elevated C_{O2} and drought. Two main processes that regulate plant growth have been studied: water relations and photosynthetic metabolism. In addition, knowing that in the future, not only drought periods will be intensified, but that they will also occur more frequently, the ability of barley to recover from drought stress in the vegetative period has been investigated, as well as the possibility of developing a priming effect when suffering another drought in anthesis.

Under future climatic conditions (700 µmol/mol CO_2 and 26/20 ° C day/night), barley plants presented a better water status at the end of the drought period in anthesis thanks to better stomatal control and less cellular dehydration, partially modulated by a specific-isoform regulation of aquaporins expression. This fact allowed plants that grew under future climatic conditions to maintain photosynthetic rates in a better state, where again the participation of an isoform-specific response of aquaporins seemed to play a key role.

Nevertheless, this positive effect on water relations and photosynthetic metabolism was not enough to maintain higher production rates since the elevated temperature had a very negative impact on the development of grains. Moreover, barley production was even lower under these future climatic conditions.

On the other hand, plants that had suffered a mild drought in the vegetative stage were not able to recover proper photosynthetic rates due to diffusional limitations, where a downregulation of leaf aquaporin *HvPIP2;1* could be involved on it, giving as a final result fewer yield. Nevertheless, it was observed that a cross-talk interaction between elevated temperature and a mild vegetative drought could have primed plants conferring an adaptation mechanism to cope with elevated temperature impairments, an issue that deserves more attention.

Finally, plants that previously suffered a mild drought in the vegetative period and suffered a subsequent drought at anthesis did not present a priming effect. However, depending on the degree of water stress caused by the anthesis drought, they even presented diffusional limitations, having developed a maladaptive memory effect, which was translated into lower yield under future climatic conditions.

Our results, hence, predict that future barley production might be compromised, threatening food security. Nevertheless, the increase presented at the vegetative tissue level open a path for breeders to exploit this potentiality, paying attention to the ability of barley to drive the extra gained assimilates to reproductive organ development and grain filling rather than to vegetative growth.

Content

CHAPTER 1

INTRODUCTION

1. Introduction

1. Introduction

Agricultural activity is under increasing pressure due to the need for productivity improvement to cope with climate change and the growing world population scenario. In this regard, an increase of 70 % in the demand for cereals –both for human and animal consumption– is estimated for 2050 (**Tester and Langridge, 2010**), needing a "Golden Revolution" in agriculture to deal with it (**Evans and Lawson, 2020**).

Grain productivity or yield is the product of genetic potential, development processes and environmental variability that occur over the complete growing period (**Slafer, 2003**). Across the years, varieties of cereals with certain characteristics or traits have been selected to increase their productivity. Thus, to increase cereal production, varieties (1) with high stomatal conductance to obtain higher photosynthetic rates, (2) with the ability to maintain the greenness of the leaves for a longer time, (3) with less tillering capacity to increase sowing density, (4) with lower height to avoid lodging risk and (5) with higher harvest index, among other characters, have been selected (**Araus et al., 2008**).

Despite the obtained new agricultural varieties, and the developed advances in technology to increase cereals yield, changes in the climate still limit this improvement (**Wang and Frei, 2011; Högy et al., 2013**)**.** In fact, cereal productivity is being influenced by complex interactions due to elevated CO_2 (ECO_2) and elevated temperature (ET) levels derived from climate change effects, and other climate-related changes such as more severe and periodic drought episodes or salinization of soils (**Clifford et al., 2000; Peng et al., 2004; Caldwell et al., 2005; Dias de Oliveira et al., 2013 and 2015a; Jagadish et al., 2014**).

It is well known that tolerance to stresses such as drought or ET is a quantitative attribute or trait, thus, selecting a single tolerance trait is difficult. Nevertheless, a special effort is being made to search for unique traits to understand which metabolic processes are essential or crucial to ensure high productivity under changing conditions (**Araus et al., 2008**). To date, it has been possible to relate higher growth rates in the pre-anthesis period (**Austin et al., 1980**), a higher concentration of soluble sugars in the stem (**Shearman et al., 2005**) and a higher total green biomass in anthesis (**Araus et al., 2008**) with higher productivity in environments with low water availability. However, it is not known whether these indicators will continue to be valid in conditions where more than one stress will occur simultaneously.

In line with the latter, it must not be forgotten that the atmospheric CO_2 concentration has been increasing since the beginning of the industrial revolution (**Stocker et al., 2011**), mainly due to anthropogenic causes, wherein the mid-long term the atmospheric CO_2 concentration is expected to continue rising (**IPCC, 2013**). Concretely, based on the intermedium scenario of the Representative Concentration Pathway (RCP6) predicted for the end of the century by the IPCC, the atmospheric CO_2 concentration will reach up to 700 ppm, leading to an average increase of land temperature about 3 °C (**IPCC, 2013**). Those changes in climate conditions are expected to exacerbate drought periods, either on the severity or on frequency (**Kebede et al., 2019**). Overall, the increase in atmospheric CO_2 concentration, together with the augmentation of temperatures and drought periods and their severity, could drive profound implications for agricultural production (**FAO, 2018**).

In this regard, one of the greatest challenges for plant physiologists, agronomists and breeders, will be to identify traits or attributes to select cereal varieties that maximize their production under climate change conditions (**Ainsworth et al., 2008 and 2012**). To achieve this goal, it will be necessary firstly (1) to understand the response of main physiological, biochemical and molecular processes to different environmental factors (ET, ECO_2, drought), individually or in combination. Secondly, (2) it is also mandatory to elucidate how the modulation of such processes to different environmental conditions along the whole plant's lifespan would determine crops growth and grain production.

Next, the studied species is described, as well as a rough summary analysis of the individual and combined effects of ET, ECO_2 and drought on plant physiology and grain production response. Afterwards, in the introduction section of Chapters 3, 4 and 5 more detailed and deep information is given.

1.1 Barley: target studied cereal

Barley (*Hordeum vulgare* L.) belongs to the monocotyledonous angiosperms, of which the genus *Hordeum* contains 32 species and 45 taxa from the Triticale tribe, encompassed in the grass family Poaceae (**Bothmer et al., 2003**). It contains seven chromosomes and is diploid (2n= 14), while other species such as wheat are tetraploid or hexaploid. In addition, the barley genome exceeds 5 Gbp (**International Barley Genome Sequencing Consortium, 2012**), being larger than for rice, but 70 % lower compared with wheat. Moreover, it is a self-pollinating species and presents large genetic resources inherited from its ancestor wild barley (*Hordeum*

spontaneum K Koch) –one of the first domesticated crop species in the Fertile Crescent which was widely distributed along many diverse climatic regions–, allowing barley to grow under different environmental conditions. For those reasons, barley has granted a top position in terms of crop model in molecular research (**Kebede et al., 2019 and references therein**).

Barley is the fourth most important cereal in terms of plant production (**FAO, 2018**). In recent years, it has reached production values of around 150 million tons per year, where the 60 % of total production recorded in 2013 for example, was accounted for in the European Union (EU). Spain is the third country of the EU with the highest production after Germany (20 %) and France (20%), accounting for 14 % of the production in 2019 (**Eurostat, 2019**).

In the EU, 80 % of the production is driven to livestock feed (forage cultivars; six-rowed) and the remaining 20 % is directed to the production of beer (malting cultivars; two-rowed), the latter generating a profit of 50 to 60 billion euros and supplying 150.000 farmers (**CLIMBAR, 2014**). Moreover, taking into account that numerous projections indicate that climate change may considerably increase barley production, at least in some regions (East Africa and West Asia, among others), this would imply a growing role for this cereal as a crop that could help to alleviate malnutrition and maintain food security in the future (**Richardson et al., 2009**). In fact, nowadays, 5 % of annual barley production is destined for human consumption in world areas where is not possible to grow other cereals and food security is threatened (**Tricase et al., 2018; Giraldo et al., 2019**).

Thus, the demand and productivity of barley are increasing owing to its genetic diversity, wide adaptability and a wide range of uses such as food, beer and feed, which adding its feature as a model crop for molecular research, it is postulated as a good candidate to focus on and that could cope with climate change constrains. Despite its contrasted relevance, to date, there are no studies that have analysed the triple interaction of ET, ECO_2 and drought in barley.

In this PhD thesis, the two-rowed malting cultivar Henley has been used as target plant species. This cultivar was selected due to its short cycle period and the obtained robust productions results –especially at drought areas–, and appropriate grain quality values for brewing, being classified as a recommended variety for malting production (**GENVCE, 2008**).

1.1.1 Spring barley growth and development

Based on the external morphological appearance of immature or mature cereals spikes, the transition of major phases on cereals –vegetative, reproductive and grain filling phases–, has

been extensively studied, being Zadocks or the Growth Stage scale the most used one (**Zadoks et al., 1974; Alqudah and Schnurbusch, 2017**). In Fig. 1.1, a simplified scheme for barley main growth phases, and in Fig. 1.2, the temporal occurrence concerning the establishment of cereal components of grain yield, respectively, are depicted.

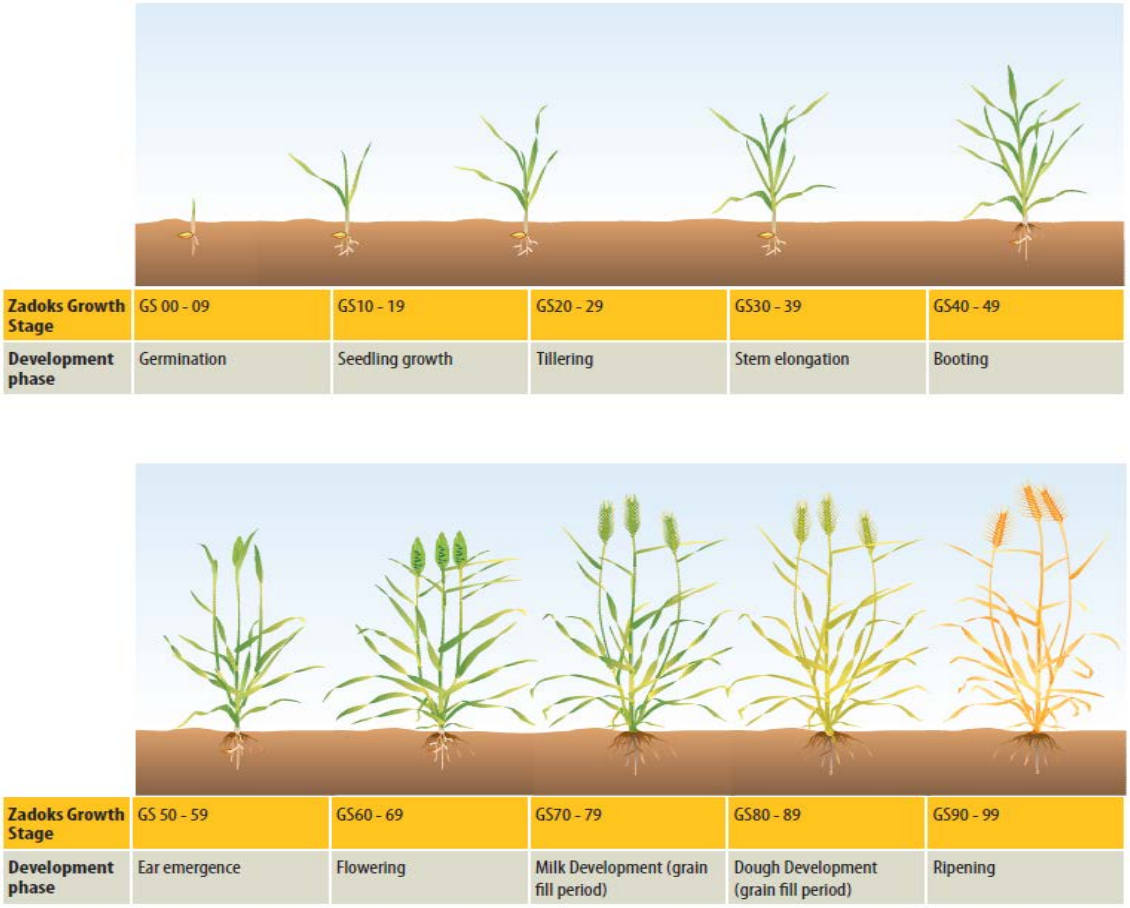

Fig. 1.1. Zadocks scale, encompassing main barley growth stages. Image derived from the GRDC (Cereal Growth Stages Guide). https://www.agric.wa.gov.au/soil-nutrients/critical-tissue-nitrogen-concentrations-diagnosis-nitrogen-deficiency-wheat.

Briefly, based on Fig. 1.2 (**Sreenivasulu and Schnurbusch, 2012**), following germination, an extended vegetative phase starts and proceeds until the collar is formed (collar initiation, CI), where subsequently the reproductive phase begins (GS30; Fig. 1.1) although vegetative tissue is still developing. The latter is divided into two phases: (1) an early-reproductive phase where spikelet or floret initiation is driven and encompasses from CI until awn primordium begins (AP; GS40) and (2) a late-reproductive phase, where spike growth and development is driven, encompassing the anthesis –where for spring barley begins with awn tipping at GS49-51–, and finished with the end of anthesis (At) and heading stage (Hs), matching with the beginning of grain filling phase (BGF; GS65). The later sub-phase is the longest developmental one and it has

a pivotal impact on grain-trait components and final grain yield (**Alqudah and Schnurbusch, 2017**). Lastly, the grain-filling phase finishes at the physiological maturity stage (GS99).

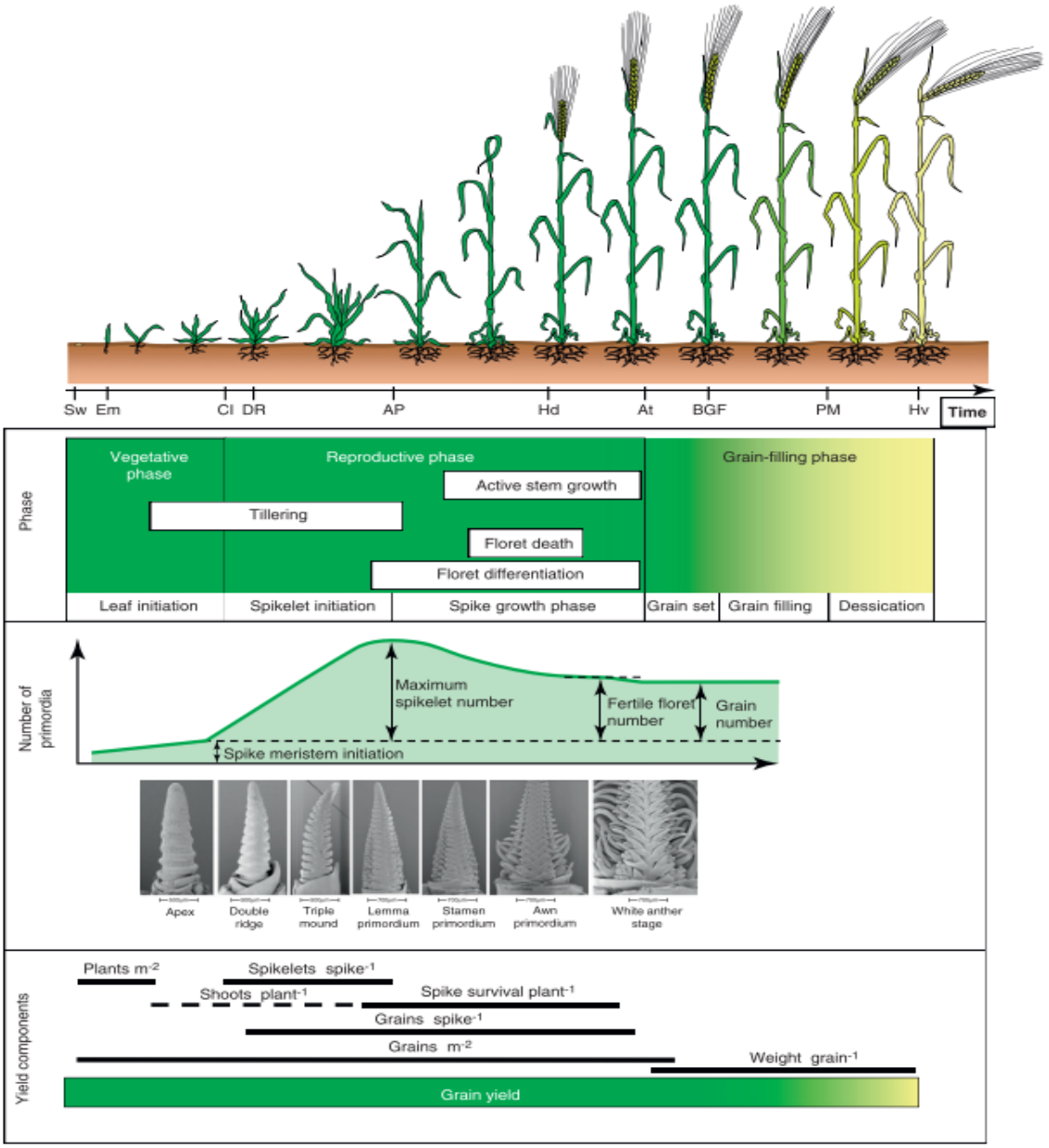

Fig. 1.2. A schematic figure depicting barley major developmental phases, their temporal occurrence concerning the establishment of components of grain yield, and a representation of spike primordial development with electron micrographs showing major barley spikelet initiation stages. Abbreviations denote: AP, awn primordium; At, anthesis; BGF, begin grain filling; CI, collar initiation; DR, double ridge; Em, seedling emergence; Hd, heading time; Hv, harvest; PM, physiological maturity; Sw, sowing. Figure taken from **Sreenivasulu and Schnurbusch** (**2012**).

1.2 Elevated temperature (ET) effects on crops physiological response and grain production

Climate change scenarios predict an increase in temperature between 0.3-4.8 °C for the end of the 21st century (**IPCC, 2013**), and the probability that plants could be exposed to extreme temperatures will be higher.

Although it could be thought that an increase in temperature could have positive effects on photosynthesis, this is not always the case, since the stimulation of net CO_2 assimilation by temperature is compensated by an increase in photorespiration. The increase in photorespiration with temperature is due, on one hand, to the greater loss of affinity of Rubisco for CO_2 than for O_2 as temperature increases and, on the other hand, to the fact that the proportion of dissolved O_2 in the medium where the Rubisco is located increases compared with the proportion of CO_2. Furthermore, as temperature increases above the optimum, negative effects are observed (**Sage and Kubien, 2007; Barnabas et al., 2008**).

Direct effects of high temperatures on the photosynthetic machinery have been detected, limiting photosynthesis. The decreases in photosynthesis were due to the thermolability of the enzyme rubisco activase and to the limitations in the electron transport of chloroplast (**Crafts-Brandner and Law, 2000; Crafts-Brandner and Salvucci, 2000; Hasanuzzaman et al., 2013**). Increases in photorespiratory and respiratory rates caused by the increase in temperature have also been observed (**Salvucci and Craftts-Brandner, 2004**). Both facts lead to lower availability of carbohydrates, resulting in a lower accumulation of biomass in the vegetative period (**Wahid et al., 2007; Barnabas et al., 2008**). On the other hand, it has also been seen that ET can directly damage plant cell membranes (**Blum, 1988**), and in the reproductive period can decrease pollen viability and reduce spikelet fertility (**Wahid et al., 2007; Barnabas et al., 2008; Jagadish et al., 2014; Tripathi et al., 2016**).

The increase of the temperature above the optimum affects the crops, accelerating their development, which is associated with lower productions. In corn, rice, wheat and barley, reductions in production between 5 and 10 % have been observed when increasing the temperature 1-2 °C above its optimum (**Baker, 2004; Barnabas et al., 2008; Högy et al., 2013**). Specifically, in barley, these decreases in production were due to a lower final concentration of starch in the grain, producing smaller grains (**Högy et al., 2013**).

1.3 Elevated CO_2 (ECO_2) effects on crops physiological response and grain production

The increases in temperature are the consequence of the growing augmentation in the concentration of greenhouse gases, mainly CO_2. In fact, the concentration of atmospheric CO_2 has been increasing rapidly since the beginning of the industrial era, and it is expected that by 2100 its concentration will have doubled, reaching levels of 700 ppm, although this value will depend on the climatic scenario that is used for its estimation (**IPCC, 2013**).

ECO_2 directly increases the photosynthetic rates in C3 plants since it increases the concentration of CO_2 near the Rubisco and, therefore, increases the carboxylation rate and decreases the O_2 competition for Rubisco that causes photorespiration (**Drake et al., 1997; Leakey et al., 2009**). In addition to increases in photosynthetic rates, the increase in CO_2 has been related to a decrease in stomatal conductance, which would reduce water consumption per unit of breathable surface (**Drake et al., 1997**). On the other hand, under conditions of ECO_2, the availability of carbohydrates is usually higher, which in some cases could cause the acclimation of photosynthesis (**Sicher and Bunce, 1997**), although in other cases the deviation of these extra carbohydrates has been observed to the synthesis of secondary compounds to reduce such acclimation (**Jaafar et al., 2012**).

In addition, either in closed or open ECO_2 systems, increases in crop productivity ranging between 15 and 30 % when the CO_2 level was increased up to 550 ppm has been shown (**Kimball, 1983; Kimball et al., 2002; Long et al., 2004; Ainsworth and Long, 2005; McGrath and Lobell, 2013**). However, unlike for ET effects, researchers have obtained more heterogeneous results depending on the species (**Jagadish et al., 2014**) or cultivar (**Ingvordsen et al., 2015; Li et al., 2019**), which could be ascribed to photosynthetic acclimation or the inability for biomass partitioning to grains filling.

1.4 Droughts effects on crops physiological response and grain production

Predictions on climate change point towards an increase in the duration and intensity of droughts during the 21st century (**IPPC, 2013**), which represents a strong impediment to the carbon balance and crop productivity (**Araus, 2004**).

The physiological responses of plants to water stress are complex since many processes are modified by drought. Drought reduces the availability of water in the soil and its uptake by the roots of the plants, causing changes in the water state (**Martínez et al., 2007; Robredo et al., 2007**), where the expression of aquaporins (*AQPs*) could play a pivotal role regulating it (**Maurel et al., 2016**; **Merlaen et al., 2019**). Moreover, it also affects carbon metabolism, decreasing net photosynthetic rates (**Robredo et al., 2007, 2010**). The decrease in photosynthesis caused by drought is mainly due to the reduction of CO_2 diffusion into the leaf by stomatal closure (**Chaves et al., 2003, Robredo et al., 2007**), although biochemical and photochemical limitations (fluorescence and pigments) could also be important when drought impairments are more severe (**Tezara et al., 1999; Lawlor and Cornic, 2002**). In most cases, these hastened effects on plants water status and photosynthetic performance give as a result, important reductions in production, reducing the number of fertile flowers and decreasing grain filling rates (**Wahid et al., 2007; Barnabas et al., 2008; Zhao et al., 2010**).

Moreover, the moment of drought imposition (vegetative, reproductive and/or grain filling), can lead to different plant physiological response and to the different mechanisms that are switched on, together with its final extent on plant production (**Barnabas et al., 2008**). In addition, plants recovery capacity is also a pivotal trait that must be considered when plants tolerance to drought is evaluated (**Gallé et al., 2009**). Besides, in recent years it has also been shown that the imposition of short drought stress in the vegetative period is beneficial to cope with subsequent drought stress (**Tripathi et al., 2016**), which could be an interesting management strategy.

1.5 Combined effects of climate change on crops physiological response and grain production

Up to now, we have described the negative and/or positive effects of each independent environmental factor on plant production and general physiology, but according to the IPCC forecasts, under future climatic conditions, all factors will manifest together.

On one hand, under future climatic conditions, it will be very likely that drought will occur under conditions of ET. In these cases, the effects of drought may be more pronounced. On the other hand, ECO_2 can interact positively or negatively with factors such as ET and drought, mitigating or magnifying the effects on the production of barley. Therefore, ET and/or drought could reduce the positive effects of ECO_2. However, if the issue is analysed from another point of view, the negative effects of the ET and/or drought may be mitigated by the ECO_2.

1.5.1 The joint action of ET and drought

Drought stress often interacts with ET, and under future climatic conditions, it will be very likely that the interaction between both factors will occur more frequently. As has been commented in previous sections, both stresses limit photosynthesis, although the mechanisms behind it differ. To some extent, the different mechanisms overlap when ET and drought stress are imposed simultaneously. Most of the works carried out until the date document that the joint action of both stresses intensifies plant growth and photosynthesis impairments. As concerns crop production, the impact of combined stresses has a significantly more negative effect compared with individual stressors effects (**Zandalinas et al., 2018**). Therefore, future cereal production in arid and semiarid regions would be more threatened (**Jagadish et al., 2014 and references therein; Cohen et al., 2021**).

However, in recent years, there have been published some works for wheat that have demonstrated that drought stress causes an increase in ET tolerance through better heat loss, triggering higher photosynthetic rates and grain yield (**Wang et al., 2015; Liu et al., 2017**).

1.5.2 The joint action of ET and ECO_2

At the physiological level, some studies show that ECO_2 mitigates the adverse effects of ET on photosynthesis, water use efficiency and plant growth (**Prasad et al., 2009; Yu et al.,**

2012). As the increase in temperature increases the oxygenation reaction of Rubisco and, therefore, photorespiration (**Jordan and Ogren, 1984**), the theoretical response of photosynthesis to ET would increase at ECO_2 since photorespiration would decrease (**Long, 1991**). **Wang et al.** (**2012**) detected this trend in a meta-analysis carried out in different plant species with C3 metabolism. It has also been shown that ECO_2 increases photosynthesis because it increases the abundance of proteins involved in light reactions, electron transport and ATP synthase (**Yu et al., 2014**). Due to these increases in photosynthesis in combined conditions, it has been proposed that biomass would also increase at ECO_2 compared with that determined at ambient CO_2.

Studies that analyse the interaction of ET and ECO_2 on grain production parameters show that in some crops the interaction of both factors is positive. For example, wheat subjected to combined conditions of ET and ECO_2 showed a greater number of spikes compared with wheat exclusively subjected to ET or ambient conditions, although the response varies depending on ET extent (**Dias de Oliveira et al., 2013**). Nevertheless, in other cases, decreases in grain production have even been detected when both conditions met together compared with ambient conditions, being only partially alleviated ET hastened effects (**Ingvordsen et al., 2015**). The joint action of both stresses response is diffusive and complex, especially when its effects are studied at the grain production level, probably due to the more heterogeneous response by plants to ECO_2, both on vegetative tissue and reproductive organs growth and development.

1.5.3 The joint action of ECO_2 and drought

As concerns plants response to drought at ECO_2 conditions, and specifically for barley, it has been observed that at the physiological level ECO_2 alleviates drought impairments improving its water status by reducing stomatal conductance and delaying its negative effects on photosynthesis (**Robredo et al., 2007**). In addition, photosynthetic parameters are less affected when barley plants grew at ECO_2 (**Robredo et al., 2010**). For other species such as wheat or festuca, it has also been shown that ECO_2 mitigates the effects of drought, allowing them to perform a greater osmotic adjustment due to higher carbohydrates (CHs) supply (**Wall et al., 2006; Chen et al., 2015**), or leading them to biomass construction due to decreased respiration (**Yu et al., 2012**).

In cereals, there are not many studies that have analysed the interaction of ECO_2 and drought on grain yield and yield-trait components. However, the information that is available – mainly for wheat (**Dias de Oliveira et al., 2013 and 2015a, b; Li et al., 2019**) and barley (**Schmid**

et al., 2016)–, go in the direction that ECO_2 mitigates drought hastened effects since higher CHs availability diminishes floral abortion, and increases spike formation and grain filling rates.

1.5.4 Future climatic scenario: ET, ECO_2 and drought

The combination of ET, ECO_2 and drought, would make plants physiological response even more complex, being more difficult to interpret developed interactions and mechanisms. Despite this difficulty, knowing that such conditions will manifest in many areas (**IPCC, 2013**) such as the Mediterranean basis (**Mitterbauer, 2017**), is of vital relevance to study them. Interactive studies between the three factors (ET, ECO_2 and drought) are scarcer than those with double interaction, and the observed responses vary from a negative effect of ECO_2 to deal with ET and drought stresses, to a positive one; furthermore, the response could vary between species (**Jagadish et al., 2014**) and cultivars (**Li et al., 2019; Abdelhakim et al., 2021**).

When this PhD thesis began (June of 2016), 51 articles investigating triple interaction were found in the Web of Science database (Table 1.1). From the analysis of these articles, it was concluded that 26 species have been analysed so far. Today (2021), about 80 studies are found, being studied up to 30 species. However, barley has not been studied yet. Some experiments have been done in growth chambers with controlled conditions, others in thermal gradient greenhouses and others in an open Free-air CO_2 enrichment system (FACE). The increases in temperature ranged from 2 °C to 12 °C. Moreover, increases in CO_2 reached up to 1000 ppm. In the case of drought, moderate to severe drought periods have been tested. On the other hand, the parameters determined have been diverse, among them, productivity, changes in phenology, growth, water relations, photosynthetic metabolism, fluorescence, pigments, antioxidant enzymes and metabolites, and some hormones. Therefore, the study scale has been diverse, analysing from agronomic characteristics (e.g., number of spikes) to molecular characteristics (e.g., changes at the transcriptional level of different genes).

Due to the disparity of results found, it is difficult to establish the basis of the interaction mechanism between ET, ECO_2 and drought. Furthermore, from these studies, it is very difficult to relate the observed specific biochemical and/or physiological changes with changes in agronomic traits, since the different changes are not usually integrated into the different scales. The source-sink relationship that is established in the different phases of crop development and how the different climatic factors affect this relationship will be crucial to understand the interaction mechanism between the three factors.

Table 1.1. Articles found on Web of Science studying the triple interaction effects on plants.

Species	[CO_2] (ppm)	Temperature (°C)	Water regimen	Studied processes	Reference
From 1996 until 2015					
Lolium perenne L. *Poa pratensis* L. *Medicago lupulina* L. *Lotus corniculatus* L.	392 ± 42 ppm 615 ± 81 ppm	Ambient +3 °C	Well-watered (FC) Withholding watering: 1 week (*M. lupulina* y *L. corniculatus*) 2 weeks (*L. perenne* y *P. pratensis*)	Growth Photosynthetic metabolism Antioxidant metabolism	**AbdElgawad et al. (2014 and 2015a, b)**
Poa pratensis L.	400 ppm 800 ppm	Ambient +15 °C	Well-watered Drought treatment with 50 % of evapotranspirated	Growth Photosynthetic metabolism Water relations	**Song and Huang (2014)**
Lotus corniculatus L.	350 ppm 700 ppm	Ambient +5 °C night +7 °C day	Well-watered Drought treatment with 60 % of evapotranspirated	Phenology Growth	**Carter et al. (1997 and 1999)**
Lolium perenne L. *Medicago lupulina* L.	375 ppm 620 ppm	Ambient +3 °C	Well-watered (FC) Drought treatment withholding watering for 2 weeks	Growth Photosynthetic metabolism Antioxidant metabolism	**Farfan-Vignolo and Asard (2012)**
Lolium perenne L. *Plantago lanceolata* L.	390 ppm 620 ppm	Ambient +3 °C	Well-watered (FC) Drought treatment withholding watering for 20 days	Growth Photosynthetic metabolism Water relations	**Naudts et al. (2013)** **Van de Velde et al. (2015)**
Calluna vulgaris L. *Deschampsia flexuosa* L.	400 ppm 510 ppm	Ambient +1,2 °C	Well-watered (Θ = 0.17 m^3/m^3) Drought treatment (Θ = 0.05 m^3/m^3)	Photosynthetic metabolism Water relations	**Albert et al. (2011a, b)** **Kongstad et al. (2012)**
Medicago sativa L.	400 ppm 720 ppm	Ambient +4 °C	Well-watered (Θ = 0.4 m^3/m^3) Drought treatment for 15 days (Θ = 0.2 m^3/m^3)	Growth Photosynthetic metabolism Water relations	**Aranjuelo et al. (2006)** **Ariz et al. (2015)**
Medicago sativa L.	400 ppm 720 ppm	Ambient +4 °C	Well-watered (Θ = 0.4 m^3/m^3) Drought treatment for 15 days (Θ = 0.2 m^3/m^3)	Growth Photosynthetic metabolism Antioxidant metabolism	**Erice et al. (2006a, b and 2007a, b)**
Eucalyptus saligna Sm.	280 ppm 400 ppm 640 ppm	Ambient +10 °C	Well-watered (gs = 0.5 m^3/m^3) Drought treatment (gs = 0.05-0.1 mol H_2O/m^2 s	Growth Photosynthetic metabolism	**Ayub et al. (2011)**
Eucalyptus radiata Sieber ex DC.	400 ppm 640 ppm	Ambient +4 °C	Well-watered (FC) Withholding watering for 2 periods	Photosynthetic metabolism Water relations	**Duan et al. (2014)**

FC, field capacity; gs, stomatal conductance; RWC, relative water content; Θ, volumetric content.

Table 1.1 continuation

Species	**[CO_2] (ppm)**	**Temperature (ºC)**	**Water regimen**	**Studied processes**	**References**
Eucalyptus globulus Labill.	Ambient +240 ppm	Ambient +3 ˚C	Well-watered (gs = 0.8 mol H_2O/m^2 s) Drought treatment (gs = 0-0.1 mol H_2O/m^2 s)	Photosynthetic metabolism	**Gauthier et al. (2014)**
Pinus sylvestris L.	Ambient 550-600 ppm	Ambient +2 ˚C	Well-watered (-0.4 MPa) Drought treatment (-1.0 MPa and -1.8 MPa)	Photosynthetic metabolism	**Kellomäki and Wang (1996)**
Pinus taeda L.	380 ppm 700 ppm	Ambient +2 ˚C	Well-watered Drought treatment (20 % of evapotranspirated)	Photosynthetic metabolism Water relations	**Wertin et al. (2010)**
Quercus rubra L.	380 ppm 700 ppm	Ambient +3 ˚C; +6 ˚C; +12 ˚C	Well-watered (Θ = 50 %) Drought treatment (Θ = 30 %)	Growth Photosynthetic metabolism	**Bauweraerts et al. (2013)**
Pinus radiata D. Don *Callitris rhomboidea* R. Br	400 ppm 640 ppm	Ambient +4 ˚C	Well-watered (FC) Withholding watering until mortality	Photosynthetic metabolism Water relations	**Duan et al. (2015)**
Phalarys arundinacea L.	370 ppm 700 ppm	Ambient +3.5 ˚C	Saturating watering (Θ = 100 %) Well-watered (Θ = 50 %) Drought treatment (Θ = 30 %)	Growth Photosynthetic metabolism	**Ge et al. (2011 and 2012)** **Zhou et al. (2011)**
Triticum aestivum L.	400 ppm 700 ppm	Ambient +2 ˚C; +4 ˚C; +6 ˚C	Well-watered (FC) Terminal drought at the onset of anthesis	Yield Phenology Growth Photosynthetic metabolism	**Dias de Oliveira et al. (2013)**
Triticum aestivum L.	400 ppm 700 ppm	Ambient +3 ˚C	Well-watered (FC) Terminal drought at the onset of anthesis	Yield Phenology Growth Photosynthetic metabolism	**Dias de Oliveira et al. (2015a, b)**
Larrea tridentata Cov.	360 ppm 500 ppm 700 ppm	Ambient +8 ˚C	Well-watered (Θ = 0.06 m^3/m^3) Drought treatment (Θ = 0.02 m^3/m^3) 15 days	Photosynthetic metabolism Water relations	**Hamerlynch et al. (2000)**
Brassica napus L.	370 ppm 740 ppm	Ambient +6 ˚C	Well-watered (FC) Drought until the wilting point	Growth Photosynthetic metabolism Hormones	**Qaderi et al. (2006)**
Vitis vinifera L.	375 ppm 700 ppm	Ambient +4 ˚C	Well-watered (FC) Drought treatment (40 % of FC)	Photosynthetic metabolism Antioxidant metabolism	**Salazar-Parra et al. (2012 and 2015)**
Festuca arundinacea L.	400 ppm 800 ppm	Ambient +10 ˚C	Well-watered (FC) Drought treatment (50 % of FC)	Water relations Photosynthetic metabolism	**Yu et al. (2012a)**
Arachis hypogaea L.	375 ppm 700 ppm	Ambient +4 ˚C	Well-watered (FC) Withholding watering for 22 days	Water relations Photosynthetic metabolism	**Clifford et al. (2000)**

FC, field capacity; gs, stomata conductance; RWC, relative water content; Θ, volumetric content

Table 1.1 continuation

Species	**[CO_2] (ppm)**	**Temperature (ºC)**	**Water regimen**	**Studied processes**	**References**
Centaurea nigra L.	380 ppm 760 ppm	Ambient +6 °C	Well-watered (FC) Drought until the wilting point	Growth Photosynthetic metabolism Hormones	**Qaderi et al. (2013)**
Apera spica-venti L.	350 ppm 700 ppm	Ambient +9 °C	Well-watered (40 % of *SWC*) Drought treatment (10 % of *SWC*)	Growth	**Sakalauskiene et al. (2013)**
Arabidopsis thaliana L.	380 ppm 730 ppm	Ambient +4 °C/+6 °C night/day for 3 days. 38/30 °C for 6 days.	Well-watered Drought treatment (45 % of plants *RWC*) Recovery	Growth Photosynthetic metabolism Antioxidant metabolism Transcriptional analysis	**Zinta et al. (2014)**
From 2016 until today (2021)					
C3 grasses *Trifolium repens*	FACE facility 390 ppm 520 ppm	+ 3.4 °C from ambient (average 12.4 °C)	Well-watered Drought treatment 1: irrigation reduced to 50 % Drought treatment 2: Full stopped irrigation	Water relations Growth Photosynthetic metabolism	**Roy et al. (2016)**
Review *Coffea arabica* *Coffea canephora*	Different regimens	Different regimens	Different regimens	Water relations Growth Photosynthetic metabolism	**DaMatta et al. (2018)**
Gossypium hirsutum L	400 ppm 640 ppm	28/17 °C 32/21 °C	Well-watered 2 progressive drought periods until *SWC* reached 30 %; 5 days of recovery between both periods	Water relations Growth Photosynthetic metabolism	**Broughton et al. (2017)**
Arabidopsis thaliana L.	380 ppm 730 ppm	8 days 26/22 °C 38/30 °C	Well-watered *Soil RWC* maintained at 45 % for 8 days	Plant metabolomics (sugars, amino acids, fatty acids) and transcriptomic analysis	**Zinta et al. (2018)**
Triticum aestivum L.	FACE facility 370 ppm 550 ppm	Ambient temperature 16.5 °C + 3-4 °C	Well-watered Semi-arid environment, 2 dry-land sites	Growth Phenology Yield	**Fitzgerald et al. (2016)**
Vitis vinifera L.	400 ppm 700 ppm	Ambient temperature + 4 °C	Well-watered Cycling drought	Growth Photosynthetic metabolism Yield	**Kizildeniz et al. (2018)**
Theobroma cacao L.	400 ppm 550 ppm 700 ppm	Ambient temperature + 3 °C	Well-watered Drought treatment (50 % of moisture)	Water relations Growth Photosynthetic metabolism	**Hebbar et al. (2020)**

FC, field capacity; gs, stomata conductance; RWC, relative water content; Θ, volumetric content; SWC, soil water content

Table 1.1 continuation

Species	**[CO_2] (ppm)**	**Temperature (ºC)**	**Water regimen**	**Studied processes**	**References**
Brassica napus	400 ppm 800 ppm	Heat wave for 7 days 21/14 °C 33/26 °C	Well-watered Drought treatment withholding water for 7 days	Water relations Growth Photosynthetic metabolism	**Diksaityte et al. (2019)**
Triticum aestivum L.	400 ppm 800 ppm	24/16 °C control For 5 days at 40/35 °C day/night	Well-watered *(SWC* of 26 %) Drought treatment (*SWC* of 16 % for 5 days)	Water relations Growth Yield Photosynthetic metabolism Hormones (ABA)	**Li et al. (2019)**
Brassica napus	Ambient ppm 1000 ppm	25/22 °C 40/22 °C	Well-watered Drought treatment withholding water for 7 days	Transcriptomic analysis	**Zhu et al. (2017)**
Gossypium hirsutum L.	400 ppm 640 ppm	28/16 °C 32/20 °C	Well-watered (*SWC* of 40-60 %) Drought treatment withholding watering until harvest	Growth Yield Photosynthetic metabolism N content	**Osanai et al. (2017)**
Arabidopsis thaliana L.	400 ppm 700 ppm	22/18 °C 28/24 °C	Well-watered Drought treatment withholding watering for 18 days	Water relations Growth Hormones Molecular analysis	**Abo Gamar et al. (2019)**
C3 and C4 grass species Non-leguminous forbs N-fixing legumes	FACE facility Ambient + 180 ppm	Ambient + 2.5 °C	Well-watered Drought treatment (- 45 % of control watering)	Water relations Photosynthetic metabolism N content	**Pastore et al. (2020)**
Pinus halepensis	420 ppm 870 ppm	25/20 °C Increasing heat for 10 days until reach 40 °C	Well-watered (*soil RWC* of 50 %) Drought treatment (*soil RWC* of 10%)	Water relations Growth Photosynthetic metabolism	**Birami et al. (2021)**
Solanum lycopersicum	400 ppm 800 ppm	23/16 °C 25/20 °C 35/30 °C	Well-watered Drought stress for 16h	Growth Photosynthetic metabolism	**Zhou et al. (2020)**
Vitis vinifera L.	400 ppm 700 ppm	Ambient (26/15 °C) + 4 °C	Well-watered Cycling drought	Growth Photosynthetic metabolism N content	**Kizildeniz et al. (2021)**
Triticum aestivum L.	400 ppm 800 ppm	23/18 °C 36/26 °C	Well-watered (*soil RWC* of 85-95 %) Drought treatments (*soil RWC* of 20-40 % for 3 days)	Water relations Growth Photosynthetic metabolism	**Abdelhakim et al. (2021)**

FC, field capacity; gs, stomata conductance; RWC, relative water content; Θ, volumetric content; SWC, soil water content

1.6 PhD thesis contextualization and justification

The demographic changes registered in recent decades, the constant increase in average life expectancy and the legitimate aspiration to enjoy a higher quality of life and health level are factors that condition, among others, the future development and well-being of society. The search and identification of physiological, biochemical and molecular characteristics that can be related to agronomic characteristics can help to adapt barley crop to climate change conditions. Therefore, this thesis addresses a global challenge that our society is facing and advances in the search for solutions capable of responding to both current and future demands, resulting from the important process of change and transformation that we are experiencing. In this way, it constitutes one of the principles of action in the design of public R + D + I policies. It was reflected in Horizon 2020 objectives and now is reflected in the 2030 climate and energy framework. Concretely it responds to the necessities picked up in the "Farm to Fork" strategy by the EU, "combating climate change" and "ensuring food security".

In this respect, different projects have been developed in the last years through the participation of researchers from different countries (EU). Among them, ClimBar (An integrated approach to evaluate and utilize genetic diversity) and WHEALBI (Wheat and barley Legacy for breeding improvement) can be found. The objective of these projects has been to evaluate and take advantage of the genetic diversity of species such as barley to obtain barley genotypes resilient to climate change conditions in the field. It is also worth mentioning the works carried out by Araus and Slafer in the selection of associated criteria for the improvement of barley and wheat yield under adverse climatic conditions (**Araus, 2004; Slafer et al., 2005**). Nevertheless, due to the complexity of studying CO_2 as a factor under field conditions, none of the mentioned projects has analysed ECO_2 as a variable. Therefore, this PhD thesis comes to make up for this lack –in part–, since it has been seen that ECO_2 has unpredictable effects on the development of plants that are subjected to stress conditions with ET and drought.

The thesis comprehends three different scales: (1) physiological-biochemical, which explains the response of barley to the interaction of the three environmental factors inquiring in two major aspects of plant physiology: water relations and photosynthetic metabolism; (2) molecular, analysing key *AQP*s expression involved in water and CO_2 transport within plants and, (3) agronomic, which analyses grain yield and yield-trait components. We consider that this type of exhaustive and integrative multiscale methodological approach will allow us to understand barley growth and yield under current and future climatic conditions.

1.7 General objectives and hypothesis

The general objective of this thesis is to analyse the combination of main climate change factors effects –elevated temperature, elevated CO_2 and drought–, on barley physiological-biochemical and molecular processes and to inquire through which mechanisms affect growth, development and grain yield. In addition, knowing that in the future drought periods will be more frequent, and that part of plants success to deal with drought impairments come from their ability to recover and to face subsequent stresses, we also have wanted to analyse this issue at different environmental conditions. To achieve these general objectives, specific objectives have been addressed, which matched with previously mentioned processes.

1- To analyse barley water relations response to the combined effect of elevated temperature, elevated CO_2 and drought applied at different stages of development (vegetative and anthesis). This objective will explain the response mechanism and the possible interactions established in the triple interaction at the water relations level.

2- To study the interaction of elevated temperature, elevated CO_2 and drought on barley photosynthetic processes, all parameters determined at different stages of development. This objective will allow us to understand the response mechanism and the possible interactions established in the triple interaction at the photosynthetic level along barley whole life span.

3- To determine barley phenology, growth, development and grain yield and yield-trait components subjected to combined conditions of elevated temperature, elevated CO_2 and drought. This objective will allow establishing relationships between the different phenological, physiological-biochemical and molecular parameters, with growth and grain yield.

We start from the hypothesis that plants at future climatic conditions (1) will reduce water loss conserving more water in the tissue due to lower stomatal conductance and (2) will keep photosynthetic rates higher (due to higher CO_2 diffusion) allowing greater availability of carbohydrates, (3) which would be diverted in higher growth, translating it in grain yield increases owed to greater spike formation and/or grain number and/or grain size. Furthermore, we think that plants will recover from the vegetative drought period irrespective of the environmental conditions, and on the other hand, that the vegetative drought period will be positive inducing a greater tolerance to face a subsequent drought period applied at anthesis.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2. Materials and methods

2.1 Experimental design

2.1.1 General growth conditions

Barley seedlings were grown in a mixture of perlite/vermiculite (3/1 v/v) in 3.1 L pots (17.1 cm in diameter and 16.4 cm in height). Fourteen seeds were sown in each pot and were watered three times per week for nine days with deionized water. Then, the eight more uniform plants were selected, reaching a final density of 350 plant/m^2. Before the PhD thesis experiment, a previous study was conducted to determine plant density optimum, from sowing until physiological maturity, for which we decided to employ that plant density.

During the experiment, plants were watered with Hoagland's solution (**Arnon and Hoagland, 1940**; Table 2.1) twice per week and were also watered with deionized water between each application of Hoagland's solution. From GS65 onwards, plants were only watered with deionized water until reached GS89. At that stage, watering was suppressed.

Plants were grown in a Conviron PGR15 controlled environment growth chamber (Conviron, Manitoba, Canada) with a daily 14 h light regimen and a relative day/night humidity of 70/80 %. During the light period, the photosynthetic photon flux density in the chamber was 400 µmol/m^2 s. The light was provided by a combination of incandescent bulbs and warm-white fluorescent lamps (Sylvania F48T12SHO/VHO, Sylvania, USA). To minimize the effects of intra-chamber environmental gradients, plants were randomly repositioned within the chamber each week **(Hymus et al., 2001)**.

The growth chamber allows us to gain full control of the different climatic parameters that we wanted to study. This facility is of special relevance to try to mimic ECO_2 conditions, where at the field is large complicated and diffusive. As long as things are done correctly and caution is exercised, obtained results could be transferable to "real conditions" as other authors have stated (**Högy et al., 2019**), which could be an optimum approach to better understand plants response when faced future environmental conditions.

Table 2.1. Chemical composition of Hoagland's solution.

Macronutrients	
Component	**Concentration**
KNO_3	6 mM
$Ca(NO_3)_2 \cdot 4H_2O$	4 mM
$NH_4H_2PO_4$	1 mM
$MgSO4 \cdot 7H_2O$	2 mM
Micronutrients	
Component	**Concentration**
$MnCl_2 \cdot 4H_2O$	9 µM
H_3BO_3	46 µM
$ZnSO_4 \cdot 7H_2O$	0.8 µM
$CuSO_4 \cdot 5H_2O$	0.3 µM
Na_2MoO_4	0.1 µM
Sequestrene (Fe)	0.01 g/l

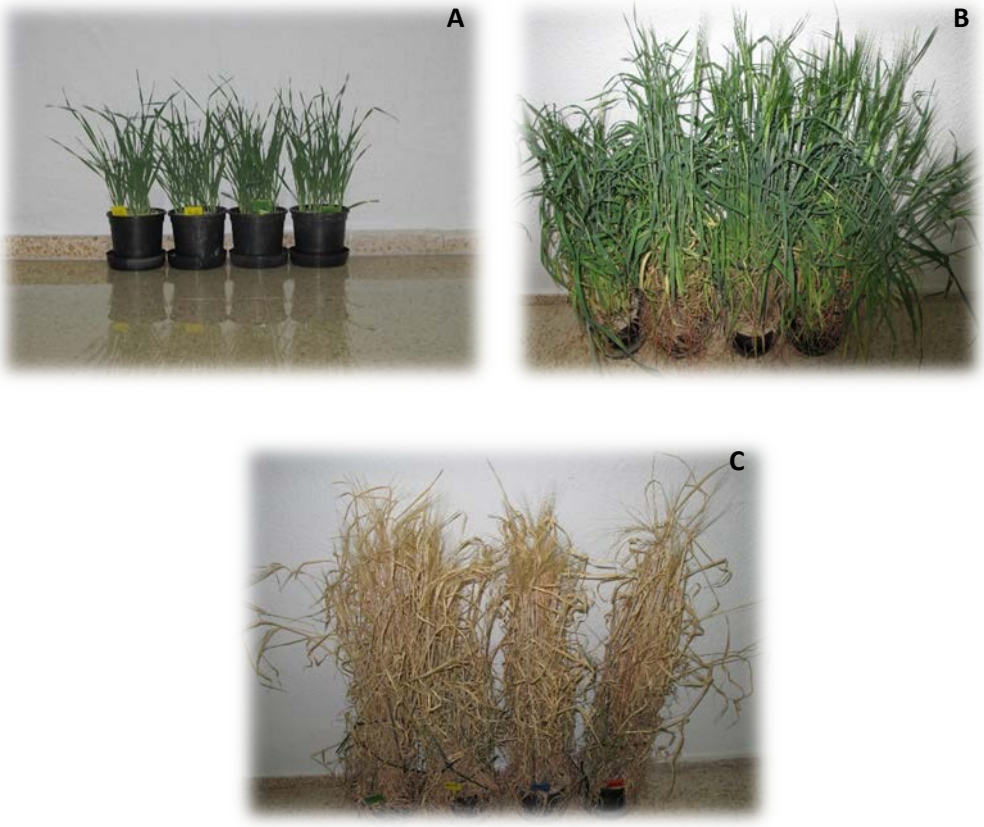

Fig. 2.1. Different barley treatment plants at the end of vegetative drought (A, GS21 + 9 days), anthesis drought (B, GS51 + 9 days) and final harvest moment (C, GS99).

2.1.2 Imposed treatments, measurements and samples recording

To mimic future environmental temperature gradient and CO_2 concentration, the intermedium scenario described by the IPCC was chosen (RCP6), which forecasts an average increase in temperatures by 3 °C and 700 ppm of CO_2 for the end of the 21st century (**IPCC, 2013**). The imposed treatments are summarized in the experimental design layout (Fig. 2.2).

2.1.2.1 Temperature treatment

To select the temperature regimen, information for the last 10 years related to April-August diurnal/nocturnal temperature regimen at Iberian Peninsula barley cultivated area was collected (**AEMET, 2016**). The obtained information was adjusted to growth chamber suitability.

The temperature treatments were: day/night temperature of 23/17 °C (TA) and day/night temperature of 26/20 °C (TE). The growth chamber temperature was maintained at ambient or elevated, from sowing throughout growth to the end of the experiment (Fig. 2.2). The software of the Conviron PGR15 chamber monitored the temperature regimen.

2.1.2.2 CO_2 treatment

The CO_2 treatments were: 400 µmol/mol air (CA) and 700 µmol/mol air (CE). The growth chamber atmosphere was maintained at ambient or elevated CO_2, from sowing throughout growth to the end of the experiment (Fig. 2.2). The software of the Conviron PGR15 chamber monitored the CO_2 concentration.

2.1.2.3 Drought treatment

2.1.2.3.1 Vegetative drought

The vegetative drought treatment started on the 21st day after sowing (DAS) when plants were at GS21 and were imposed for 9 days. The beginning of the drought treatment marked day 0 of the experiment, where all pots were taken to field capacity. Then, the vegetative drought treatment (VD) consisted of withholding-water plants, while the well-watered plants (VC) were watered with 100% of their daily evapotranspiration. Half of the pots were conducted for each treatment (Fig. 2.2). During the drought period, the watering of VC

treatments was alternated daily between Hoagland's solution and deionized water. After 9 days of drought and until the subsequent drought period at anthesis, all treatments were well-watered by alternating Hoagland´s solution and deionized water.

2.1.2.3.2 Anthesis drought

The anthesis drought treatment started when half of the plants of each pot had reached GS49-51 (anthesis onset; Fig. 1.1). Differences between treatments were recorded, although for each treatment the variability on the time to reach anthesis was low (Table 5.2). The anthesis drought was imposed for 9 days. The beginning of the drought treatment marked day 0 of the experiment, where all pots were taken to field capacity. Then, the drought treatment consisted of limiting the watering to 50 % of the daily evapotranspiration of the plants, while the well-watered plants were watered with 100 % of their daily evapotranspiration. Drought treatments were watered with Hoagland´s solution, whereas control treatments were watered by alternating Hoagland´s solution and deionized water.

Half of the pots that came from being VC were conducted to control at anthesis too (RC), whereas the other half were led to anthesis drought treatment (RD). The same was done for plants that at the vegetative stage suffered a VD, driving half to control at anthesis (VR) and the other half to drought (DD).

After 9 days of drought and until plants reached GS65 (half of the plants of the pot had the spikes out of the panicle; 7 days after anthesis drought period approximately), all the treatments were well-watered by alternating Hoagland´s solution and deionized water. All the combinations of treatments are summarized in Fig. 2.2.

2.1.2.4 In vivo, in vitro measurements and samples recording

In vivo measurements were carried out at the onset and the end of drought periods; GS21, GS21 + 9 days and GS51 + 9 days. *In vitro* measurements were only done at GS21 and the end of drought periods; GS21 + 9 days and GS51 + 9 days. For those analyses, the samples were harvested and stored at -80 °C. Finally, plant harvest driven for biomass and growth determination was obtained along the full life span of plants; GS21, GS21 + 9 days, GS51 + 9 days and GS99.

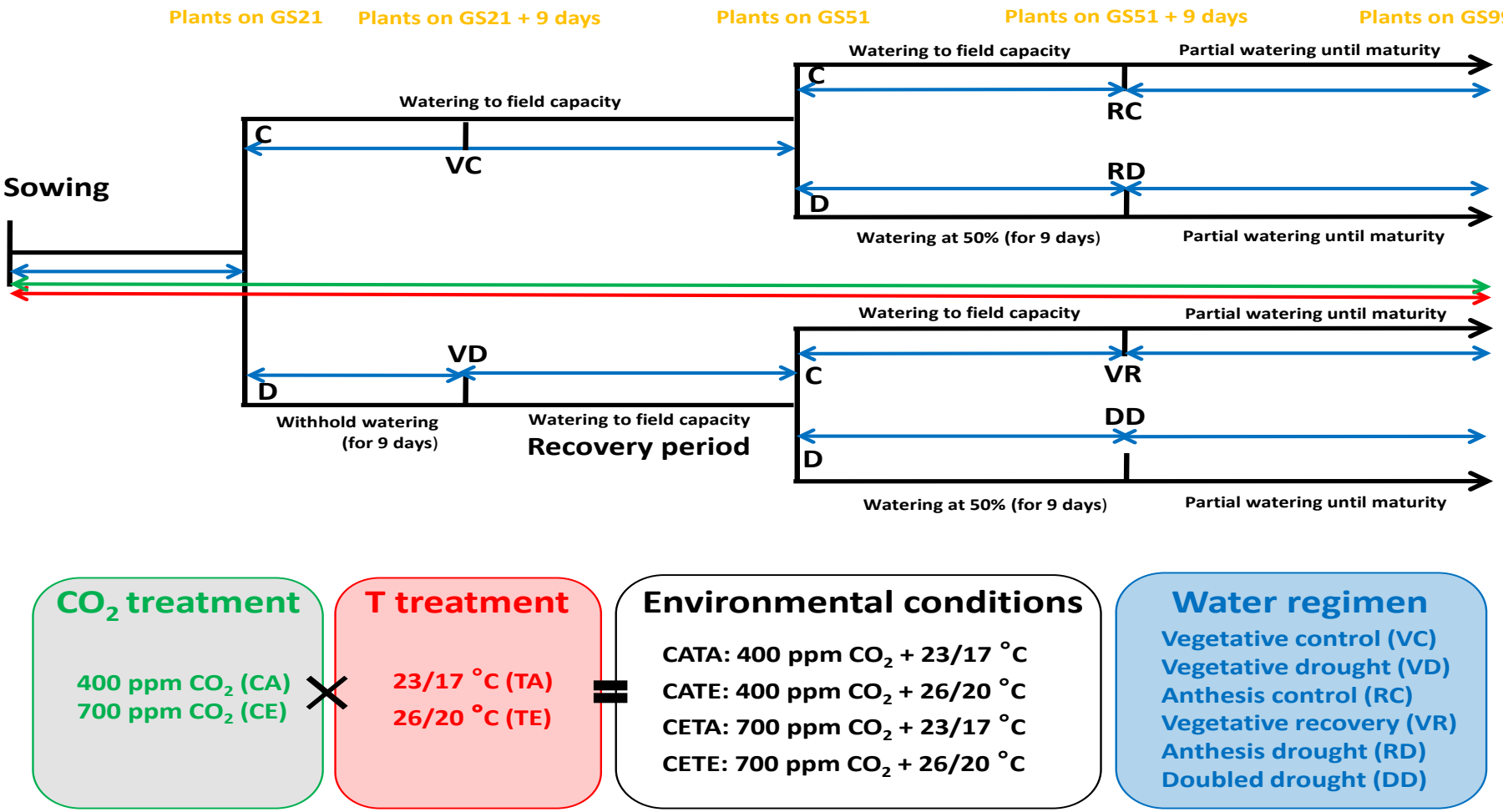

Fig. 2.2. Experimental design layout. DAS denote days after sowing. The rest of the abbreviations are explained in the picture.

2.2 Water relations

2.2.1 Soil Volumetric Water Content (*SVWC*)

The soil volumetric water content (*SVWC*) is the volume of water per unit volume of soil. For that, an average estimation of the water content that the substrate of pots could retain at field capacity without plants was carried out. We checked the homogeneity of that measure throughout five replicates. This value was taken as 100% of the *SVWC*. As drought was going on, pot weight was measured each day. At the end of the drought treatment, the same procedure as the abovementioned was used to calculate the *SVWC* of drought pots. The values of *SVWC* are given in percentage as they are calculated compared to the values measured at field capacity.

2.2.2 Leaf Relative Water Content (*LRWC*)

The leaf relative water content (*LRWC*) was measured by gravimetric methods. Firstly, the fresh weight (*FW*) of the second full expanded youngest leaf from the principal tiller was determined at the harvest. Secondly, using the same leaf as for *FW* determination, the turgid weight (*TW*) was calculated by incubating it for 24 h in deionized water and storing it in dark at 4 °C. Lastly, the dry weight (*DW*) was subsequently obtained by drying the tissue for at least 48 h in an oven at 80 °C, to constant weight. It was calculated by the following formula:

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

2.2.3 Water potential (Ψw)

Following the procedure described by **Scholander et al.** (**1965**) the leaf water potential (Ψw) was determined with a Scholander chamber (Plant Water Status Console, Model 3005, Soil Moisture equipment corp., CA, USA). For this, a clean-cut was made in the apical portion of the leaf, putting the excised material in a rubber-sealing gasket. To avoid leaf breaking when pressure was applied, a sheet of filter paper was put between the leaf and the rubber-sealing gasket. After that, the chamber was sealed and N_2 was applied progressively, where the surface of the cut was constantly monitored with a magnifying glass until the xylematic fluid appeared. At that moment, the N_2 flow was closed and the pressure reached was written down. This

pressure corresponds to the leaf water potential and is considered the pressure balance; it is the one that balances the negative pressure that retains water in the xylem. The leaf water potential measures were carried out before dawn (Ψw_{pd}) and six hours after the start of the photoperiod, at midday (Ψw_{md}).

2.2.4 Osmotic potential (Ψo)

Taking advance from the procedure of the freezing point of the cellular sap, the osmotic potential (Ψo) was measured in the same leaf that was used for the water potential determination. For that, an osmometer (Osmomat 030, Gonotec, Germany) was used, which was calibrated with a standard solution of NaCl (Gonotec) of 0.3 osmol/kg.

To obtain the cellular sap the used procedure was the next. Leaf cuts of the same length were done and kept in a previously perforated 0.5 mL Eppendorf tube, which was fitted in other Eppendorf tube of 1.5 mL of volume to facilitate the subsequent centrifugation and extraction of cellular sap. Quickly, they were frozen in nitrogen liquid and stored at -80 °C. The freezing process applied to the sample allows breaking plant cells and cell walls, thereby negating the turgor potential. Thus, the measured potential is only for the osmotic potential.

Moreover, on the day where the extraction of the cellular sap was carried out, firstly the leaf segments were thawed and immediately centrifuged at 13200 *g* for 5 minutes, discarding the pellet. Subsequently, 25 µL of the cellular sap were taken and osmolarity was measured by osmometer. The osmotic potential was calculated according to **Wyn Jones and Gorham** (**1983**) by Van't Hoff equation:

$$\Psi_o\ (\mathrm{MPa}) = -n \times R \times T$$

where,

n = osmol/kg (direct measure from osmometer)

R = Ideal gas constant, 0.083 kg MPa/mol K

T = Absolute temperature of the sample, in Kelvin degrees (K)

As it was explained for leaf water potential determination, the leaf osmotic potential measures were carried out before dawn (Ψo_{pd}) and six hours after the start of the photoperiod, at midday (Ψo_{md}).

2.2.5 Turgor potential (Ψt)

The turgor potential (Ψt) was obtained by the difference between the leaf water potential and the leaf osmotic potential for both predawn (Ψt_{pd}) and midday (Ψt_{md}) measurement periods.

$$\Psi_t \text{ (MPa)} = \Psi_w - \Psi_o$$

2.2.6 Dehydration (DH)

The dehydration (*DH*) was calculated by the following formula:

$$DH \text{ (MPa)} = \Psi_o^{100} - \Psi_o$$

To obtain the osmotic potential at full turgor (Ψo^{100}), the same procedure as explained for the calculation of *TW* was carried out.

2.2.7 Osmotic Adjustment (OA)

The osmotic adjustment was obtained by the difference between the Ψo^{100} of the control and droughted plants.

$$OA \text{ (MPa)} = \Psi_o^{100} \text{ control} - \Psi_o^{100} \text{ drought}$$

2.2.8 Cell-wall volumetric elasticity modulus (ε)

Throughout the method of **Rivelli et al.** (**2002**), assuming a near-linear relationship between Ψt and *LRWC*, the volumetric elasticity modulus (ε) was estimated. Concretely by:

$$\varepsilon(\text{MPa}) = \frac{\Delta P}{\Delta V / V}$$

where,

ΔP is the variation in Ψt between growth conditions and full turgor conditions.

$$\Delta P = (\Psi_o^{100} = \Psi_t^{100}) - \Psi_t$$

ΔV/V is approximated from values for the difference in *LRWC* between fresh and fully hydrated tissue (*LRWC*^{100}).

$$\Delta V/V = \frac{(LRWC^{100} - LRWC)}{100}$$

2.2.9 Plant transpiration during drought period (T)

Whole-plant cumulated transpiration during drought (*T*) was calculated by the gravimetric method. Each pot was weighed daily at the same time, before and after watering (**De Luis et al., 1999**). Thus, the water loss in 24 hours per pot, along the 9 days, was obtained.

2.2.10 Plant hydraulic conductance (K)

Plant hydraulic conductance (*K*) was estimated according to **Johnson et al.** (**2002**). Specifically, the instantaneous transpiration rate (*E*) was divided by the difference between the measured midday leaf water potential (Ψw_{pd}) minus the predawn leaf water potential (Ψw_{md}).

$$K\left(\frac{\mathrm{gH_2O}}{\mathrm{cm^2\ d\ MPa}}\right) = \frac{E}{\Psi w_{md} - \Psi w_{pd}}$$

2.2.11 Leaf and root aquaporin genes expression

2.2.11.1 Samples grounding and preservation

Leaf and root samples that were stored (within a maximum period of three weeks) in a freezer at -80 °C, were lyophilized to avoid RNA degradation or quality loss. Subsequently, the lyophilized material was milled with a vibration mill (Model MM301, Fisher Bio block Scientific) with a continuous shaken 30 s for 3 minutes.

2.2.11.2 RNA extraction

To extract the RNA of each root and leaf powder, an RNeasy Plant Mini Kit (Qiagen) was used. All the material used was RNase-free. Approximately 10 mg of powder samples were used for each extraction, which was led following the manufacturer´s instructions. To elute the RNA, 30 µL of RNase-free water was used. Once the RNA extraction was obtained, special precaution

was taken to maintain the cold chain. In the day, until the synthesis of the cDNA was not performed, the extraction samples were stored in ice. Then, the leftover RNA was stored at -80 °C in the freezer and the cDNA at -20 °C.

2.2.11.3 RNA concentration and purity determination

To determine the RNA concentration and purity of samples extraction a NanoDrop spectrophotometer (NanoDrop Lite Spectrophotometer, ThermoFisher Scientific, Massachusetts, USA) was used. The concentration of nucleic acid and protein was determined by the difference in their maximum absorbance at different wavelengths of light (260 nm for nucleic acid and 280 nm for protein). The concentration of contaminants such as carbohydrates and phenols were determined by the maximum absorbance at 230 nm. The contamination of the RNA samples with protein was determined by the 260/280 nm absorbance ratio. All the obtained values were about 2.0, considering them as ¨pure¨.

2.2.11.4 DNase treatment

To remove the residual genomic DNA from our RNA samples, a DNase treatment (Deoxyribonuclease Amplification Grade I, Invitrogen) was carried out. Exactly 1 µg of RNA, 1.1 µL of 10X DNase reaction buffer and 1 µL of DNase enzyme were made up to a total volume of 11 µL using autoclaved water. To carry out the DNase treatment manufacturer´s instruction was used.

2.2.11.5 cDNA synthesis

Following the DNAse treatment, cDNA was synthesised from the RNA samples using SuperScript II Reverse Transcriptase (Invitrogen, San Diego, California, USA) and a PCR machine (G-Storm, Gene Technologies, Limited, Rayne, Essex, UK). Used volumes are summarised in Table 2.2 and 2.3.

Table 2.2. Used volumes in the first step of cDNA synthesis.

Total volume	14 uL (mix gently)
Random Primers (Promega, *10x diluted*)	1 µL
dNTP mix (Thermo Scientific, 2 mM each)	4 µL
RNA sample after DNase treatment	9 µL

Subsequently, incubation for 2 min at 25 °C in the PCR machine, the following volumes were added to the PCR tubes, where the reaction start was driven by the RT-enzyme addition:

Table 2.3. Used volumes in the second step of cDNA synthesis.

Total volume of the reaction mix	20.9 µL (mix gently)
5X First Strand Buffer	4 µL
DTT (0.1 M)	2 µL
Superscript reverse transcriptase (RT-enzyme)	0.9 µL

After that, samples were subjected to the next programme of PCR machine explained in Table 2.4:

Table 2.4. cDNA synthesis programme run.

10 min at 25 °C	annealing of RT-enzyme
50 min at 42 °C	amplification of cDNA
10 min at 72 °C	denaturation of RT-enzyme

Once the programme was finished, the obtained cDNA samples were made up to 100 µL using autoclaved water. In addition, to check if DNase treatment worked, 1 µL of remained DNase treatment extraction was made up to 9 µL using autoclaved water to use as a negative control in the subsequent step.

2.2.11.6 PCR and agarose gel electrophoresis

To check that the different treatments worked, a PCR was undergone for both the cDNA samples and the DNase treatment negative controls above-mentioned, using the GoTaq G2 Flexi DNA Polymerase (Promega, USA) and the PCR machine. The manufacturer's indications were followed. To make up this test, it was used specific gene primers pairs with proven efficiency and quality. Briefly, the PCR sample setup is summarised in Table 2.5 and the PCR programme run in Table 2.6.

Table 2.5. PCR setup.

Total volume	15 µL
H_2O (autoclaved)	7.2 µL
cDNA template or DNase-control	2 µL
5X Green GoTaq Flexi Buffer	3 µL
$MgCl_2$ (provided in kit)	0.9 µL
dNTP (2 mM each)	0.35 µL
Gene-specific primers, forward and reverse (each 5 µM)	0.75 µL
GoTaq DNA polymerase (Reaction start)	0.05 µL

Table 2.6. PCR programme run.

Programme steps	Purpose
(i) 4 min at 95 °C	Heat activation of polymerase
(ii) 30 cycles of	
45 seconds at 95 °C	Denaturation of DNA separating strands
30 seconds at 59 °C	Annealing of strands
1 min and 30 seconds at 72 °C	Elongation of strands
(iii) 7 min at 72 °C	Final elongation
(iv) infinite at 4 °C	Nucleotide acids conservation

After that, to visualize the PCR products, a standard agarose gel electrophoresis was carried out. Briefly, the gel was made up of 1.1 % of agarose, using a 0.5 Tris-acetate-EDTA (TAE) buffer and loading 1 kb ladder as bands control. The gel was run at 100 V for 30 min, where subsequently was stored in a bathtub with ethidium bromide at dark conditions for 30 min. The gel was viewed under UV light and the image was given by the Quantity One v.4.5 software. For all the negative controls, no bands or negligible ones were recorded, and the cDNA samples presented a clear homogeneous band.

2.2.11.7 Determination of the reference and AQPs candidate genes for qPCR analysis

Through a normal PCR first, and then by a qPCR with serial cDNA dilutions, we test 13 different primers pairs to determine which were the ones that worked best.

For the housekeeping (reference genes), we test seven primers pairs: *Hordeum vulgare* Elongation Factor-1 (*HvEF-1*), Plasma membrane proton-ATPase (*H-ATPase*), Glycerinaldehyde-6P-dehydrogenase (*GAPDH*), *Tubulin, Ubiquitin, Cyclophilin and* Heat shock protein of 70 KDa *(HSP70)*.

For *AQP* genes, based on the works carried out for barley by Dr. Katsuhara group (**Katsuhara et al., 2002; Katsuhara and Hanba, 2008; Horie et al., 2011; Mori et al., 2014**) and Dr. Wieland group (**Knipfer and Fricke, 2010; Besse et al., 2011; Knipfer et al., 2011 and 2021**), in which they analyse *AQPs* implication on water flow either at root and leaf and on CO_2 transport of leaves, we decided to check *HvPIP1;3, 2;1, 2;2, 2;3, 2;5 and HvTIP1;1.*

Based on the test runs results, we chose to work with three reference genes: *H-ATPase, GAPDH* and *HvEF-1*, and five *AQPs* genes: *HvPIP1;3, 2;1, 2;2, 2;5* and *HvTP1;1*. Primers used and their efficiency is summarised in Table 2.7.

Table 2.7. Primers used for qPCR expression of candidates AQPs and housekeeping genes together with their efficiencies (E) obtained by the qPCR dilution test. Sequences facilitated by Dr. Wieland Fricke.

Name	Forward	Reverse	E
HvPIP1;3	CCTGTTCAAGTCTGCGAGTTTCAG	ATTAAATGGCGTGCGTGGTACTGG	1.78
HvPIP2;1	AGATATGTGCGAAGAAGAAGGCCG	ATATGCACAAGCCGAGGAACGGTA	1.89
HvPIP2;2	TCCTTGTCGCCCTTAATGTTGTCG	ATTGCAGCACTTGTCACTCACAGC	1.85
HvPIP2;5	GCAAGATTGAAGCAATGGCGACCT	CGAATTACAACACACGGCACGCAT	1.83
HvTIP1;1	TCCGTCCGTGTGGTCGAT	TCGTCACAGGTTTCACAGCACCA	1.76
HvEF-1	AAGGCTGCCATCAAGAAGAA	CAGAAGCATCCATGTTTTCCC	1.87
H^+-ATPase	ACATCGACACCATCAACCAA	ACAACTAGGGGCTGGTCAGA	1.81
GADPH	GTGAGGCTGGTGCTGATTACG	TGGTGCAGCTAGCATTTGAGAC	1.83

2.2.11.8 qPCR analysis

Through qPCR analysis, the level of expression of candidate *AQPs* compared with the level of expression of housekeeping genes was carried out. To be sure that the results were reproducible, two technical replicates were conducted for each *AQP* gene and sample. Analysis was performed in 96-well qPCR plates, using TB Green Premix Ex Taq II (Takara, Japan) and Step One Plus Real-Time PCR System (Applied Biosystems). qPCR sample setup is summarised in Table 2.8 and the qPCR programme run in Table 2.9.

Table 2.8. qPCR setup.

Total volume	10 µL
cDNA template	1 µL
H_2O (autoclaved)	2.8 µL
TYBR Green	5 µL
Gene-specific primers, forward and reverse (each 5)	0.5 µL
ROX	0.2 µL

Table 2.9. qPCR programme run.

Programme steps	Purpose
(i) 10 min at 95 °C	Hold stage
(ii) 40 cycles of	PCR stage
15 seconds at 95 °C	Denaturation of DNA separating strands
1 min at 60 °C	Annealing of strands
(iii) 60-95 °C	Melting curve (one fluorescence read every 0.3 ℃)

Lastly, the relative expression of candidate *AQP* genes for each sample was calculated using the ΔCycle threshold (ΔCt) method (**Pfaffl, 2001; Mestdgah et al., 2009**). Results were expressed by $2^{-\Delta Ct}$.

2.3 Photosynthetic metabolism

2.3.1 Gas-exchange parameters

Through the gas analyser in the infrared (IRGA) in open system Li-6400 (Li-Cor Inc., Lincoln, NE, USA) gas-exchange parameters were determined. Measurements were performed between 2-3 h after dawn in the full expanded youngest leaf of the principal tiller. The employed cuvette conditions mimicked the defined ones at each environmental growth conditions; 23 or 26 °C, 400 or 700 ppm CO_2, relative humidity of about 60 % and a photosynthetic photon flux density of 400 µmol/m^2 s provided by a red/blue LED light source (model Li 6400-02B, Li-Cor Inc.). The measurement record was made when the equilibrium of water and CO_2 exchange (steady-state) was reached, a condition that was obtained after 10 minutes. Net photosynthetic rate (A_{net}), stomatal conductance (gs), intercellular CO_2 concentration (Ci) and the instantaneous transpiration rate (E) were determined according to the method of **von Caemmerer and Farquhar** (**1981**). The parameters were calculated based on the following formulas:

$$Anet\ (\mu\text{mol CO}_2/\text{m}^2\ \text{s}) = \frac{F \cdot (C_r - C_s)}{100S} - (C_s \cdot E)$$

$$gs\ (\text{mol H}_2\text{O}/\text{m}^2\ \text{s}) = \frac{1}{\left(\frac{1}{g_{tw}} - \frac{k_f}{g_{bw}}\right)}$$

$$Ci\ (\mu\text{mol CO}_2/\text{mol air}) = \frac{\left(g_{tc} - \frac{E}{2}\right) \cdot C_S - A}{g_{tc} + \frac{E}{2}}$$

$$E\ (\text{mmol H}_2\text{O}/\text{m}^2\ \text{s}) = \frac{F \cdot (W_s - W_r)}{100 \cdot S \cdot (1000 - W_s)}$$

where,

F = air flow rate (µmol/s)

C_r = reference CO_2 concentration (µmol CO_2/mol air)

C_s = sample CO_2 concentration (µmol CO_2/mol air)

g_{tw} = total conductance to water vapour (mol H_2O/m^2 s)

g_{bw} = boundary layer conductance to water vapour (mol H_2O/m^2 s)

k_f = $(K^2+1)/(K+1)^2$. Being K a dimensionless coefficient that estimates the fraction of conductance of one side of the leaf to the other.

g_{tc} = total conductance to CO_2 (mol CO_2/m^2 s)

S = leaf area (cm^2)

W_r = reference water mole fraction (mmol H_2O/mol air)

W_s = sample water mole fraction (mmol H_2O/mol air)

In addition, night respiration rate (*Rn*) was also determined before dawn at the same humidity and CO_2 conditions, but the PAR was maintained at 0 µmol/m^2 s and the employed temperatures were the ones that concern night temperatures of the experiment (17 °C at CATA and CETA, and 20 °C at a CATE and CETE).

2.3.2 Stomata related parameters

To be able to analyse stomatal properties (leaf density, *LD*; total stomata size, *TSS*), leaf imprints were taken both at adaxial and abaxial leaf surface due to barley´s amphiestomatic leaf type. The measurements were made in the same leaf for gas-exchange determination, taking the prints from the centre of the leaf. For that, a cyanoacrylate glue (Loctite Superglue-3, Loctite Corporation, Henkel Ibérica, S.A., Barcelona) was applied on the leaf, which was subsequently pushed onto a glass microscope slide for a few seconds. After that, the leaf was removed obtaining the final impression on the glass slide. In addition, to prevent prints degradation, they were stored in darkness.

Once the prints were obtained, using a Nikon ECLIPSE 50i fluorescence microscope (Nikon Corporation, Japan) with a Leica DFC 420C camera (Leica Microsystems, Germany) several images were taken. For each epidermal print, four images were acquired through LAS V3.7 program (Leica Microsystems, Germany), both at 4x and 10x magnifications. Concretely, to analyse the variability that could be in the sample itself, the epidermal prints were divided into four squares, and for each square one image was taken at x4 and the other at x10 magnification. After that, to delimit the area in the image for assessment of stomatal traits, the epidermal idioblast cells (siliceous and suberose cells) were identified on the print. The images and analyses that were taken at the 10x magnification comprised the stomata between two rows of epidermal idioblast cells (Fig. 2.3). LabelStoma tool, an open-source graphical user interface that employs the YOLO model, was used for stomata traits measurements (**Casado-García et al., 2020**).

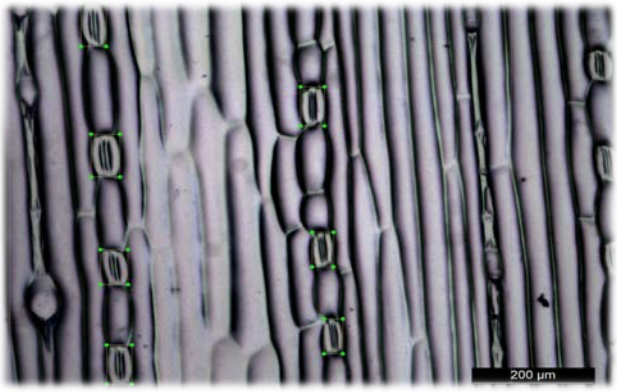

Fig. 2.3. Acquired image at 10x magnification and analysed by LabelStoma tool. The detected stomata between the idioblasts are enclosed with green boxes.

2.3.3 Chlorophyll *a* fluorescence

To determine chlorophyll *a* fluorescence, the same open gas-exchange system as for gas-exchange parameters determination was used (Li-6400; Li-Cor Inc., Lincoln, NE, USA), adding an integrated fluorescence chamber head (Li-6400-40; Li-Cor Inc.).

Plants were adapted to darkness and all energy-dependent fluorescence quenching coefficients were relaxed when measurements were done. To obtain those requirements, all plants chlorophyll *a* fluorescence determination was made before dawn. In addition, as concerns the measurement process, the leaves were exposed to different light pulses. First, the basal dark-adapted fluorescence signal (F_0) was determined with a 660 nm output of 0.25 µmol quanta/m^2 s set at a frequency of 500 Hz. Later, the maximum dark-adapted fluorescence (*Fm*) was measured with a saturating flash of 7800 µmol quanta/m^2 s for 0.8 s. Actinic illumination was provided by light at 400 µmol quanta/m^2 s. During exposure to actinic illumination, we induced a transient closure of PSII photochemical reaction centres by applying saturating pulses every 15 s until a steady state of variable fluorescence (F_t) was achieved and the maximum light-adapted fluorescence (*Fm'*) was recorded. At that point, to allow maximum oxidation of the PSII electron acceptor, the actinic light was switched off and subsequently, the basal light-adapted fluorescence (F_0') by applying a far-red (735 nm) light intensity of 5.88 mW/s for 10 s. The following parameters were calculated:

The photochemical efficiency of PSII in dark-adapted leaves (Fv/Fm): This parameter indicates the maximum proportion of light absorbed by the antenna that reaches the reaction centre in dark-adapted conditions, where all the photochemical components are oxidized. In addition, it determines the photoinhibition of PSII (**Krause and Weis, 1991).**

$$Fv/Fm = \frac{(Fm - F_0)}{Fm}$$

The photochemical efficiency of PSII in light-adapted leaves (Fv'/Fm'): This parameter indicates the proportion of light absorbed by the antenna that reaches the reaction centre in light-adapted conditions. Since some of the components are reduced at these conditions, the obtained values are lower.

$$Fv'/Fm' = \frac{(Fm' - F_0')}{Fm'}$$

The photochemical quenching (qP): This parameter indicates the proportion of energy in the reaction centre that is used in photochemical processes. Therefore, it measures the redox state of the electron transporters (**Demmig-Adams and Adams, 1996**).

$$qP = \frac{\left(Fm' - F_t\right)}{\left(Fm' - F_0'\right)}$$

The non-photochemical quenching (NPQ): This parameter indicates the level of non-radiative energy dissipation (principally as heat) in the light-harvesting antenna of PSII (**Demming-Adams and Adams, 1996**).

$$NPQ = \frac{\left(Fm - Fm'\right)}{Fm'}$$

The actual quantum yield of PSII (ΦPSII): This parameter indicates the proportion of light absorbed by the antenna that reaches the reaction centre and is used in photochemical processes. Therefore, it is equivalent to the proportion of energy used in the photochemical processes of photosynthesis (**Genty et al., 1989**).

$$\Phi PSII = qP \times \frac{Fv'}{Fm'} = \frac{\left(Fm' - F_t\right)}{Fm'}$$

The electron transport rate (ETR): This parameter indicates the proportion of light used in photochemical processes from the total energy reaching the leaf per unit of time and surface (PPFD). Following **Genty et al.** (**1989**), to determine this parameter, the absorption coefficient

of the leaves was taken as 0.85 and the fraction of the excitation energy distributed to PSII was considered as 0.5.

$$ETR = \Phi PSII \times PPFD \times 0.85 \times 0.5$$

2.3.4 Photosynthetic pigment

To quantify photosynthetic pigments content, three leaves fragments of 0.5 cm long were used and the width of each was recorded. Leaves samples were weighed too. To carry out chlorophylls and carotenoids extraction, the procedure described by **Barnes et al.** (**1992**) was conducted. The samples were incubated in 2 mL of dimethylsulfoxide (DMSO) for 2 h in the dark and in an oven at 80 °C. After that, the supernatant was poured into spectrophotometer cuvettes and the absorbance was measured at 750, 665, 649 and 480 nm. The former absorbance (750 nm) was used to control the degree of turbidity of extract control. To take the measures valid, the value has to be less than 0.02, otherwise, they were discarded. The following formulas were used (**Wellburn, 1994**):

$$Chlorophyll\ a\ (\mu g/mL) = 12{,}47A_{665} - 3{,}62A_{649}$$

$$Chlorophyll\ b\ (\mu g/mL) = 25{,}06A_{649} - 6{,}5A_{665}$$

$$Carotenoids\ (\mu g/mL) = \frac{(1000A_{480} - 1{,}29Chla - 53{,}78Chlb)}{220}$$

The chlorophylls and carotenoid concentration were expressed on a leaf surface basis, g cm^{-2}.

2.3.5 *A/Ci* curves related parameters

Applying *A/Ci* curves, different parameters related to plants gas-exchange, but principally with photosynthetic biochemistry, were obtained. The measurements are determined using the same open gas-exchange system as for gas-exchange parameters determination was used (Li-6400; Li-Cor Inc., Lincoln, NE, USA).

A/Ci curves were measured under saturating photosynthetic flux density of 1200 µmol/m^2 s and 60 % humidity. The *Ca* values applied to construct the curves were 80, 100, 150, 250, 350, 550, 700, 850, 1000, 1300, 1600, and 2000 µmol CO_2/mol air, being the initial measurement the one that corresponds to the *Ca* growth concentration. That point of measurement was recorded three times along the curves as the control point. The employed temperature matched with the used for gas-exchange measurements, depending on the temperature treatment. In addition, the time lag between two consecutive measurements at different *Ca* levels were restricted to 2–4 min, so each curve was completed in 50-60 min. The *Ci* at each *Ca* was calculated with the equations of **von Caemmerer and Farquhar** (**1981**). Moreover, through a curve-fitting method proposed by **Ethier and Livingston** (**2004**), based on the model proposed by **Farquhar et al.** (**1980**), the maximum carboxylation rate (Vc_{max}), maximum electron transport rate (J_{max}), triose phosphate utilization (*TPU*), the rate of mitochondrial respiration in the light not related to photorespiration (*Rd*) and photorespiration (R_{photo}) were determined. The basis for these determinations come through the photosynthesis limitation carboxylation process, which could be the slowest of the three following steps: (1) Rubisco activity (Rubisco limited); (2) RuBP regeneration (RuBP limited); or (3) triose-phosphate utilization (*TPU* limited).

In addition, taking advance from the curve-fitting method proposed by **Ethier and Livingston** (**2004**), the CO_2 mesophyll conductance (*gm*) was also estimated. The determination of this parameter leads us to determine the CO_2 concentration at chloroplasts (*Cc*) by the following equation defined by **Flexas et al.** (**2013a**):

$$gm = A_{net} / (C_i - C_c)$$

2.3.6 Leaf nitrogen concentration (*leaf N*)

The leaf nitrogen concentration (*leaf N*) was determined in a homogenous mix of at least 3 independent plants with 3 replicates per treatment. The dry material was milled with a vibration mill (Model MM301, Fisher Bio block Scientific) with a continuous shaken 30 s for 3 min. 2 mg *DW* were determined using an elemental analyser (FlashEA 1112; Thermo Finnigan, Germany).

2.4 Plant growth, development and yield

2.4.1 Absolute growth parameters

When plants reached 21 and 30 DAS, it is, at early-vegetative (GS21) and vegetative (GS21 + 9 days) stages, they were harvested and separated into leaves, stems, and roots. Moreover, after the anthesis drought period (GS51 + 9 days) and at physiological maturity (GS99) it was proceeded in the same way, together with tiller and spike counting and spike harvest. At each growth period and for each treatment, the fresh and dry weight of different organs were measured. To determine the dry weight of organs (*DW*), samples were dried at 80 °C for 48 h in an oven. In addition, total leaf area and green leaf area were also determined.

On the same day of harvest, before plant samples were put in the oven, several images of leaves were taken by a scanner (Epson expression 10000 XL). The total leaf area (*TLA*) and green leaf area (*GLA*) were determined using Winfolia software (Regent Instruments Inc., Canada).

Besides, *biomass partitioning* to vegetative tissue or reproductive tissue between anthesis and physiological maturity stages was estimated. For that, firstly the absolute gained biomass was determined by diminishing final plant *DW* biomass at physiological maturity minus plant *DW* biomass at anthesis. Secondly, the difference for *shoot DW* (leaf + stem) and *spike DW* between both growth periods was calculated. The final results are presented in percentage.

2.4.2 Yield and yield-trait parameters

At physiological maturity, apart from *biomass DW* determination and tiller (*FTN*) and spike (*FSN*) counting, the spikes were threshed by hand to separate the grains per spike and plant. Grains were divided into filled and non-filled, counting and weighing them. The obtained parameters were: *grain yield*, total grain number per spike (*TGNS*), grain filled percentage (*GF*) and individual grain weight (*IGW*).

2.5 Statistical analysis

The results presented in this PhD thesis are the mean ± standard error (SE) of different independent replicates, which varied depending on the growth stage. For data obtained at GS21 and GS21 + 9 days, two independent experiments were carried out, obtaining a final six independent biological replicates (n=6 pots) for each treatment at each environmental condition. Within each pot, different technical replicates were conducted for physiological parameters measurements, which were pooled to give a final biological replicate value. In the case of data obtained for anthesis (GS51 + 9 days) and physiological maturity (GS99) stages, one experiment was performed, driven four pots (n=4) for each treatment at each environmental condition. As for the above-mentioned growth stages, different technical replicates were conducted for physiological parameters measurements, which were pooled to give a final biological replicate value.

Data analyses were performed using the SPSS 24.0 software package (Chicago, IL). Three-way analysis of variance (ANOVA) was used to evaluate the main effects of elevated temperature (ET), elevated CO_2 (ECO_2), drought and their interactions on all dependent variables. Means were compared using Duncan's multiple range test. *P*-values ≤ of 0.05 were considered statistically significant. Before analyses, we tested whether the assumptions of an ANOVA, homogeneity of variances and normally distributed errors were achieved. The homogeneity of variances for all the studied parameters was evaluated by Levene's test and the distribution of the residuals was assessed by Kolmogorov–Smirnov test.

Principal component analysis (PCA) and correlation matrixes (Corplots) were performed using the open-source computer software R v4.0.3 and RStudio v1.1.456 and the pRocessomics R package facilitated by Dr. Valledor and colleagues from the University of Oviedo.

CHAPTER 3

BARLEY'S WATER RELATIONS

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3. Barley's water relations

3.1 Introduction

As it is well known, drought provokes a reduction in soil volumetric water content (*SVWC*). A lower *SVWC* reduces the amount of water in the soil or substrate that is available to be taken by plants through the roots (**Taiz et al., 2014**). When the availability of water is scarce, the physiological and metabolic processes of the plant are affected and consequently its growth. It is, hence, essential to know and quantify the plant water status to investigate its ability to cope with stress conditions. Relative water content (*RWC*), and water potential (Ψ_w), in this case also their components: osmotic potential (Ψ_o) and pressure potential (Ψ_t), give information about the water status of the plant.

It is necessary to keep in mind that water stress develops when the loss of water through transpiration exceeds its intake by the roots. Nevertheless, plants can trigger different adaptative mechanism to deal with water shortage. One possibility is to increase the capacity to uptake water by the roots decreasing the water potential and/or increasing root biomass (**Robredo et al., 2007; Markestejin and Poorter, 2009**).

Another possibility is to decrease the amount of water that is lost by the leaves. For that, a well-documented trait is the reduction of the stomatal conductance (*gs*) through stomata aperture/closeness control and/or stomatal physical properties (e.g. density, size) modulation (**Harrison et al., 2020**). Furthermore, if such mechanisms were not enough, plants to avoid a massive water loss could also modify their leaf anatomical properties reducing the leaf area (**Farooq et al., 2009**).

Another mechanism that has been seen to be involved in plant response to drought is the alteration of the hydraulic conductance (*K*) of the tissues, being the aquaporins (*AQPs*) activity regulation a key trait (**Maurel et al., 2016; Merlaen et al., 2019**). Depending on the tissue and cell type, development stage, and in response to a variety of environmental factors, *AQPs* expression pattern may be varied (**Groszmann et al., 2017**).

Moreover, it must be borne in mind that water within the plant tissues can move through two main pathways: by the apoplastic path through the outside of cells and by the cell-to-cell path, which its contribution to the overall water movement depends on the species, organ, developmental stage and environmental conditions, among others (**Maurel et al., 2016**).

The latter path involves the plasmodesmata and the transcellular path across the cell membranes, although, until today, it has not been determined experimentally each participation.

For barley, the cell-to-cell pathway through the aquaporin activity could imply almost 100 % of root hydraulic conductance (*Lpr*) (**Knipfer et al., 2011 and 2021**). *AQPs* are integral membrane proteins from a larger family of major intrinsic proteins (*MIPs*) that form pores in the membranes of biological cells, facilitating the transport of water between cells (**Maurel et al., 2008**).

Currently, at least forty *AQP* genes have been identified in barley; 19 plasma membrane intrinsic proteins (*PIPs*), 11 tonoplast intrinsic proteins (*TIPs*), 8 nodulin intrinsic proteins (*NIPs*) and two small intrinsic proteins (*SIPs*) (**Hove et al., 2015**). For some of them, it has been possible to verify their role in the water transport along with roots and leaves. The most important families involved in such water transport are *PIPs* and *TIPs* (**Katsuhara and Hanba, 2008; Knipfer and Fricke, 2010; Besse et al., 2011; Horie et al., 2011; Knipfer et al., 2011 and 2021**).

Nevertheless, little literature exists concerning barley aquaporin participation on water balance under drought conditions (**Kurowska, 2021**). Only a few works, **Dhanagond et al.** (**2019**) through a *QTL* study on roots, and **Kurowska et al.** (**2019**) focusing on *TIPs* response in leaves, have analysed their expression. Besides, **Veselov et al.** (**2018**) through an increased evaporative demand treatment, also studied the response of root *AQPs* on the *Lpr*, enquiring also in this case on the ABA implication.

Taking all this in mind, the balance between the uptake and loss of water processes will define plant *RWC*, contributing thus to an adequate plant water status. Therefore, plants try to modify the processes related to the uptake and loss of water to better adapt to water deficit. It is known that (1) the activation of a concrete mechanism to deal with water deficit and (2) the relevance of each activated mechanism can change with the age of the plant (**Tripathi et al., 2016**). Besides, it has been observed that drought might be more stressful at the early vegetative stage, where plants are more sensitive to stresses (**Gray and Brady, 2016**). At this stage, drought reduces leaf area, shoot elongation and tillering by decreasing *gs* and net photosynthetic rates (A_{net}), and simultaneously reduces the *RWC* of plants. However, other authors have demonstrated that the effects of drought are more severe when the drought stress occurs at the anthesis stage since plants have higher transpiration area and hence, the risk of water loss is increased (**Izanloo et al., 2008**). On the other hand, the mechanisms activated to cope with water stress might also be modified when the suppression of the water supply occurs in

interaction with other environmental agents, such as elevated temperature (ET) and elevated CO_2 (ECO_2), triggered by climate change (**Jagadish et al., 2014**).

Drought, combined with high temperature, are the most commonly co-occurring stressors. In this respect, it has been shown that warmer atmospheres could increase water losses reducing the capacity of plants to tolerate water stress (**Qaderi et al., 2019 and references therein; Sadok et al., 2020**). On the other hand, **Robredo et al.** (**2007**) stated that elevated CO_2 could allow a better tolerance to water stress in barley, increasing the capacity of plants to uptake water —due to greater root biomass and minimizing Ψ_w reduction—, and also avoiding water losses through better stomatal control. Furthermore, **Li et al.** (**2020a**) have observed that an overexpression of *AQPs* genes on plants grown at ECO_2 allowed them to cope better with drought impairments.

The effect on the plant water relations when drought, elevated temperature and elevated CO_2 are applied together has been far less studied. Until now, studies done in different species reveal that the response is very dependent on the species, on the magnitude of temperature increase and drought severity, and the developmental phase when stresses are applied (**Aranjuelo et al., 2006; Dias de Oliveira et al., 2013 and 2015a, b; Broughton et al., 2017**). Besides, in these studies, the water movement through the soil-plant-atmosphere continuum (SPAC) has not been analysed deeply, which is fundamental to understand the plant water relations under future environmental conditions.

Apart from the commented diverse effects of drought on plant water status, it is also important to study if, after a drought episode, the water replenishment may contribute to the posterior plant survival. In this respect, the analysis of the effects of re-watering after a withholding water period is crucial for understanding future plant performance. Moreover, the recovery period after re-watering is presented as the key phase for developing a stress memory, or instead, a resetting or forgetfulness, dictating plants ability to recover from vegetative drought effects (**Crisp et al., 2016**). To develop a stress memory significates that plants kept turned on the activated drought tolerance mechanisms or metabolic or biochemical signature after stress cessation. Resetting and forgetfulness, however, implies that the mechanism or processes that plants turned on to deal with the vegetative drought went back to control treatment levels after stress cessation (**Crisp et al., 2016; Martínez-Medina et al., 2016**).

In line with the plant memory effect, it has been shown that a mild drought period at the vegetative stage could prime plants against subsequent drought stress conferring them a positive stress memory, the so-called priming or hardening (**Martínez-Medina et al., 2016**). In

this regard, different works have been carried out for wheat, analysing the recovery capacity and priming effect on plant water relations, photosynthetic metabolism and final plant growth and grain yield. Through that works, it is concluded that plants response depends on the extent of the first and/or subsequent stresses, the interaction with other environmental agents (temperature) and genetic resources (**Wang et al., 2015; Abid et al., 2016; Liu et al., 2017; Mendanha et al., 2020**).

Therefore, intending to palliate the lack of knowledge above-exposed, throughout this Chapter we have studied the general mechanisms that barley plant activates under drought and how these mechanisms change with the moment of stress application and under future environmental conditions. This general objective has been divided into four specific objectives:

1. To elucidate if the moment of the water stress application (vegetative or anthesis stage) affects drought extent, determining the mechanisms that barley plants activate to respond to drought.
    - ✓ We hypothesize that drought applied at anthesis will be more stressful than the one applied at the vegetative stage, due to plants higher water demand and loss capacity in the former stage.

2. To study if future environmental conditions (elevated temperature and CO_2 applied together; CETE) improve or worsen the activated tolerance mechanisms.
    - ✓ We expect that plants grown at CETE conditions will respond better to drought, mainly owed to the beneficial effects of the elevated air CO_2 concentration on water loss control, finally permitting a better water status.

3. To analyse the response of different root and leaf aquaporin isoform expression under the studied water regimen treatments under CATA (current) and CETE (future) conditions, and to integrate that response with the one observed for the physiological data.
    - ✓ We hypothesize an isoform-specific expression response of the candidate *AQPs* genes along with the different water treatments. In addition, for drought treatments, higher relative expression values in CETE treatment plants would afford them to face better drought constraints compared with CATA drought treatment plants.

4. To investigate if plants that had suffered a vegetative drought treatment, on one hand, recovered proper water status, and on the other hand, developed a possible priming effect when suffered a subsequent drought period at anthesis. For both issues, we wanted to observe if the response of plants was or not dependent on the environmental conditions.
    - ✓ We hypothesise that irrespective of the environmental conditions, plants that suffered a drought period at the vegetative stage will be recovered from a water status point of view.
    - ✓ Otherwise, plants that suffered a vegetative drought will be primed, allowing them to cope better with a subsequent drought at anthesis.

3.2 Results

3.2.1 Soil and plant water status

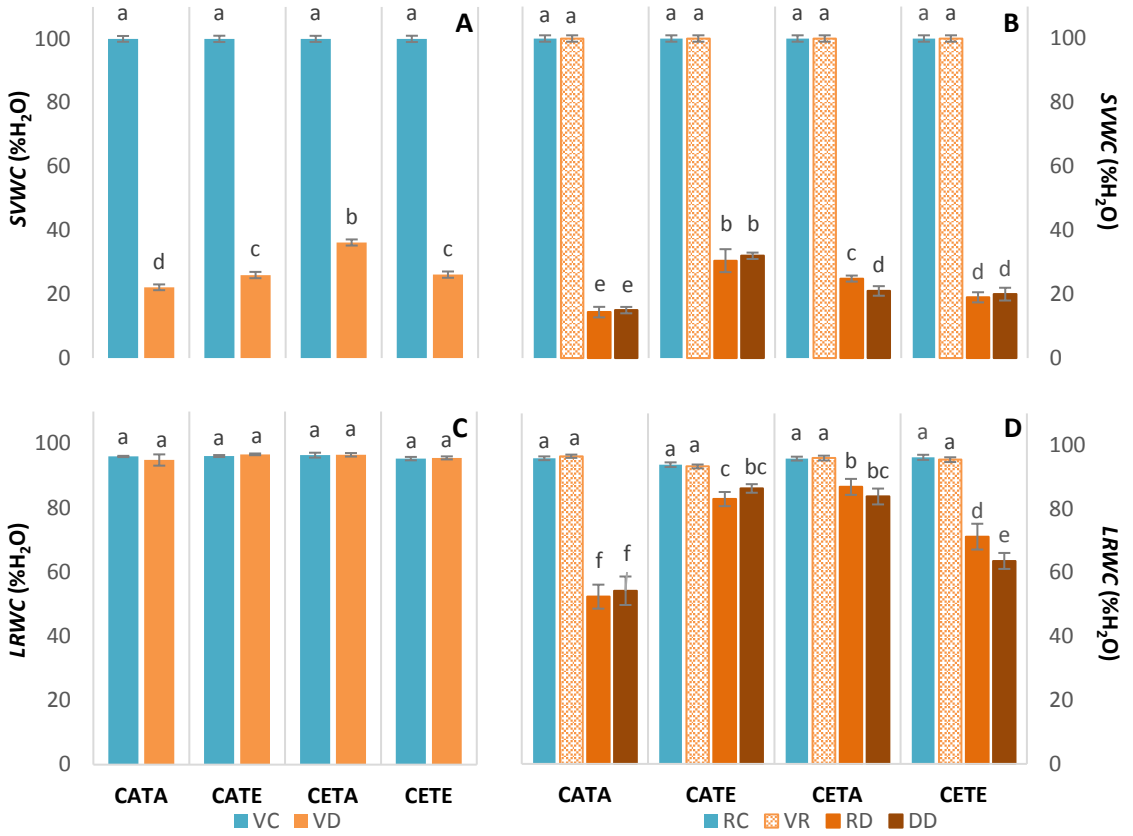

Fig. 3.1. Soil volumetric water content (*SVWC*; A-B) and leaf relative water content (*LRWC*; C-D) at the vegetative (GS21 + 9 days; A, C) and the anthesis (GS51 + 9days; B, D) stages in *Hordeum vulgare* cv. Henley plants subjected to different environmental conditions (CATA, ambient CO_2 and temperature; CATE, ambient CO_2 and elevated temperature; CETA, elevated CO_2 and ambient temperature and CETE, elevated CO_2 and temperature) and water regimens. Abbreviations for water regimen treatments denote VC, vegetative control; VD, vegetative drought; RC, anthesis control; VR, vegetative recovery; RD, anthesis drought; DD, double drought. Legend for water regime is depicted in the figure. Values represent mean ± SE values of 6 plants per treatment for the vegetative stage and 4 plants per treatment for the anthesis stage. Different letters indicate significant differences ($P \leq 0.05$) between water regimen treatments along with the different environmental conditions.

Regarding soil volumetric water content (*SVWC*), the decrease provoked by drought varied depending on the growth stage and the environmental conditions. The drought applied at the vegetative stage (VD) reduced *SVWC* compared with vegetative control treatments (VC), reaching values that were reduced of 22 %, 26 % at CATA and CETE, and of 36 % and 26 % at CATE and CETA conditions, respectively (Fig. 3.1A). VD treatment did not affect leaf relative water content (*LRWC)* in any environmental condition (Fig. 3.1C). The drought applied at

anthesis (RD) provoked a higher decrease of *SVWC* compared with anthesis control treatments (RC), detecting values for CATA and CETE conditions of 14 % and 22 %, and 25 % and 30 % for CATE and CETA (Fig. 3.1B), respectively. At this growth stage, such decreases in the *SVWC* gave, as a result, a reduction in the *LRWC* values. Concretely, at CATA and CETE conditions, *LRWC* values of 55 % and 70 % were registered, whilst at CATE and CETA conditions values around 80 % were recorded (Fig. 3.1D).

3.2.2 Water uptake processes

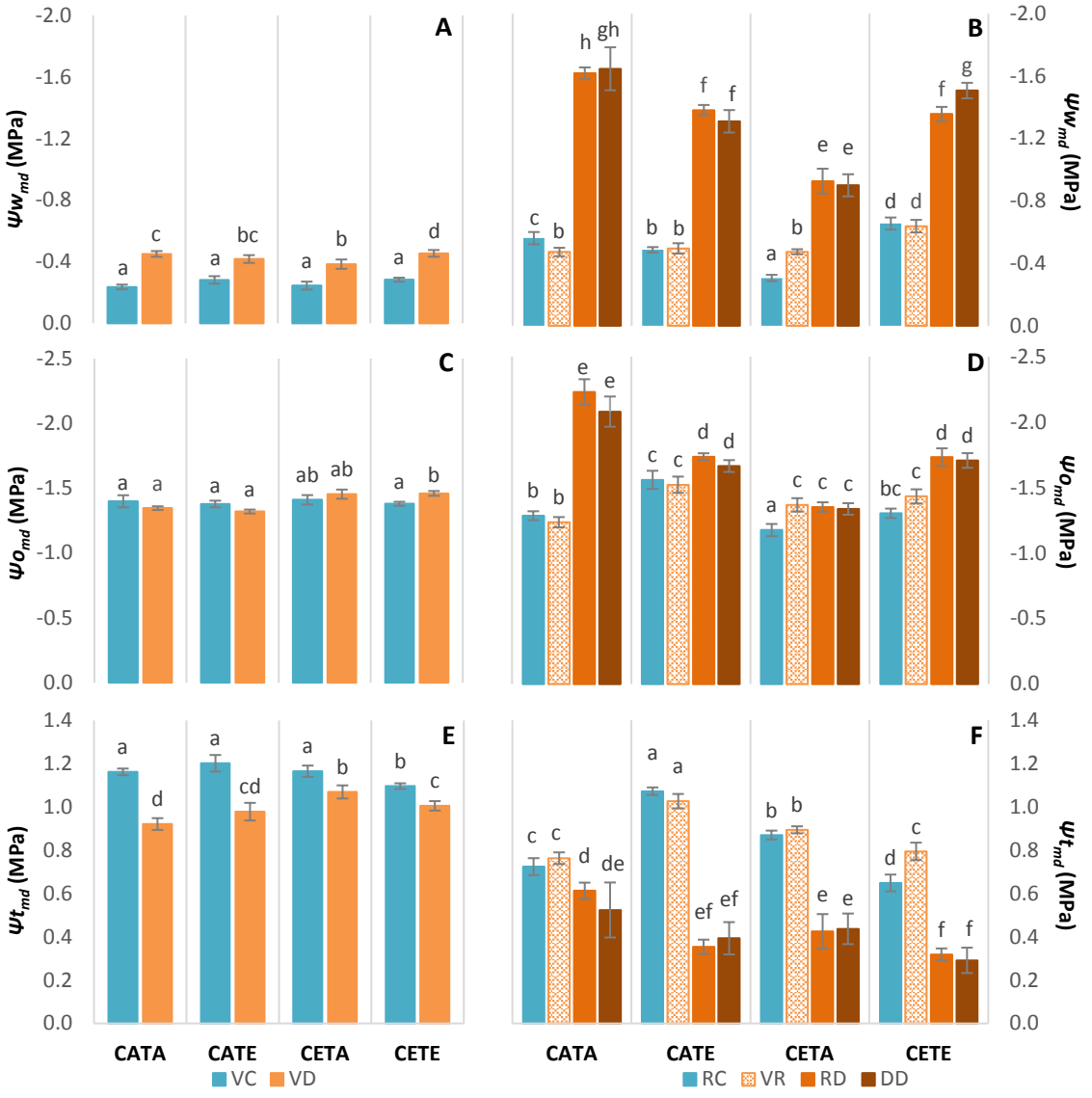

Fig. 3.2. Midday water potential (Ψw_{md}; A-B), osmotic potential (Ψo_{md}; C-D) and turgor potential (Ψt_{md}; E-F) at the vegetative (A, C, E) and the anthesis (B, D, F) stages. Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.

The balance between the uptake and loss of water determines the *LRWC* of the plant. As concerns the former, one of the main process that defines *LRWC* is the water potential (Ψw_{md}), a value that indicates the capacity of the plants to take up water, which is composed by the osmotic (Ψo_{md}) and turgor potential (Ψt_{md}). Changes in Ψw_{md}, Ψo_{md} and Ψt_{md} are depicted in Fig. 3.2.

As concerns the well-watered treatment and at the vegetative stage, except for the turgor potential (Ψt_{md}), no differences between the different environmental conditions were observed concerning water potential components. Conversely, at anthesis and for the RC treatment, slight differences were observed between environmental conditions, highlighting the higher Ψw_{md} at CETA and Ψo_{md} and Ψt_{md} at CATE conditions, respectively.

As regards drought treatments and Ψw_{md} (Fig. 3.2A-B) and Ψo_{md} (Fig. 3.2C-D), regardless of the environmental conditions, more negative values at anthesis compared with vegetative stage were observed. On the contrary, in the case of Ψt_{md} (Fig. 3.2E-F), lower values were recorded at anthesis, irrespective of the water regimen.

Focusing on the vegetative stage, slightly lower values for Ψw_{md} and Ψo_{md} were shown by CETE VD treatment compared with the same treatment at CATA conditions. Nevertheless, as concerns the Ψt_{md}, at CETE conditions higher values, together with a lesser reduction respect its control treatment, were observed compared with CATA conditions. No differences were observed for CATE and CETA conditions concerning water potential and its components.

At anthesis, the Ψw_{md} at RD treatment was more negative in CATA (-1.62 MPa) than in CETE (-1.36 MPa) conditions. Besides, different trends were observed for Ψo_{md} and Ψt_{md}. For the former and at CATA conditions, values around -2.2 MPa were reached, whereas at CETE conditions were around -1.7 MPa. For the latter, at CATA conditions the RD treatment slightly reduced it, while at CETE conditions it was reduced by 50 %.

Lastly, it is remarkable that the RD treatment at CETA conditions presented the highest Ψw_{md} and Ψo_{md} values, but lower Ψt_{md}, whilst at CATE conditions same values as for CETE conditions were registered.

Besides, when plants are under water stress it is necessary to maintain cell turgor as the water potential decreases to sustain as far as possible cell elongation and growth. In this respect, as soil water availability declined, midday water potential tended to decrease as we have observed in Fig. 3.2, so the water uptake continues.

Table 3.1. Effects of environmental conditions and water regimens on dehydration (*DH,* MPa), osmotic adjustment (*OA,* MPa) and cell-wall volumetric elasticity modulus (*ε,* MPa). Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1. Abbreviations denote GS, growth stage; EC, environmental conditions; WR, water regimen; VEG, vegetative; ANTH, anthesis.

GS	EC	WR	*DH*	*OA*	*ε*
VEG	**CATA**	**VC**	0.08 ± 0.01 c	- ± -	4.29 ± 0.27 c
		VD	0.19 ± 0.02 a	0.1 ± 0.03 a	6.58 ± 0.34 b
	CATE	**VC**	0.15 ± 0.04 a	- ± -	3.35 ± 0.63 d
		VD	0.10 ± 0.02 c	0.01 ± 0.05 a	9.41 ± 0.76 a
	CETA	**VC**	0.09 ± 0.01 c	- ± -	6.05 ± 0.73 b
		VD	0.11 ± 0.01 c	0.04 ± 0.03 a	7.90 ± 0.87 a
	CETE	**VC**	0.10 ± 0.01 c	- ± -	4.16 ± 0.23 c
		VD	0.15 ± 0.01 b	0.03 ± 0.03 a	8.13 ± 0.48 a
ANTH	**CATA**	**RC**	0.07 ± 0.00 f	- ± -	13.83 ± 0.42 a
		VR	0.01 ± 0.00 g	- ± -	13.47 ± 0.78 ab
		RD	1.10 ± 0.05 a	0.05 ± 0.05 a	1.22 ± 0.12 h
		DD	1.00 ± 0.06 a	0.03 ± 0.02 a	1.22 ± 0.28 h
	CATE	**RC**	0.04 ± 0.00 f	- ± -	7.28 ± 0.28 c
		VR	0.13 ± 0.00 e	- ± -	5.51 ± 0.50 de
		RD	0.40 ± 0.01 b	0.00 ± 0.05 a	4.46 ± 0.15 e
		DD	0.26 ± 0.01 c	0.02 ± 0.02 a	7.67 ± 0.56 c
	CETA	**RC**	0.08 ± 0.00 f	- ± -	5.31 ± 0.49 de
		VR	0.20 ± 0.01 d	- ± -	6.91 ± 0.41 cd
		RD	0.14 ± 0.00 e	0.12 ± 0.07 a	6.01 ± 0.61 d
		DD	0.12 ± 0.00 e	0.04 ± 0.03 a	4.83 ± 0.44 e
	CETE	**RC**	0.04 ± 0.00 f	- ± -	14.95 ± 0.55 a
		VR	0.06 ± 0.00 f	- ± -	12.85 ± 0.89 b
		RD	0.43 ± 0.02 b	0.00 ± 0.05 a	3.76 ± 0.12 f
		DD	0.41 ± 0.01 b	0.02 ± 0.01 a	2.84 ± 0.16 g

The active (osmotic adjustment; *OA*) or passive (dehydration; *DH*) osmolyte accumulation contributes to increase the osmotic pressure and reduce the water potential. These mechanisms, altogether with the elastic adjustment of the cell wall (*ε*), can contribute to maintaining the cell turgor under drought stress (Table 3.1).

As a general trend, regardless of the environmental conditions and the growth stage, it is observed that our barley plants did not perform *OA* under water stress treatment. In addition, irrespective of the growth stage, lower *DH* values were recorded in drought treatments at CETE conditions –and even lower at CATE but mainly at CETA– compared with the ones at CATA conditions. Furthermore, it is worth noting the observed values at anthesis, since at CATA conditions the RD treatment reached 1.10 MPa, compared with the 0.43 MPa at CETE, 0.40 MPa at CATE and 0.14 MPa at CETA conditions, respectively.

Moreover, focussing on *ε* and compared with control treatments, the VD treatment increased it by 50 % under CATA conditions, whereas at CETE conditions the increase was by 95 %. Besides, at anthesis and both environmental conditions, the RD treatment presented lower values compared with RC treatments, where the *ε* for RD treatment at CETE was 150 % higher concerning the same treatment at CATA conditions. Ultimately, as concerns CATE and CETA conditions, it is of interest to point out that the registered values for *ε* by RD treatments were higher than the ones commented for the above-mentioned environmental conditions.

Otherwise, plants can also change the root to shoot growth (*root/shoot ratio*) in an attempt to keep ensuring water uptake at drought conditions, a fact that was recorded by our drought treatments plants.

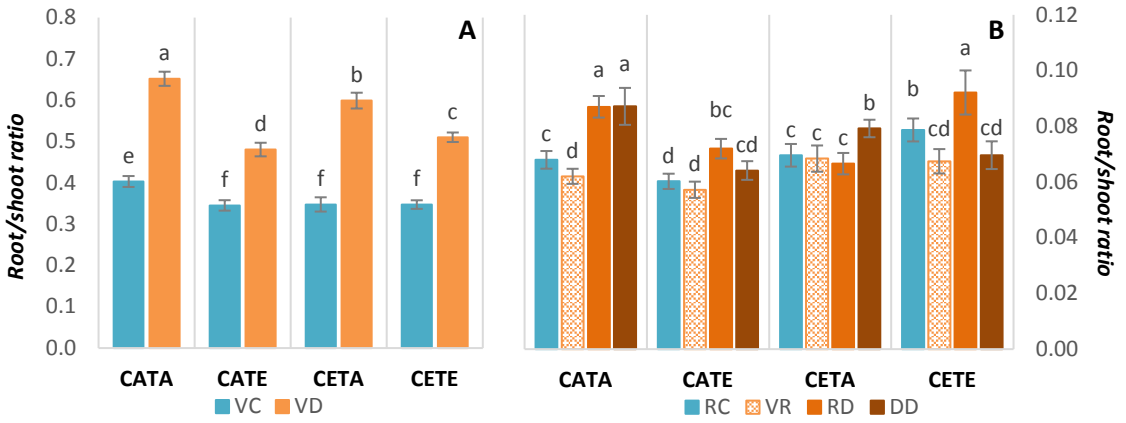

Fig. 3.3. *Root/shoot ratio* at the vegetative (A) and the anthesis (B) stages. Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.

At the vegetative stage (Fig. 3.3A), the VD treatment at CATA conditions increased the *root/shoot ratio* by 60 % compared with its control treatment, whilst at CETE conditions the increase was to a lesser extent, by 40 %, recording at the same time lower *root/shoot ratio* values than at CATA conditions. Nevertheless, at anthesis (Fig. 3.3B), no differences were shown between CATA and CETE conditions *root/shoot ratio* for the RD treatment, whereas the

increasing percentage respect the RC treatment at CATA conditions was higher (28 %) than for CETE (16 %).

In the case of CATE conditions, an increase respect to its control treatment was observed for either VD and RD treatments, being this increase of lesser magnitude and recording lower absolute values compared with the above-mentioned environmental conditions. Lastly, at CETA conditions, an increase of the same order to CATA on *root/shoot ratio* was observed at VD treatment, whilst at RD treatment no larger differences were observed compared with its RC treatment.

3.2.3 Water loss processes

In response to drought, as abovementioned, plants develop some strategies to maintain an adequate water status that allow them to resist until the conditions return to suitable conditions for resume their growth, being water loss by transpiration another key process.

Irrespective of the environmental conditions and water treatment, the cumulative transpiration per plant (*T*) at anthesis (Fig. 3.4B) was higher than at the vegetative stage (Fig. 3.4A), mainly owed to the greater green leaf area (*GLA*; Fig. 3.4C-D).

As regards well-watered treatments, either at vegetative or anthesis stage, CETE treatments presented around 10 % lower *T* values compared with CATA conditions, whereas the *GLA* values were increased by VC, but remained unchanged by RC treatment. Moreover, lower *T* values were recorded too for CATE and CETE conditions at both growth stages. Concerning *GLA* and the CATE conditions, it was increased by VC but reduced by RC. In the case of CETA conditions, no differences were observed for the *GLA* neither at vegetative nor at anthesis stages.

As concerns drought effects on *T*, it is of highlight that all treatments reduced it –to a higher or lesser extent–, together with the *GLA*. At CETE conditions slightly statistically significant lower *T* values were registered compared with CATA conditions for both VD and RD treatments, whilst the *GLA* presented the opposite trend. Furthermore, the trend observed at CATE conditions was dependent on the growth stage. Being this way, at the vegetative stage, the VD treatment did not modify *T* –but increased *GLA*– compared with CATA, whereas the percentage of drop caused by RD to its RC treatment was to a lesser extent at CATE conditions, matching with the *GLA* values. Lastly, in the case of CETA conditions, on one hand, it is worth noting that the VD treatment presented the lowest *T* and *GLA* values. On the other hand, the RD

treatment at these environmental conditions provoked the lowest reduction in *T* values compared with its control treatment.

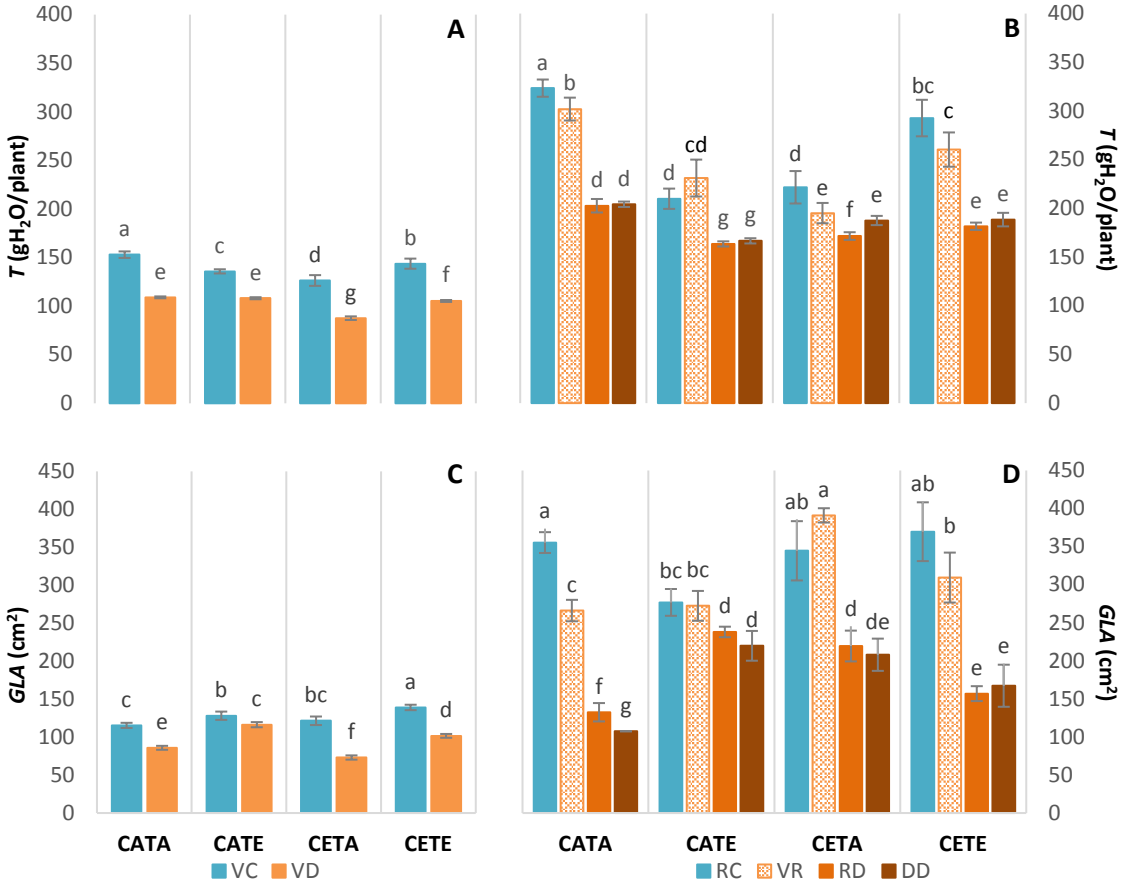

Fig. 3.4. Accumulated transpiration along with water stress period (*T*, A-B) and green leaf area (*GLA*, C-D) at the vegetative (A, C) and the anthesis (B, D) stages. Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.In addition to the general parameters related to the plant water loss described in Fig. 3.4, the stomatal conductance (*gs*), instantaneous transpiration (*E*) and plant hydraulic conductance (*K*) add more specific information about the water flow within the plant (Fig. 3.5).

As regards well-watered treatments, *gs* (Fig. 3.5A-B) and *E* (Fig. 3.5C-D) were higher at anthesis, whilst *K* (Fig. 3.5E-F) presented higher values at the vegetative stage irrespective of the environmental conditions. Moreover, under CETE conditions and both at VC and RC treatments, those parameters presented statistically significant lower values compared with CATA.

If data for CATE and CETA conditions are analysed –the individual environmental conditions that form CETE conditions–, different trends were observed. In the former, the VC treatment increased *gs* and *E* but did not modify *K*, whereas at RC it reduced the first two parameters and increased the latest. As for CETA conditions, either VC or RC treatments

presented the same trend explained for CETE, adding that the values recorded for *E* were even lower.

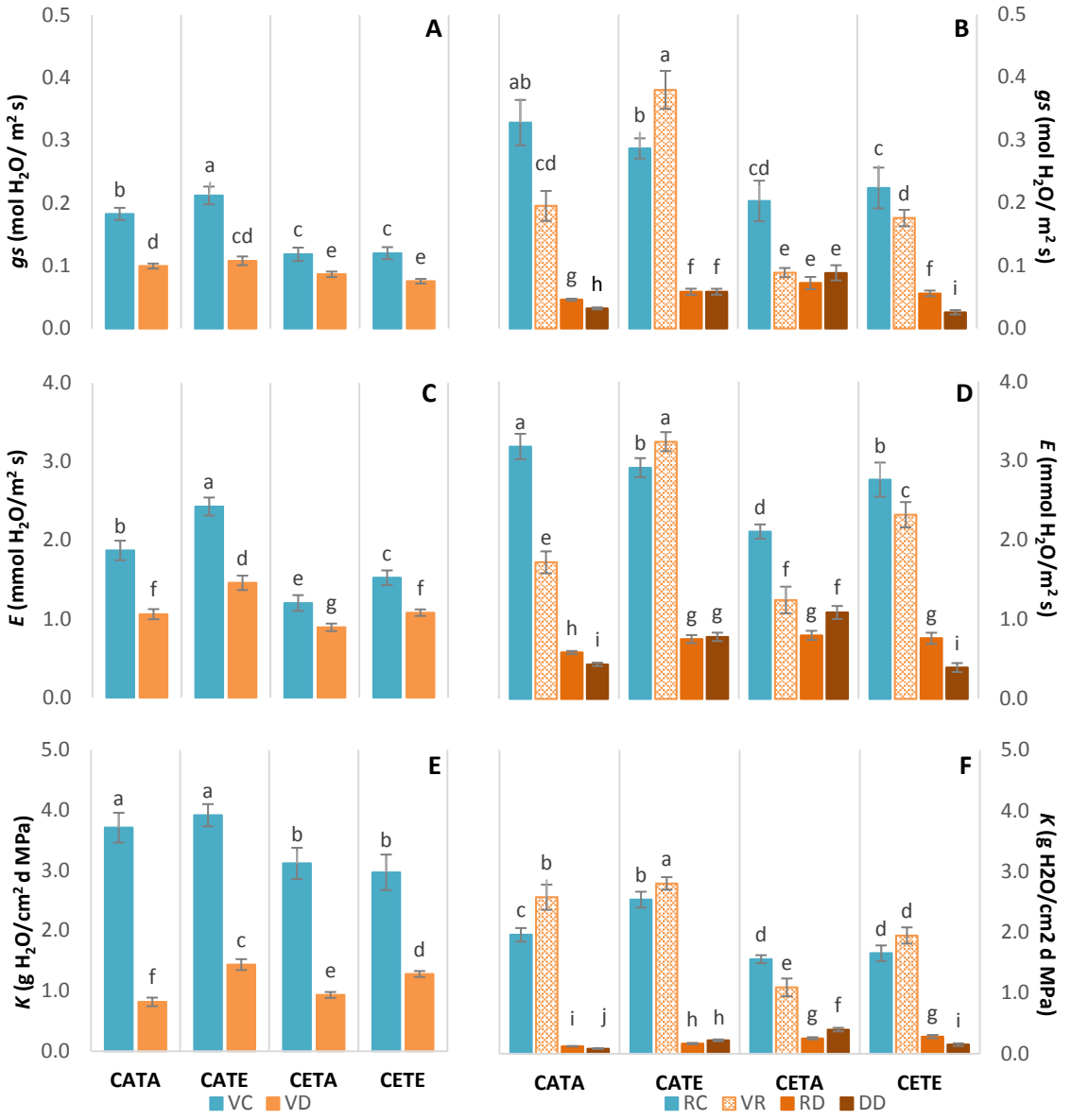

Fig. 3.5. Stomatal conductance (*gs*, A-B), instantaneous transpiration (*E*, C-D) and plant hydraulic conductance (*K*, E-F) at the vegetative (A, C, D) and the anthesis (B, D, F) stages. Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.

Besides, and in line with the commented facts in Fig. 3.4, regardless of the environmental conditions, in drought treatments the decreases of *gs*, *E* and *K* values were statistically significant, being the observed reductions more marked in RD than in VD treatments. Nevertheless, the registered response to drought between the different environmental conditions varied with the growth stage (Fig. 3.5).

At the vegetative stage, VD treatment at CETE conditions presented 22 % lower *gs*, same *E* and 55 % higher *K* values compared with CATA VD, whilst RD treatment at CETE conditions recorded statistically significant higher values for all the parameters depicted in Fig. 3.5.

Otherwise, the response of drought treatments at CATE and CETA conditions compared with CATA was also unique. In the case of CATE conditions, both VD and RD treatments presented higher *gs*, *E* and *K* values. As regards CETA conditions, the VD treatment recorded lower *gs* and *E* but higher *K* values, whereas the RD treatment increased them compared with the same treatment values at CATA conditions.

Lastly, through the PCA-biplot presented in Fig. 3.6, it is possible to see which of the variables studied –growth stage, water regimen and environmental conditions–, had the largest effect on studied water-related parameters for current (CATA) and future (CETE) environmental conditions.

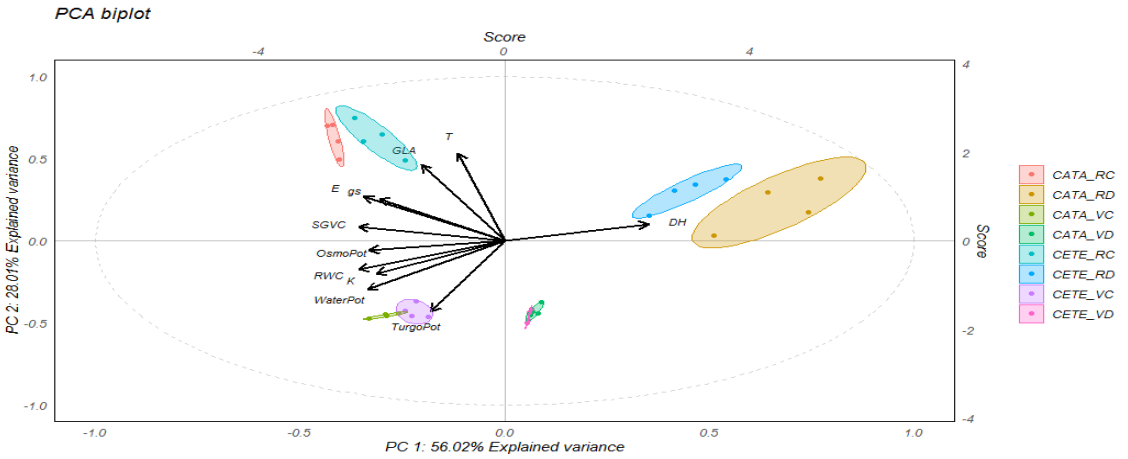

Fig. 3.6. Principal component analysis (PCA-biplot) based on PC1 and PC2 for key physiological parameters along with different water regimens under current (CATA) and future (CETE) environmental conditions. Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.Projections of the arrows on the axes indicate with which variables the PCs are linked the most. The arrow closeness indicates how much correlation exists between the variables. For abbreviations, *WaterPot*, *OsmoPot* and *TurgoPot* denote Ψw_{md}, Ψo_{md} and Ψt_{md}, respectively. The rest of the abbreviations are the same described previously.

The PC1 explained the 56.02 % of the variance, separating drought treatments and control treatments irrespective of the growth stage. On one hand, the parameter that most correlated positively with the PC1 was *DH*. On the other hand, the rest of the parameters presented a negative correlation pattern with the PC1, where Ψo_{md}, *K* and *LRWC* seemed to be the ones which more explained such behaviour, being the opposite to *DH*.

As concerns the PC2, it explained the 26.01 % of the variance, which evidenced the different behaviour between vegetative and anthesis treatments. In this case, the parameters that most correlated positively with PC2 were *T* and *GLA*.

It is of highlight that, for the same water regimen treatment under the same growth stage, the environmental conditions barely affected the observed behaviour. However, regardless of the water regimen, at anthesis, a clear clustering pattern was developed between CATA and CETE treatments, especially important for RD treatments since CETE one was nearest grouped from control treatments.

3.2.4 *AQPs* expression pattern

Overall, non-statistically significant differences were recorded for each *housekeeping gene* between treatments, which strengthens its suitability to be employed as reference genes for our experiment.

Regarding the results obtained for candidate *AQPs* genes, in general, no large expression values differences were observed between vegetative (Fig. 3.7A-B) and anthesis (Fig. 3.8A-B) treatments values, except for *HvPIP2;5*, where, specially in roots, the expression values at anthesis were much lower. In addition, it is remarkable that both at vegetative and anthesis, the expression values of *HvPIP1;3* isoform were higher in leaf than in root.

Focusing at the vegetative stage, both in leaf (Fig. 3.7A) and in the root (Fig. 3.7B), *HvPIP1;3*, *HvPIP2;5* and *HvTIP1;1* isoforms were the ones with the highest expression values.

Under well-watered regimen (VC), an isoform-specific response was observed for leaf expression data. Firstly, and compared with CATA conditions, leaf *HvPIP1;3* and *HvPIP2;1* genes presented a decrease by 30 % and 45 %, respectively, at CETE conditions. Secondly, non-statistically significant differences were registered for leaf *HvPIP2;2* and *HvTIP1;1* isoforms, whereas an increase by 100 % was recorded for leaf *HvPIP2;5* isoform at CETE conditions. As concerns root expression data, no differences were observed for any aquaporin isoform between CATA and CETE conditions.

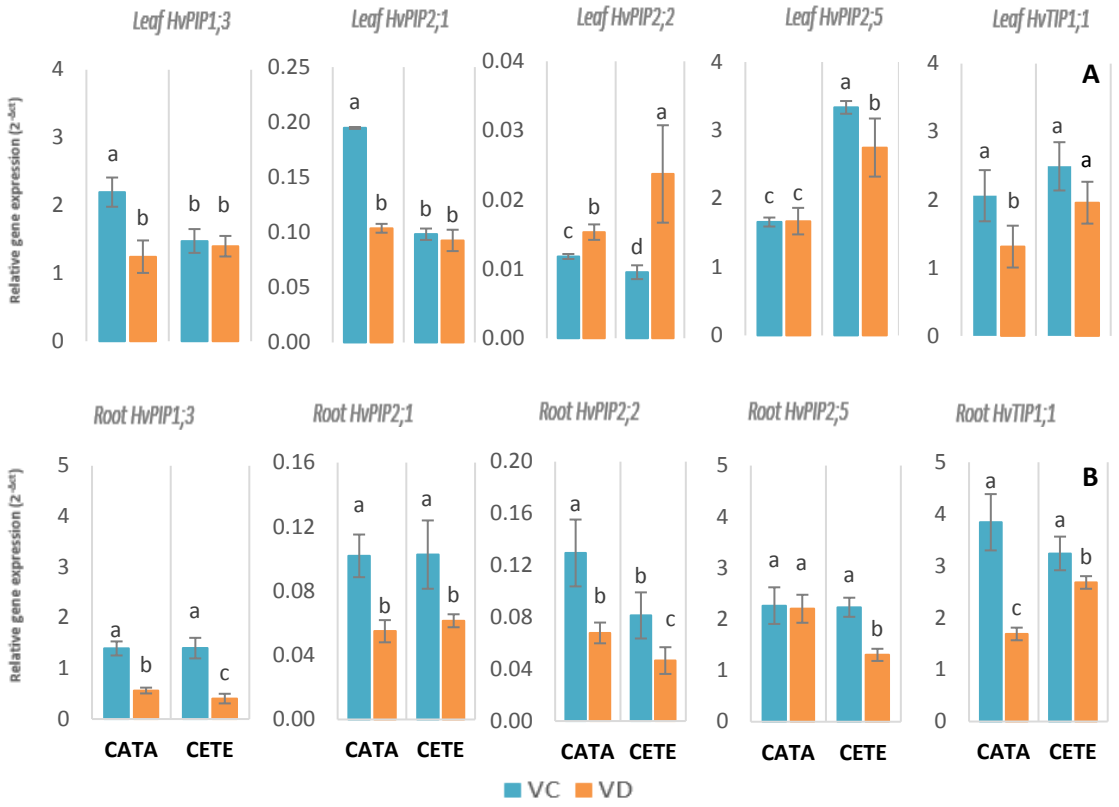

Fig. 3.7. Leaf (A) and root (B) *AQPs* expression data under current (CATA) and future (CETE) environmental conditions at the vegetative stage. Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.

Drought treatment at the vegetative stage (VD) also triggered an isoform-specific response in leaves. At CATA conditions, it reduced by 45 % the expression of leaf *HvPIP1;3* and *HvPIP2;1* and by 40 % and 20 % the expression of *HvTIP1;1* and *HvPIP2;5*, respectively, while it slightly increased the *HvPIP2;2* isoform. Nevertheless, at CETE conditions, no reduction was observed for any given isoform compared with its VC treatment, and only an increase for *HvPIP2;2* was shown. In roots, the expression of aquaporin isoforms decreased, except for CATA *HvPIP2;5* isoform, which remained unchanged.

As regards leaf and root expression data at anthesis, different trends that the ones commented for the vegetative stage were detected. On one hand, for control treatments, there were not differences between the expression of any leaf *PIP* candidate genes between CATA and CETE conditions (Fig. 3.8A), whereas, in the case of roots, only *HvPIP2;5* presented lower values at CETE conditions (Fig. 3.8B). Concerning *HvTIP1;1* isoform, it was reduced in leaves by CETE conditions.

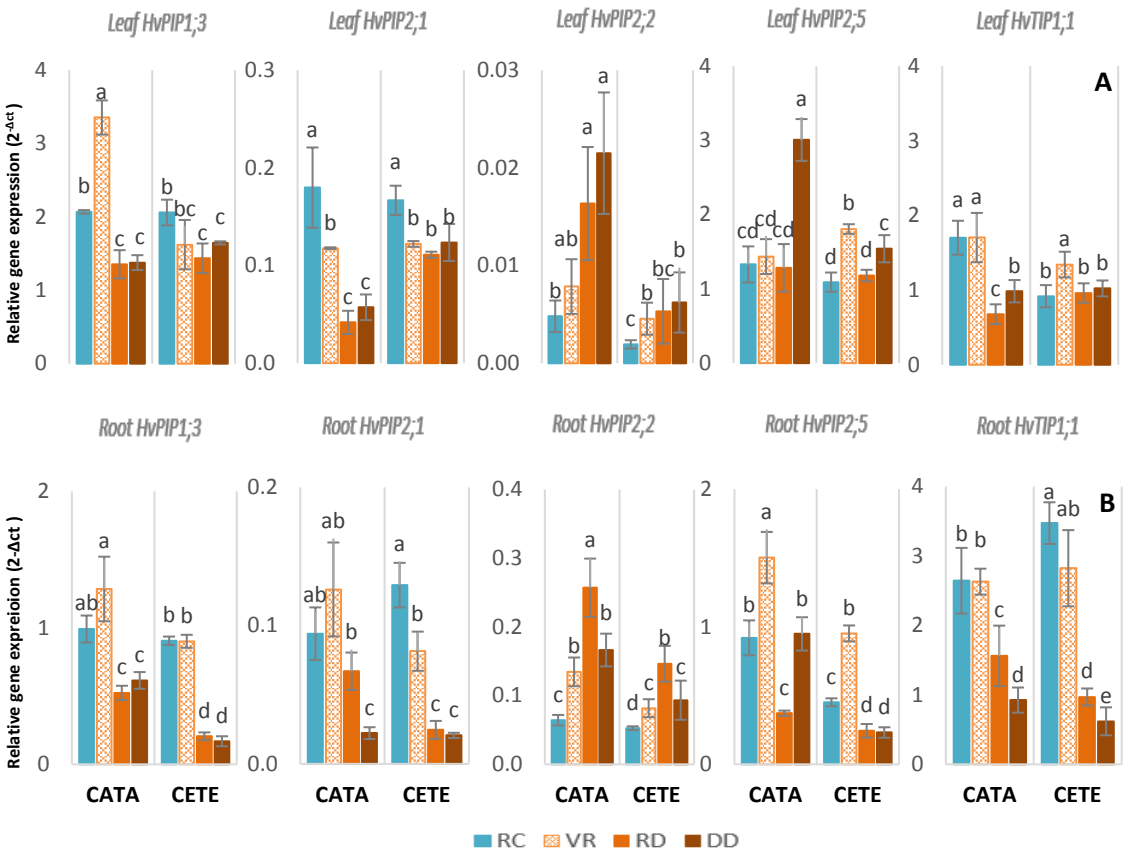

Fig. 3.8. Leaf (A) and root (B) *AQPs* expression data under current (CATA) and future (CETE) environmental conditions at the anthesis stage. Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.

On the other hand, the response of leaf genes expression to the anthesis drought was also isoform-specific. Thus, both at CATA and CETE conditions, the RD treatment decreased the expression of *HvPIP1;3* and *HvPIP2;*1 isoforms, although for the latter isoform the reduction at CETE conditions, respect its RC treatment, was much lower compared with the one at CATA conditions (35 % *vs.* 75 %). Regardless of the environmental conditions, RD treatment did not modify the expression of *HvPIP2;5* isoform, whereas for leaf *HvTIP1;1* isoform, it reduced by 60 % at CATA conditions but not at CETE conditions. The leaf *HvPIP2;2* isoform was increased at both environmental conditions, although at CETE conditions was not statistically significant.

Lastly, as it happened at the vegetative stage, in general, the anthesis drought treatment reduced the expression of root aquaporin isoforms except for *HvPIP2;2*, which it increased. The rest of the *PIP* isoforms showed lower values at CETE than at CATA conditions.

With the aim of ensuring the role of those isoforms in the water balance, we performed different correlation matrixes between the water flow related parameters and aquaporin

relative expression data under the current and future environmental conditions (Fig. S1). Those correlations are summarized in Table 3.2.

Table 3.2. Summary table for the main division correlations between water-related physiological parameters (*K, E and gs*) and leaf and root *AQP*s expression data that met the requirement of r> ±0.55 and P< 0.05. All; all treatments (VC, VD, RC, RC). Controls; all well-watered treatments (VC, RC). Drought; all drought treatments (VD, RD). Veg; all vegetative treatments (VC, VD). Anthesis; all treatments at anthesis (RC, RD). CATA; all treatments at CATA conditions (VC, VD, RC, RD). CETE; all treatments at CETE conditions VC, VD, RC, RD). Plant memory; all treatments at anthesis (RC, VR, RD, DD). Recovery; well-watered treatments at anthesis (RC, VR). Priming; drought treatments at anthesis (RD, DD). *P* refers to a positive and *N* to a negative correlation.

Divisions	*Leaf AQP*	P/N	Parameter	*Root AQP*	P/N	Parameter
All	*HvPIP1;3*	P	*gs, E*	*HvPIP1;3*	P	*K*
	HvPIP2;1	P	*K, gs, E*	*HvPIP2;1*	P	*K, gs, E*
	HvTIP1;1	P	*K*	*HvPIP2;5*	P	*K*
				HvTIP1;1	P	*K, E*
Controls	*HvPIP1;3*	P	*E*	*HvPIP1;3*	P	*K, E (N)*
	HvPIP2;2	P	*K, E(N)*	*HvPIP2;2*	P	*K*
	HvPIP2;5	N	*gs, E*	*HvPIP2;5*	P	*K, E (N)*
	HvTIP1;1	P	*K, E (N)*			
Drought	*HvPIP2;1*	P	*E,*	*HvPIP2;2*	N	*K, gs, E*
	HvPIP2;5	P	*K, E*	*HvPIP2;5*	P	*K, gs, E*
	HvTIP1;1	P	*K, gs, E*	*HvTIP1;1*	P	*K*
Veg	*HvPIP1;3*	P	*K, gs, E*	*HvPIP1;3*	P	*K, gs, E*
	HvPIP2;1	P	*K, gs, E*	*HvPIP2;1*	P	*K, gs, E*
	HvTIP1;1	P	*K*	*HvPIP2;2*	P	*K, gs, E*
				HvTIP1;1	P	*K, gs, E*
Anthesis	*HvPIP1;3*	P	*K, gs, E*	*HvPIP1;3*	P	*K, gs, E*
	HvPIP2;1	P	*K, gs, E*	*HvPIP2;1*	P	*K, gs, E*
	HvTIP1;1	P	*K, gs, E*	*HvPIP2;2*	N	*K, gs, E*
				HvPIP2;5	P	*K, gs, E*
				HvTIP1;1	P	*K, E*
CATA	*HvPIP1;3*	P	*K, gs, E*	*HvPIP1;3*	P	*K, E*
	HvPIP2;1	P	*K, E, gs*	*HvPIP2;1*	P	*K, gs*
	HvPIP2;2	N	*gs, E*	*HvPIP2;5*	P	*K*
	HvTIP1;1	P	*K, E, gs*	*HvTIP1;1*	P	*K*
CETE	*HvPIP1;3*	P	*gs, E*	*HvPIP1;3*	P	*K*
	HvPIP2;1	P	*gs, E*	*HvPIP2;1*	P	*K, gs, E*
	HvPIP2;5	P	*K*	*HvPIP2;5*	P	*K*
	HvTIP1;1	P	*K*	*HvTIP1;1*	P	*K, gs, E*
Plant memory	*HvPIP1;3*	P	*K*	*HvPIP2;1*	P	*K, gs, E*
	HvPIP2;1	P	*gs, E*	*HvPIP2;2*	N	*E*
	HvTIP1;1	P	*K, gs*	*HvPIP2;5*	P	*K*
				HvTIP1;1	P	*K, gs, E*
Recovery	*HvPIP1;3*	P	*K*	*HvPIP2;2*	P/N	*K (P), E (N)*
				HvPIP2;5	P/N	*K (P), E (N)*
Priming	*HvPIP2;2*	N	*K*	*HvPIP1;3*	N	*K, gs, E*
	HvPIP2;5	N	*K*	*HvPIP2;5*	N	*K*

To verify the correlations between the aquaporin genes and physiological data (water and gas-exchange parameters), a negative control was carried out for one of the housekeeping genes, the *H⁺-ATPase* (Fig. S1). The leaf and root *H⁺-ATPase* did not present either positive nor

negative correlations with the analysed physiological data, which would strengthen the obtained correlations.

Thus, the most outstanding information obtained by the correlation matrixes for the water transport function of our candidate *AQPs* is that in general, positive correlations were registered both for leaf and root *AQPs* candidates with physiological data, except for *HvPIP2;2*, which presented negative correlations. At leaves, the most highlighting correlations along the different categories were observed for *HvPIP1;3*, *HvPIP2;1* and *HvTIP1;1*, whereas *HvPIP2;5* seemed to be more erratic. In the case of roots, all the isoforms were found to be positively correlated with water-related parameters except the commented *HvPIP2;2*.

3.2.5 Plant water relations recovery and possible priming effect

If *LRWC* is taken as a final plant water status parameter, and the recovery capacity of plants that had suffered a vegetative drought and were subsequently re-watered (VR) is analysed (comparing them with RC), VR plants presented the same value as RC plants, a logic trend since VD treatment did not modify it (Fig. 3.1D). Furthermore, a similar trend was observed for water potential data and its components (Fig. 3.2), whilst it is of interest to point out that at CATA and CETE conditions VR treated plants presented statistically lower *root/shoot ratio* values compared with RC CATA (Fig. 3.3B). In line with the last fact, and as concerns water loss parameters, the VR treatment plants presented different values compared with RC ones. The response varied depending on the environmental conditions at which plants were grown.

At CATA conditions, the VR treatment presented statistically significant 7 % lower *T* values (Fig. 3.4B), where the *GLA*, *gs* and *E* matched with such reduction through 25 %, 40 % and 45 % lower values, respectively, whilst the *K* presented 32% higher ones (Fig. 3.4D and Fig. 3.5B, D, F). Moreover, at CETE, the VR treatment did also reduce the *T* and *GLA by* around 11 % (although they were not statistically significant) and *gs* and *E* by 15 %, keeping constant *K* values. In this point, it should be noted that under CATE conditions a trend towards higher *T* values at VR treatment compared with RC treatment was observed, where the greater values for *gs*, *E* and *K* matched with it. Moreover, at CETA conditions and as concerns the water loss related parameters, the opposite trend was shown (Fig. 3.4 and 3.5).

In line with the water loss and water flow processes within the plant, the expression pattern of the *AQPs* genes were also studied for the VR treatment at CATA and CETE conditions (Fig. 3.8). In that sense, an isoform specific-response was observed, which varied between CATA

and CETE conditions. On one hand, at CATA conditions and for leaves, the expression of *HvPIP1;3* was increased compared with RC treatment, whereas the expression of *HvPIP2;1* was diminished and the expression of the rest *AQPs* remained unchanged. Furthermore, in the case of root *AQPs* expression, it is worth noting that higher values were recorded for VR treatment by *HvPIP2;2* and *HvPIP2;5* isoforms.

On the other hand, at CETE conditions, the expression of leaf *HvPIP1;3* was not modified compared with its RC treatment, whereas the *HvPIP2;1* expression decreased and the *HvPIP2;2, HvPIP2;5* and *HvTP1;1* expression were increased. A similar trend to leaf was observed for root *AQPs* expression pattern between VR and RC treatments, except that leaf and root *HvTIP1;1* isoforms remained unchanged.

Otherwise, to inquire about the possible priming effect of plants that had suffered a vegetative drought and subsequently suffered another drought period at anthesis (DD), the obtained results for plant water relations were compared with RD treatment ones. In that sense, a unique response pattern was developed for either CATA or CETE. For the former, no longer differences were observed for water status and uptake studied parameters between RD and DD treatments. Nevertheless, at CETE, changes in *LRWC*, Ψw_{md} and *root/shoot ratio* were observed.

Besides, when water flow within plant-related parameters is analysed, it is observed that at both environmental conditions the DD treatment presented statistically significant lower *gs*, *E* and *K* values. In line with this, it is worth noting that at CATA conditions the expression of leaf and root *HvPIP2;5* isoforms were doubled at DD treatment compared with RD treatment, whereas leaf *HvTIP1;1* was slightly increased, but root *HvTIP1;1* decreased (Fig. 3.8A-B). At CETE conditions, a similar response to the above-mentioned for CATA was observed for *AQPs* expression genes, although the increase of leaf *HvPIP2;5* was more moderated and the one at root kept constant.

Lastly, regarding the possible priming effect by DD treatment at CATE and CETA conditions, no differences were recorded for the former compared with RD treatment at any given water-related parameter. Otherwise, at CETA conditions, no differences were shown for final water status and water uptake parameters, although the water loss parameters presented higher values at DD treatment compared with RD treatment.

3.3 Discussion

3.3.1 Water status of drought treatments under CATA and CETE conditions

The balance between the water loss and uptake processes determined the rate of decrease of soil *SVWC*. This, in turn, depending on the growth stage, affected in different way *LRWC* values, a parameter that indicates the water status of the leaf (**Yamasaki and Dillenburg, 1999**).

As concerns the VD treatment, *SVWC* decreased reaching values around 25 % of maximum soil water content both at CATA and at CETE conditions (Fig. 3.1A). This diminution did not modify the *LRWC* in any of the environmental conditions (Fig. 3.1C). The reason for the maintenance of *LRWC* could lie in the fact that *LRWC* declines only when *SVWC* has dropped below a threshold value (**Polley et al., 2012**), that for the vegetative drought treatments of our barley plants was not reached. Similarities in *LRWC* between environmental conditions have been observed for alfalfa (**Aranjuelo et al., 2006**), loblolly pine (**Wertin et al., 2010**) and different wheat genotypes (**Abdelhakim et al., 2021**). The similarity in *LRWC* between CATA and CETE conditions is indicating a similar balance between water uptake and loss capacity in both environmental conditions.

As regards the RD treatment and regardless of the environmental conditions, firstly, the *SVWC* of RD treatments decreased more –detecting values around 14 % of the controls at CATA– , compared with the *SVWC* of VD treatments (Fig. 3.1A-B). Secondly, lower *LRWC* values were registered at RD treatment plants compared with VD plants (Fig. 3.1C-D). These results indicate that the effect of the water stress period on the soil and plant water status was much higher than the one observed during the vegetative drought period.

Otherwise, unlike for VD treatment, the effects of RD treatment were different between CATA and CETE conditions; at CETE conditions plants presented higher *LRWC* values compared with the ones detected at CATA conditions (70 % *vs.* 55 %). This result indicates that the water stress was more severe in CATA conditions, thus, elevated CO_2 together with high temperatures delay the negative effects of water stress. **Yu et al.** (**2012**) in tall fescue, a grassland species, also observed a greater *LRWC* at CETE than at CATA conditions in plants subjected to drought. Despite this fact, they did not demonstrate the reason behind this trend; they suggested that a

greater root system at CETE conditions could be involved in it. Moreover, **Li et al.** (**2019**) also observed higher *LRWC* values on wheat plants that suffered a drought period at the reproductive stage and were subjected to future environmental conditions, attributing it in that case to the positive effect of ECO_2 on water loss control by *gs* reduction, being involved the leaf ABA concentration.

To try to disengage the observed response for *LRWC* between CATA and CETE conditions, we analysed the response pattern of *LRWC* of RD treatments grown at CATE and CETA; higher values at these conditions can be shown, specially at CETA (Fig. 3.1D). This fact would imply that the response of plants to drought at future environmental conditions cannot be elucidated by the analysis of single environmental agents (**Rampino et al., 2012**), which strengths the need to carry out works like ours to try to shed some light as concerns this issue.

Therefore, to explain the differences observed in barley plants response between vegetative and anthesis drought (specific objective 1), and also regarding the observed differences at RD treatment concerning *LRWC* between CATA and CETE conditions (specific objective 2), we will focus on the analysis of the activated mechanisms to increase water uptake and to reduce the water losses. To facilitate the discussion of results concerning the specific objective 2, data of CATA and CETE conditions will be compared, referring only to CATE and CETA results when appropriate.

3.3.2 Plants water uptake processes

Water uptake capacity is partially defined (i) by the water potential (Ψw) difference between the soil and the plant and (ii) by the root biomass itself. Under CATA conditions and at the vegetative stage, VD reduced Ψw_{md} until -0.44 MPa (Fig. 3.2A) explained by a decrease in turgor potential (Fig. 3.2E), while at anthesis Ψw_{md} was reduced till -1.62 MPa (Fig. 3.2B) explained mainly by a massive reduction in osmotic potential (Fig. 3.2D). Thus, the mechanisms activated to reduce Ψw_{md} at vegetative and anthesis are different. When the water stress is not severe, the plant can afford to reduce turgor potential to some extent, while when the water stress is more severe the plant reduces the osmotic potential maintaining high the turgor potential which is necessary to an adequate growth (**Farooq et al., 2009 and references therein**).

Furthermore, at CETE conditions, the reduction on Ψw_{md} was statistically similar to CATA conditions at the vegetative (-0.45 MPa) stage but lower at the anthesis (-1.36 MPa) stage.

Besides, the relevance of the mechanisms responsible for the behaviour was different between both environmental conditions. At the vegetative stage, where the stress was mild, the Ψw_{md} at CATA VD treatment was decreased due to a reduction of turgor potential as above-mentioned, while for the same treatment at CETE conditions the reduction was ascribed both to the reduction of osmotic and turgor potentials. At anthesis, where the stress was more severe, the Ψw_{md} was reduced by CETE RD treatment owed to a decrease of both osmotic and turgor potential, while in CATA the decrease was only ascribed to a massive decrease of osmotic potential.

Generally, irrespective of the treatment, the decreases of Ψo_{md} were due to increases in *DH*, since no *OA* was detected (Table 3.1), as **Robredo et al.** (**2007**) also observed in barley. In addition, CATA RD treatment plants presented the highest cell *DH*, which was negatively correlated with *LRWC* as can be observed in the PCA-biplot (Fig. 3.6). Nevertheless, despite the lower water content of those plants, their cell walls became extremely elastic. The activation of this mechanism by those plants allowed them to adjust better to the new cell volume and kept driving an optimum Ψt_{md} (Fig. 3.2F) as **Pérez-López et al.** (**2009**) for barley and **Miranda-Apodaca et al.** (**2018**) for different grass species suggested. As plant growth is in part determined by cell expansion, which involves turgor potential (**Farooq et al., 2009 and references therein**), this strategy might be ascribed to an anisohydric behaviour for growth maintaining (**Sade et al., 2012; Locke and Ort, 2015**) as it will be explained in Chapter 5.

Otherwise, CETE RD treatment plants were able to avoid a massive cell *DH*, although it was at the expense of higher reduction on turgor potential despite their adjusted –to a lesser extent– cell wall elasticity. The latter strategy developed by CETE RD plants allowed them to ensure a better leaf cell water status (Fig. 3.2D) and probably better maintenance of the biochemical and metabolic processes too (**Serraj and Sinclair, 2002; Miranda-Apodaca et al., 2018**). This fact was reflected by the better performance on the photosynthetic metabolism by these plants (Chapter 4), which could be reflecting an isohydric behaviour (Chapter 5) (**Sade et al., 2012**).

As aforementioned, water uptake capacity is also dependent on root biomass. At the vegetative stage, water stress provoked increases in biomass allocation to root biomass in both CATA and CETE conditions (Fig. 3.3A). In this respect, **Markestejin and Poorter** (**2009**) proposed this feature as a drought-tolerant mechanism to deal with the water stress, since it enables a greater water uptake. On the other side, the increase compared with control values both at VD

and RD treatments was higher at CATA than at CETE conditions, which might be another evidence of a higher water stress perception by roots in the former (**Farooq et al., 2009**).

3.3.3 Plants water loss processes

As we have seen before, the plant water status not only depends on the capacity of water uptake by roots, but it also depends on water loss related to stomatal control and leaf area.

In this sense, and regardless of the environmental conditions and water regimen treatment, the *T* (Fig. 3.4A-B) at the end of the drought period at anthesis was higher than at the vegetative stage due to a larger *GLA* (Fig. 3.4C-D) and greater *gs* (Fig. 3.5A-B) and *E* (Fig. 3.5C-D).

For the same reason, drought treatment plants presented lower *T* values compared with well-watered treatments. Concretely, the lower *gs* –a common response by plants to deal with drought stress (**Farooq et al., 2009 and references therein**) which is triggered through a complex cascade of signalization by ABA (**Li et al., 2020b**)–, leaded to reduced *E* and subsequently *K* values (Fig. 3.5E-F), accelerating leaf senescence processes too (**Abdelhakim et al., 2021**) and reducing *GLA*. In agreement with our results, **Izanloo et al.** (**2008**), **Wang et al.** (**2015**) and **Liu et al.** (**2017**) for wheat did also record higher drought effects at anthesis than at the vegetative stage. In addition, the PCA-biplot (Fig. 3.6) corroborated the observed trend, since the above-mentioned parameters were clustered with anthesis treatments at PC2, and at the same time, developed a correlation pattern with *T*, the variable which more explained the observed different behaviour between vegetative and anthesis treatments.

On the other hand, when we compared the effect of temperature and CO_2 on recorded *T* values of drought treatment plants (CETE *vs.* CATA conditions), at CETE VD lower *T* than at CATA VD was shown; the same happened when the drought was applied at the anthesis stage (RD). However, we detected –as **Dias de Oliveira et al.** (**2015a**) did it for wheat–, absolute higher values for *GLA*, *gs*, *E* and *K* in CETE RD plants at the end of the drought period. To explain this fact, we can turn to the model described by **Li et al.** (**2020b**), which comes to say that plants grown at ECO_2 develop a delay in drought constraints due to basal lower *gs* levels before water stress treatment is imposed, leading to a more conservative (isohydric) strategy (**Domec et al., 2017**). Because of that, knowing that our CETE RD plants on the onset of the drought period started from lower *gs* values (see RC treatment) and that CETA plants did the same, we could

say that the response at future environmental conditions was governed by the effect of ECO_2, being, therefore, explained the observed better water status of the plants.

Moreover, in agreement with the explanation for the water uptake process concerning the turgor potential, it also seems that CATA RD treatment plants have driven an anisohydric behaviour and were more focused to keep growth rates (Chapter 5). Plants would achieve it by the maintenance of the stomata opened permitting higher photosynthetic rates until a *SVWC* threshold value was exceeded, at this moment probably leading to an abrupt hydraulic failure and triggering the observed massive stomatal closure (Fig. 3.4B), evidencing such strategy as a risk-taking one (**Locke and Ort, 2015**). Our hypothesis would be supported by the lack of difference in *GLA* at RC treatments between CATA and CETE (Fig. 3.4B), suggesting that the difference in *T* was owed exclusively to the different stomatal conductance regulation.

In this regard, if we go back to the PCA-biplot again, it can be observed that *K* was positively correlated with *LRWC*, and negatively with *DH*. Therefore, it is acceptable to postulate that the higher values registered for *K* at CETE drought treatment plants –specially for RD since drought effects at anthesis were more severe–, played an important role in the avoidance of cell massive *DH* and a *LRWC* better maintenance. In that sense, as plant hydraulic conductance has been observed to be regulated by *AQPs* activity (**Kaldenhoff et al., 2008; Besse et al., 2011; Chaumont and Tyerman, 2014; Maurel et al., 2016; Merlaen et al., 2019**), we decided to go more deeply by studying different key barley *AQPs* expression response pattern. The aim was to inquire about their involvement in the commented physiological response, which was carried out through specific objective 3.

3.3.4 *AQPs* expression data and its involvement in the physiological response

Firstly, we want to check for well-watered treatment plants how the future environmental conditions (CETE) modulated *AQPs* candidate expression response both at vegetative and anthesis, and analyse its implication in the plant water relations to better understand the results obtained in drought treatment plants.

3.3.4.1 Well-watered treatments

At the vegetative stage, a downregulation for leaf *HvPIP1;3* and *HvPIP2;1* isoforms, and an increase for *HvPIP2;5*, were observed for VC treatment plants at CETE conditions (Fig. 3.7.A), whereas no differences were observed for root *AQPs* expression between CATA and CETE conditions (Fig. 3.7B). In this regard, **Zaghdoud et al.** (**2013**) for broccoli both in roots and leaves, and **Secchi et al.** (**2016**) for tobacco leaves, also observed a downregulation of different *AQPs* isoforms at ECO_2. In addition, and more specifically, **Fang et al.** (**2019**) in tomato also observed that leaf *PIP1;3* and *PIP2;1* (among others) were reduced at ECO_2, contributing to the reduction of plant hydraulic conductance.

Therefore, CETE plants to match with the observed lower *E* –which was governed by the effect of the ECO_2 on *gs* reduction as stated before–, downregulated both leaf *HvPIP1;3* and *HvPIP2;1* aquaporin isoforms, probably participating in the adjustment of *K* (Fig. 3.5E-F). **Fang et al.** (**2019**) suggested the same trend in tomato for actual temperature and elevated CO_2 conditions (CETA), adding that ABA could mediate the regulation of *gs* and leaf (*Kleaf*) and root (*Lpr*) hydraulic conductance –through a downregulation of aquaporin expression–, coordinating whole-plant hydraulics and water balance. Moreover, the overexpression of *HvPIP2;5* could be more related to its putative CO_2 transport function as will be discussed in Chapter 4.

However, at anthesis, no differences were registered for any given *PIP* isoform at leaves at CETE compared with CATA conditions (Fig. 3.8A), but a decrease in root *HvPIP2;5* isoform expression was shown (Fig. 3.8B). **Fang et al.** (**2019**), apart from the observed lower leaf *AQPs* expression values, did also observe a downregulation in root *HvPIP2*;5, which suggested its participation in the lower *K* at ECO_2.

In line with our results, but for current environmental conditions, other authors have also observed that water relations can be regulated by root hydraulic modulation. In this regard, **Vandeleur et al.** (**2009**) commented that they observed in grapevine a lower root hydraulic conductance that could alter leaf water relations. **Ehlert et al.** (**2009**) observed in maize that, under high water evaporative demand, the reduction of the aquaporin activity –and hence *Lpr*– , triggered a reduction of *E* by > 50 %. Moreover, **Sakurai-Ishikawa et al.** (**2011**) in rice turned around the cause-effect argument, stating that the lower transpiration demand was responsible for the downregulation of the root aquaporin(s) expression to reduce the *Lpr*, adjusting to such transpiration demand.

In our case, it seems that the reduction of plant hydraulic conductance at CETE conditions was strongly leaded by root *AQPs* downregulation. Nevertheless, it is worth noting out that the mechanism underlying the coordination between plant transpiration and shoot and root aquaporin activity (**Maurel et al., 2016**), and the control of water adjustment by aquaporin expression at elevated CO_2 conditions remains unknown, although it is proposed that ABA could be playing an important role (**Fang et al., 2019**).

3.3.4.2 Drought treatments

In the case of drought treatments and specifically for VD treatment, an isoform-specific pattern response was shown depending on the environmental conditions at which plant were grown. Thus, at CATA conditions, both leaf *HvPIP1;3* and *HvPIP2;1* isoforms were downregulated compared with their VC treatments (Fig. 3.7A), which presented positive correlations with the physiological parameters related to water movement (Table 3.2). Therefore, the downregulation in the expression of those leaf isoforms at CATA VD might explain the observed decrease on *K*, which was triggered to match with the lower *E* caused by the stomatal closure (Fig. 3.5) as other authors for different species have stated (**Kaldenhoff et al., 2008; Maurel et al., 2016; Merlaen et al., 2019**). In the case of the VD treatment at CETE conditions, a decrease in *K* compared with VC treatment was also recorded, but to a lesser extent than at CATA conditions. In this regard, for VD treatment at CETE conditions, same expression levels as for the VC treatment were observed for these two leaf isoforms, which together with the higher leaf *HvPIP2;5* expression values, could lead to the better maintenance of *K* compared with CATA conditions.

Additionally, the lower expression of leaf *HvTIP1;1* under CATA conditions for VD treatment could decrease the water permeability of membranes, which could be corroborated by the results obtained for barley by **Kurowska et al.** (**2019**) after having analysed different *HvTIP* isoforms under drought conditions. However, as *AQP*s have a bidirectional path, the maintenance of leaf *HvTIP1;1* expression level at CETE VD treatment compared with CATA VD probably led to keep more stable the turgor potential (Fig. 3.2E), and hence, growth. It is worth noting that this isoform is catalogued as a "housekeeping" gene due to its abundance and widespread high expression pattern in barley leaves and roots, which indicates a reference level for the hydraulic conductance of the tonoplast, ensuring the rapid osmotic equilibrium between the vacuole and cytosol (**Maurel et al., 1993**).

Moreover, irrespective of the environmental conditions, VD treatment plants presented in general lower root aquaporin relative expression compared with VC treatments (Fig. 3.7B). This lower root aquaporin expression under drought conditions led probably to the decrease of root water uptake and root hydraulic conductance as **Merlaen et al.** (**2019**) and **Li et al.** (**2020a**) commented for strawberry and cucumber, respectively, matching with the registered lower transpiration demand. Thus, both the downregulation of leaf and root *AQP* expression allowed drought treatment plants to adjust hydraulic conductance at the plant level. Nevertheless, it is of interest to highlight that for the VD treatment at CATA conditions the expression of root *HvPIP2;5* was kept stable, whereas at CETE conditions a reduction was recorded, which would follow the same pattern commented for RC treatment at CETE conditions.

Besides, at the RD treatment, a similar trend that the above-mentioned for leaf *AQPs* expression at VD treatment was shown (Fig. 3.7A and Fig. 3.8A). Concretely, the expression levels of leaf *HvPIP2;1* and *HvTIP1;1* at CETE RD treatment were less affected than at CATA RD treatment compared with their RC treatments. Overall, this trend probably allowed the better *LRWC* at RD CETE compared with CATA conditions when drought was more severe, thus, participating in the previously commented more conservative isohydric behaviour, the most appropriate strategy for plant survival when drought stress is severe (**Sade et al., 2012**).

In addition, the above-commented trend was triggered by the positive effect that ECO_2 had on stomatal control, since as they started from a lower *gs*, they presented a range with greater flexibility, delaying drought effects as **Li et al.** (**2020b**) suggested in their model. Furthermore, RD CETE treated plants could avoid the massive cell *DH* (Table 3.1), presenting higher *K*, *E* and *gs* values at the end of the drought period as previously was suggested. This, probably, was also in part due to the higher expression levels of the above-mentioned leaf *AQPs* and its role in not only the water flow within the plant but also in the stomata movement as **Moshelion et al.** (**2015**) and **Maurel et al.** (**2016**) commented in their respective reviews, and **Abdelhakam et al.** (**2021**) analysed in sunflower more recently. In this regard, **Li et al.** (**2020a**) in a water stress experiment conducted in cucumber seedlings observed a greater root-specific aquaporin isoform expression under ECO_2 conditions compared with ambient CO_2 conditions, which resulted in a better hydraulic conductance and lesser stress effect at plant level. These results would support partially the trend observed for our data, where the higher expression of leaf *AQPs* at future environmental conditions allowed them to cope better with the water stress.

Therefore, the greater expression levels of such leaf *AQPs* allowed CETE plants probably to maintain the biochemical and metabolic processes in a better status at the end of the drought

period, participating –directly or indirectly due to the possible CO_2 transport function of some *PIP AQPs* as it will be seen later–, in higher photosynthetic rates (Chapter 4). Nevertheless, at RD treatment and specially for *HvTIP1;1* aquaporin, the higher expression values were not translated in a higher turgor potential and anishohydric behaviour as other authors have suggested (**Sade et al., 2009 and 2012; Maurel et al., 2016 and references therein**). This trend could be owed to the fact that the turgor potential is not only defined by vacuole volume since it is also defined by the capacity of plants to adjust the cell wall elasticity to the new volume. In this regard, CETE RD plants become the cell wall more elastic compared with its RC treatment but did not reach the extremely lower ε values observed in CATA RD (Table 3.1).

Otherwise, regardless of the environmental conditions, all root isoforms which presented a positive correlation pattern with water flow (Table 3.2), were downregulated for RD treatments compared with RC treatments (Fig. 3.8B). Even more, under severe drought conditions as those observed in our data, the downregulation of aquaporin expression could avoid water loss to the environment as other authors have stated (**Aroca et al., 2012; Surbanovski et al., 2013**).

Besides, in an opposite way to the one observed at the vegetative stage, for all the isoforms analysed, at anthesis minor root *AQPs* expression values were registered for RD treatment plants at CETE conditions compared with the same treatment at CATA conditions. However, *K* at CETE RD treatment was higher than at CATA conditions, which would be explained by the above mentioned better stomatal control due to the ECO_2, causing the delay of drought effects. Therefore, on one hand, this fact could be indicative of the observed better plant water status for these plants. On the other hand, it could strengthen the above suggested possible trend for CETE plants, it is, the downregulation of plant hydraulics would be more driven by root hydraulic reduction due to root *AQPs* expression decrease. Ultimately, this fact might be a consequence of the possible higher necessity of plants grown at ECO_2 conditions to deal with the higher CO_2 concentration (**Zhang et al., 2021b**), where leaf *AQPs* could be playing an important role in it as it will be discussed on Chapter 4.

3.3.5 Plant water relations recovery and possible priming effect: plant memory as the keystone

The last specific objective of this Chapter, was, firstly, to study the recovery of plants that had suffered a vegetative drought (VD) and subsequently had been well-watered (VR *vs.*

RC). Secondly, we also wanted to inquire if those plants (VD) responded better to a second anthesis drought (DD) compared with the plants that only suffered an anthesis drought (RD), that is to say if the vegetative treatment induced the so-called priming (DD *vs.* RD). For both objectives, plant memory is the cornerstone.

3.3.5.1 Plant water relations recovery

Focusing on the *LRWC* (Fig. 3.1D) and taking it as the parameter that measures the final water status of the plant, regardless of the environmental conditions, the same values as for control treatments were recorded for VR treatment plants. This result was logical since VD treatment did not alter plants water status as previously was commented (Fig. 3.1C). Furthermore, in line with the observed trend for barley by **Robredo et al.** (**2007**) too, VR treatment plants presented similar leaf Ψw_{md} values as RC treatment ones (Fig. 3.2B). Therefore, as concerns barley VR treatment plants water uptake capacity, it could be said that a resetting and forgetfulness was developed (**Mendhana et al., 2020**) and that a complete recovery was achieved.

Nevertheless, when data for the water loss processes were analysed, some differences arose between VR and RC treatments, which the variable of temperature regimen played an important role. On one hand, at CATA and CETA conditions –the ones grown at current temperature–, the VR treatments presented lower *T* (Fig. 3.4B) owed to lower *GLA* –only at CATA (Fig.3.4D)–, *gs* (Fig. 3.5B) and *E* (Fig. 3.5D). On the other hand, no differences were recorded for *T* between VR and RC treatments at CATE and CETE conditions –the ones grown at elevated temperature–, although at CATE conditions the VR treatment presented higher values for water flow parameters, whereas at CETE the opposite trend was shown (Fig. 3.5).

At this point, it must be highlighted that other authors as **Wang et al.** (**2015**), **Liu et al.** (**2017**) and **Mendhana et al.** (**2020**) working with wheat, had also analysed plants performance at anthesis after having suffered a drought period at the early-vegetative stage. Through those works, the researchers were able to observe that plants that suffered a vegetative drought at current environmental conditions, did not recover *gs* values at anthesis –although the effect was dependent on the applied drought extent–, whilst the plants that suffered the same drought treatment but were exposed to an elevated temperature treatment (ET), presented higher *gs*. In this regard and for both cases, it could be said that a plant memory effect was developed, and its effects we will see more in detail in the subsequent Chapters for photosynthesis (Chapter 4)

and growth and grain yield (Chapter 5). As we will see, in the case of plants with lower *gs*, it could be said that they paid the allocation costs of developing a memory effect, namely maladaptive plant memory effect, since the remained alterations were detrimental. Nevertheless, in the case of plants that presented higher *gs*, a priming effect was developed (**Crisp et al., 2016; Martínez-Medina et al., 2016**).

Besides, in an opposite way to the above-mentioned trait for the water loss and flow parameters, and focusing again on current and future environmental conditions, at CATA higher *K* was observed at VR treatments plants (Fig. 3.5F). This fact could be due to an attempt by barley plants to revert in part the commented higher water diffusional resistance, but also to recover cell water status as **Pérez-Martín et al.** (**2014**) stated. Moreover, as concerns CETE conditions, similar *K* was observed between VR and RC treatments.

In addition, knowing the possible implication of *AQPs* in water relations, and specially in plant hydraulic conductance, we checked out the literature to inquire about their possible implication on recovery. In agreement with this idea, **Jang et al.** (**2013**) and **Pawlowicz et al.** (**2017**) for jatropha and festuca species, respectively, and **Secchi et al.** (**2017**) and **Kumar et al.** (**2020**) through their respective reviews, stated that some aquaporins presented higher expression values at VR treatment plants, demonstrating its relevance in water homeostasis recovery after stress cessation.

Regarding our results, in CATA the leaf *HvPIP1;3* and in CETE *HvPIP2;5 AQPs* expression levels were higher for VR treatment than for RC treatment (Fig. 3.8A), corroborating in both cases its implication in plant water balance as had been verified by **Besse et al.** (**2011**) and **Horie et al.** (**2011**). Furthermore, at the root level, the *HvPIP2;5* isoform was also increased under both environmental conditions (Fig. 3.8B), where it is thought to be a key isoform in the barley radial water movement (**Knipfer et al., 2011**). In addition, our correlation matrixes for plant memory and recovery divisions might also strengthen its implication in the water movement (Table 3.2). Lastly, it is worth noting that **Parent et al.** (**2009**) also observed that maize plants that had passed drought stress, kept greater *K* values despite lower *gs*, leading to that complex situation.

Therefore, it seems that, on one hand, the above-mentioned leaf and root *AQPs* isoform-specific response could be involved in the proper water status recovery of VR plants, enabling the observed higher *K* rates and, in this way, proper cell hydration recovery. On the other hand, as it will be seen in Chapter 4, it cannot be ruled out the hypothesis that the higher *AQPs* expression values might be a compensation mechanism to try to mitigate the negative effects triggered by *gs* reduction to CO_2 diffusion within the plant.

3.3.5.2 Possible priming effect

Currently, it is widely accepted that plants that have suffered a mild stress exposure (*e.g.* drought) before a future more severe stress, may become primed, promoting an acclimated state that could persist until a subsequent exposure (**Crisp et al., 2016**).

Regarding our data, , it is observed that DD treatment at CATA and CETA conditions presented similar leaf water status as RD treatment (Fig. 3.1D). If we go deeper, no larger differences were recorded neither for water uptake nor for water loss parameters under these environmental conditions. Therefore, from a water relations point of view, and in agreement with the observed trend in wheat by **Liu et al.** (**2017**) and **Mendhana et al.** (**2020**), DD treated plants that were grown at current environmental temperature conditions did not present a priming effect.

Nevertheless, at CETE conditions lower *LRWC* and Ψw_{md} (Fig. 3.2B) were recorded for DD treatment compared with RD treatment, denoting a maladaptive priming effect, whilst at CATE were kept constant. In a deeper analysis of water relations, when we compared both ET conditions with CATA condition, a lower *root/shoot ratio* was recorded (Fig. 3.3B). Thus, the lack of ability of DD treatment plants to drive more carbohydrates to root growth under CATE and CETE conditions might become an important deficit to deal with anthesis drought impairments, which could be a consequence of the higher resources driven to leaf development for increasing the heat loss (**Gray and Brady, 2016**). Moreover, the different water extent of anthesis drought effects between CATE and CETE could be explained by the observed different response behaviour concerning the transpiration extent between them (Fig. 3.4B). In line with this, **Wang et al.** (**2015**) in wheat stated that the degree of the effect of the previous drought or another stress could probably be essential in the subsequent plant memory response, matching with the observed trend by us.

Lastly, we must reveal that on one hand, either at CATA or CETE conditions, the water flow parameters for DD treatment presented lower values compared with RD treatment one, mainly owed to the developed constraints on *gs* and *K*, which also jeopardized photosynthetic performance (Chapter 4), and growth and yield-trait components (Chapter 5). On the other hand, the percentage of *gs*, *E* and *K* decreases were higher at CETE conditions (Fig. 3.5). Therefore, apart from the above-mentioned more negative Ψw_{md} and lower *root/shoot ratio* between DD and RD treatments at CETE conditions compared with CATA, the greater observed

impairment on plant hydraulic conductance could also play an important role in the different final leaf water status.

In this regard, the information that can be extrapolated by the measured leaf *AQPs* expression levels is quite diffusive, since an isoform-specific response was developed. Attending to CATA conditions, both the leaf and root *HvPIP2;5* presented higher expression values at DD treatment, whereas at CETE higher expression values were only recorded for leaf *HvPIP2;5* (Fig. 3.8A-B). However, a negative correlation was registered with water flow related parameters (Fig. 3.2), which might mean that the activation state of those isoforms was very low so that the higher transcript levels could be an attempt to palliate it (**Yepes-Molina et al., 2020**). In this respect, although aquaporin gene expression is one possible mechanism to regulate its activity (**Merlaen et al., 2019**), it is necessary to remember that *AQPs* present post-transcriptional modifications such as changes in gating, trafficking or turnover, which neither proteins levels nor aquaporin activity must be ineludibly correlated with the expression data (**Yepes-Molina et al., 2020**). Thus, these results should be analysed carefully.

At this point, it is worth noting that only a few works have studied the priming maladaptive effects, not going deep into it. For instance, **Skiryzc and Inzé** (**2010**) observed that repeated drought stress could result in a higher sensitivity to adverse effects, decreasing photosynthesis and delaying the growth and development of the plants. In the same way, **Soja et al.** (**1997**) in grapevine treated with ozone along different years, observed that plants were sensitive to the ozone over the years. However, the causes behind this maladaptive plant memory are poorly studied, hence it deserves more attention.

3.4 Conclusions

- Regardless of the environmental conditions, our drought treatment applied at the vegetative stage did not constrain plants water status despite *SVWC* decreased to values around 25%.
- The effects on the soil and plant water status caused by water stress imposed at anthesis (RD) were much higher than the ones observed when drought occurred during the vegetative period. The reason for this lies in the fact that at the anthesis stage, on the onset of water stress treatment, plants had both greater leaf area and stomatal conductance, so the water losses through transpiration were higher.
- The behaviour and mechanism that plants turned on to face drought period at anthesis varied among environmental conditions.
- At CATA conditions, plants carried out a risk-taking behaviour (anisohydric) putting growth maintenance before biochemical and metabolic status maintenance. For that purpose, on one hand, CATA RD plants developed extremely elastic cell walls to maintain turgor potential at expense of massive cell dehydration. On the other hand, those plants maintained the stomata opened until a *SVWC* threshold was exceeded, triggering finally a hydraulic failure.
- At CETE conditions, plants carried out a conservative behaviour (isohydric), avoiding massive cell dehydration, controlling better the water loss due to ECO_2 effects on stomatal conductance and maintaining higher water flow within the plant. This response was partially driven by a leaf and root *AQP* isoform-specific response, where it seems that its trend and behaviour varied depending on the requirement of each organ.
- Irrespective of the environmental conditions, plants that had suffered a vegetative drought period and were re-watered along the rest of their life span (VR), presented an optimum water status and a recovery of water uptake capacity and cell hydration, supported by a greater hydraulic conductance led by an *AQPs* isoform-specific response at each environmental conditions.
- Concerning the water loss processes, VR treatment plants presented a plant memory effect. In addition, the temperature regimen at which plants were grown seemed to had an important influence on it, since under current temperature conditions (CATA and CETA) VR treatment plants presented lower transpiration and stomatal conductance, while at CATE increased and at CETE was almost recovered.

- Irrespective of the environmental conditions, plants that had suffered a vegetative drought period and subsequently passed a drought period at anthesis (DD), did not present a priming effect. Moreover, at CETE conditions, a maladaptive plant memory effect was developed, where the lack of biomass allocation pattern to roots and the hastened plant hydraulic conductance are postulated as the principal causes.

CHAPTER 4

BARLEY'S PHOTOSYNTHETIC METABOLISM

4. BARLEY'S PHOTOSYNTHETIC METABOLISM

4. Barley's photosynthetic metabolism

4.1 Introduction

Photosynthesis is the major biological process that can use solar energy (**Taiz et al., 2014**). It is estimated that each year approximately 200 billion tons of CO_2 are converted into biomass through photosynthesis. Consequently, life on earth depends on energy derived from the sun and captured by photosynthetic organisms.

Photosynthesis covers the capture of energy by photosynthetic pigments and its conversion into chemical energy in the form of NADPH and ATP, until the use of these products by the Rubisco enzyme for the fixation of the CO_2, the latter absorbed through the stomata.

Concretely, solar energy is captured in the chloroplast of the leaves by the photosynthetic pigments that are located in the antenna complexes, part of the photosystems. Plants have two photosystems, PSI and PSII. The main photosynthetic pigments are chlorophylls *a* and *b*, although carotenoids, called accessories pigments, can also capture and transmit solar energy to chlorophylls. Furthermore, as abovementioned, the photosynthetic pigments are arranged in two antenna complexes (LHCI and LHCII), each located in a photosystem (PSI and PSII), being responsible for the energy capture and transmission to the respective reaction centres in the photosystems, with an efficiency of 95 %. The mechanism that allowed it is called resonance transfer (**Pullerits and Sundström, 1996**).

The photons channelled by the antenna complex excite a specialized chlorophyll of the PSII reaction centre, which losses an electron that is transported through thylakoids membranes finally reducing $NADP^+$ to NADPH. The passage of the electron through cytochrome $b_6/_f$, which is located in the thylakoid membrane, together with the lysis of the water itself, allows the generation of a proton gradient in the lumen which will ultimately be leveraged by an ATPase for ATP production.

These two products of the light phase of photosynthesis (NADPH and ATP) are used in the Calvin-Benson cycle to obtain triose phosphates, which one-sixth are used for the synthesis of sugars, and the rest five-sixths are used for the regeneration of ribulose 1,5-bisphosphate (RuBP), the molecule where Rubisco fixes the CO_2. Lastly, the stored energy in the photoassimilates can be used later for various cellular processes such as growth, the formation of reserve structures, respiration and/or antioxidant compounds. However, the performance

and efficiency of the photosynthetic machinery depends on the actual and future environmental conditions such as temperature, water availability and CO_2 concentration, among others.

In this respect and regarding future environmental conditions, plants will cope with different abiotic factors, being the most important ones the elevated temperature (ET), elevated air CO_2 concentration (ECO_2), and more frequent and intense drought periods (**IPCC, 2013**).

Photosynthesis can be increased with an augmentation of air temperature, but when a threshold value is exceeded, a reduction in photosynthesis rates due to a decreased Rubisco activity uses to happen (**Crafts-Brandner and Salvucci, 2000**). The decrease in the carboxylation efficiency could be principally related to the thermo-sensitive of the Rubisco activase enzyme to the elevated temperatures (**Sage et al., 2008; Dusenge et al., 2019)**.

Besides, barley photosynthesis, as in other C3 species, is limited by CO_2 (**Leakey et al., 2009**). Therefore, any CO_2 increase may enhance CO_2 fixation rates (**Long, 1991**). However, with long-term exposure to elevated CO_2 or other limitations, the downregulation of the photosynthetic capacity or the so-called photosynthetic acclimation can occur, reducing the beneficial effect of the elevated CO_2 on photosynthesis. This photosynthetic acclimation will depend on the species, plant developmental stage and environmental conditions (**Moore et al., 1999; Urban et al., 2012; Sanz-Sáez et al., 2013**), where for example it has been shown that the positive effect of ECO_2 is strong at the earliest stages of the plant, whereas with the time can be diluted (**Tausz-Posch et al., 2020**). Moreover, one concept that would define plants response to the elevated air CO_2 concentration and it should be kept in mind is the sink (strength), which was defined by **Ainsworth et al.** (**2004**) "as the parts of the plants that, at a given stage of development, are utilizing photosynthates in construction, storage or respiration".

On the other hand, when water stress is imposed, usually a reduction in the net photosynthetic rate (A_{net}) is shown (**Feller, 2016**). This reduction on the A_{net} can be driven by diffusional and/or non-diffusional limitations (**Long et al., 2004; Hu et al., 2010**), which will become more or less important depending on the severity of the stress (**Flexas et al., 2006**). The diffusional limitations belong to the stomatal closure and/or the reduction of the mesophyll conductance (**Pérez-Martín et al., 2014**), whereas the non-diffusional limitations are caused by metabolic impairment, such as reduced photochemical and carboxylation efficiency (**Flexas and Medrano, 2002a; Long et al., 2004**).

Moreover, as the periods and frequency that plants will have to deal with drought stress would increase (**IPCC, 2013**), the capacity to manage it will be a key factor. In that sense, the critical period for determining the plant capacity for resetting, or to develop a memory effect

after having passed for example a drought period in the vegetative stage, is the recovery period (**Crisp et al., 2016**). In this regard, in recent years different researchers have analysed the photosynthetic metabolism recovery capacity of plants (**Gallé et al., 2009; Pérez-Martín et al., 2014**), understanding it as a key feature that needs to be taken into account when plants ability to face drought is analysed. On the other hand, as it has been commented for plant water relations, it has also been studied the priming effect as a management strategy to deal with subsequent drought stress at the reproductive stage, even analysing the cross-talk effects with ET (**Wang et al., 2015; Liu et al., 2017; Mendhana et al., 2020**). From that works, either for the recovery capacity or for the priming effect, it is deduced that plants diffusional limitations could play a pivotal role, where the response would vary depending on the other environmental stimuli such as ET or the applied drought magnitude effects on plants water relations.

In addition, in the last years, the importance of the *AQPs* in the CO_2 transport within the plants has been studied. In this regards, different works have been carried out to elucidate its implication in the CO_2 transport, and definitively, in photosynthetic response (**Uehlein et al., 2003 and 2008; Hanba et al., 2004; Flexas et al., 2006; Heckwolf et al., 2011; Kawase et al., 2013**). As concerns barley, it has been demonstrated the participation of different *PIP2 AQPs* in the CO_2 transport, **(Katsuhara and Hanba, 2008; Mori et al., 2014**) and photosynthesis regulation (**Hanba et al., 2004**), being therefore classified as dual channel proteins (**Katsuhara et al., 2008; Horie et al., 2011).** Recently, for tomato, it has also been possible to test that the relative importance of *PIP*-channels in determining membrane permeability and mesophyll conductance could largely depend on the ambient and intercellular CO_2 concentration (**Zhang et al., 2021b**). Thus, *AQPs* expression response analysis along different environmental conditions and water regimens could grant key information about the CO_2 diffusional path and plant photosynthetic performance.

Otherwise, knowing that the different abiotic factors described above will act simultaneously in the near future, until the date, a significant number of works have studied their interactive effects on photosynthesis. In this way, **Jagadish et al.** (**2014**) reviewed the double interaction of those abiotic factors in cereals. Moreover, more recently **Li et al.** (**2019**) and **Abdelhakim et al.** (**2021**) have inquired on it analysing different wheat cultivars.

In general, when high temperatures and drought act together, the impact on photosynthetic processes, growth and productivity of cereals uses to be more harmful than the one of each stress applied individually (**Jagadish et al., 2014 and references therein**). However, other works have stated that drought stress can increase the tolerance of photosynthesis to

elevated temperature stress by increasing heat loss conferring them a priming status (**Wang et al., 2015; Liu et al., 2017**). In the case of the combination of elevated CO_2 and elevated temperature effect on photosynthesis, increased, unchanged and lower rates have been shown (**Jagadish et al., 2014 and references therein; Li et al., 2019; Abdelhakim et al., 2021**). Meanwhile, when plants grown at elevated CO_2 conditions suffer a drought period, the most common observed response is to present better leaf photosynthesis compared with drought individually conditions, in part due to the better stomatal control (**Robredo et al., 2007**) and the delay of the stress in other photosynthetic related processes (**Widodo et al., 2003**). However, a higher biomass production and leaf area could negate this feature by increasing water use (**Dias de Oliveira et al., 2015a**).

Nevertheless, the studies that have analysed the triple interaction effect on photosynthesis are scarce. **Naudts et al.** (**2013**) and **Song and Huang.** (**2014**) in different grass species, and **AbdElgawad et al.** (**2015b**) in two grass and two legume species observed that in general, the elevated CO_2 alleviated partially the adverse effects caused by the elevated temperature and drought, although the response magnitude was species-specific. Besides, **Dias de Oliveira et al.** (**2013**) in a reproductive drought experiment conducted on wheat, concluded that the beneficial effect of the elevated CO_2 only appeared when the temperature treatment did not exceed + 2 °C from the ambient one, whilst **Li et al.** (**2019**) and **Abdelhakim et al.** (**2021**) observed a cultivar-specific response. Thus, the response of the photosynthesis processes to the triple interaction is not clear and it will depend on the species, cultivar, growth development and the type of stress imposition (**Zhang et al., 2021a**).

Therefore, it seems necessary to shed some light on this important issue. In this context, through this Chapter, the response of the photosynthetic metabolism to the different environmental conditions that englobes climate change along barley full life span has been studied, both inquiring on well-watered regimen and drought treatment plants. Specifically, this objective has been divided into three specific objectives:

1. To analyse the response of the different mechanisms associated with photosynthetic metabolism that barley activates to take advantage of growing under future environmental conditions (elevated temperature and elevated CO_2) under the well-watered regimen and if the activated mechanisms are dependent on the growth stage of the plant.
    - ✓ We hypothesize that at CETE conditions, the elevated CO_2 effect will improve photosynthetic metabolism response and it will alleviate elevated temperature negative effects.

2. To compare the response of the photosynthetic metabolism of plants that have suffered a water stress period at vegetative or anthesis stage, and to inquire if the activated mechanisms to cope with it will vary under future environmental conditions.
    - ✓ We hypothesize that anthesis drought treatment will jeopardize to a higher extent than the one applied at vegetative stage barley plants photosynthetic metabolism, and that future environmental conditions will alleviate this negative effect due to delayed effects of drought and better water status.

3. To study if plants that had suffered a water stress period at the vegetative stage can recover their photosynthetic metabolism proper functioning and if these recovered plants present a priming effect to deal with a subsequent anthesis drought period. Besides, we want to address if this recovery and priming effect is dependent on the environmental conditions that plants have been growing.
    - ✓ In the case of recovery, the observed memory effect at stomatal conductance for each environmental conditions will define in part the capacity to recover the proper function of photosynthetic metabolism.
    - ✓ In the case of plants that have suffered a double drought, these plants will have a priming effect due to the developed plant memory capacity, presenting higher net photosynthetic rates than the plants that only had suffered a drought period at anthesis for the first time.

4.2 Results

Unless the opposite is said, to facilitate results description, for well-watered treatments all the comparisons and percentages will be related to CATA treatments at each growth stage. In the case of drought treatments, the comparisons and percentages will be done to its control treatment at each environmental conditions and growth stage.

4.2.1 Gas-exchange process

4.2.1.1 Well-watered treatments

Plants capacity to perform gas-exchange is pivotal to ensure optimum net photosynthetic rates (A_{net}). In Fig. 4.1A, 4.2A and 4.3A data of A_{net} along the early-vegetative, vegetative and anthesis stages, respectively, are depicted.

Under the well-watered regimen, for all the environmental conditions, the higher A_{net} was achieved at the early-vegetative stage, observing reductions as the plants continued their life-cycle. The reduction over time was more marked in CATE and CETA conditions. In CATE, the A_{net} ranged from 14.2 at the early-vegetative stage to 10.7 at anthesis, whereas in CETA, the A_{net} ranged from 17.2 to 14.3.

Moreover, we observed that the ET alone (CATE) decreased A_{net} by 10% at the vegetative stage (VC) and by 20 % at anthesis (RC). Under ECO_2 alone (CETA), at the vegetative stage, similar values to CATA conditions were detected, whilst at anthesis, the values were 7 % higher, although not statistically significant. Lastly, when elevated CO_2 and elevated temperature were applied simultaneously (CETE), the A_{net} was 12-13 % higher at both vegetative and anthesis stages.

One of the most important parameters that determine the A_{net} is the stomatal conductance (*gs*) which is defined by its conductance per stomata (*gs/stomata*), stomata density (*SD*) and total stomata size (*TSS*). The mesophyll conductance (*gm*) and the external CO_2 concentration are also of relevant importance. Besides, the processes related to carbon consumption (Table 4.1) as night respiration (*Rn*) and its balance with the net photosynthetic rates (Rn/A_{net}), day respiration (*Rd*) and photorespiration (R_{photo}) are also of vital importance to better understand plants gas-exchange capacity.

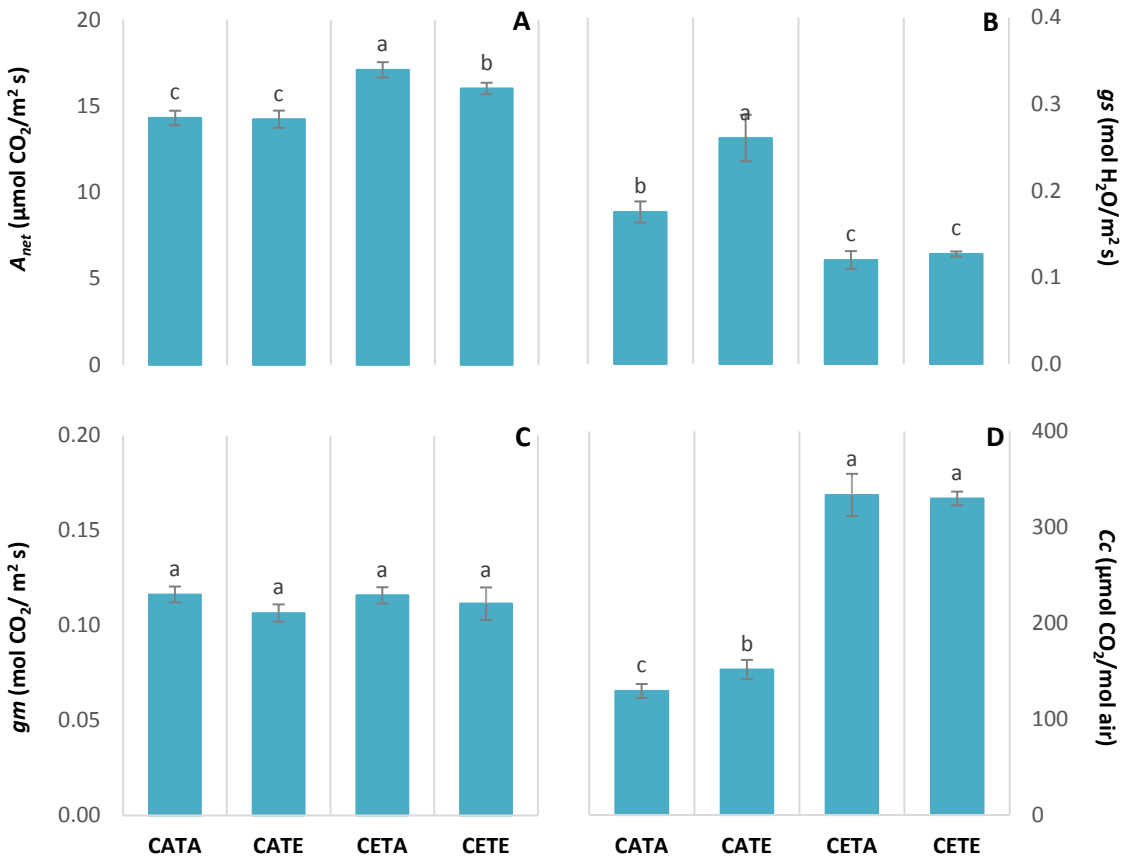

Fig. 4.1. Net photosynthetic rates (A_{net}; A), stomatal conductance (*gs*; B), mesophyll conductance (*gm*; C) and chloroplast CO_2 concentration (*Cc;* D) for the early-vegetative stage control treatment (EVC). Growth environmental conditions and statistical analysis are as described in Fig. 3.1.

At CATE conditions and in the early-vegetative stage, *gs* (Fig. 4.1B) and *Cc* (Fig. 4.1D) were increased by 49 % and 14 %, respectively, *gm* not being modified (Fig. 4.1C). Besides, *Rn* and Rn/A_{net} were reduced by about 33 %, whilst *Rd* and R_{photo} kept constant (Table 4.1). On the other side, at the vegetative stage, on one hand, a statistically significant increase on *gs/stomata* was recorded (Fig. 4.2B), -where *SD* (Fig. 4.2C) and *TSS* (Fig. 4.2D) kept constant-, and on the other hand, a reduction on *gm* by 18 % (Fig. 4.2E) and an increase on *Cc* (Fig. 4.2F) values by 14 % were observed. In addition, lower *Rn*, *Rd* and R_{photo} values were shown. However, different to the rest of the growth stages, at anthesis a statistically significant reduction either for *gs/stomata*, *SD* and *TSS* were shown (Fig. 4.3B-D), together with a lower *gm* (Fig. 4.3E), whilst *Cc* presented higher values (Fig. 4.3F). Furthermore, lower *Rn* and Rn/A_{net} values were observed, not being altered the *Rd*, whereas the R_{photo} presented a reduction of 27 % (Table 4.1).

In the case of gas-exchange parameters at CETA and CETE conditions, the ones at ECO_2, it is noticed that *gs* (Fig. 4.1B and Fig. 3.5A-B) was decreased about 30 % along with the plants life-cycle compared with CATA conditions, owed to lower *gs/stomata* (Fig. 4.2B and 4.3B). In addition, the observed absolute values for *Cc* were also about 170 % higher at elevated CO_2

treatments (Fig. 4.1D, Fig. 4.2F and Fig. 4.3F), whereas in general, the *gm* was not modified. Nevertheless, when values recorded at the vegetative stage for *gm* and *Cc* are analysed, it is worth noting that 20 % lower *gm* was registered at CETA compared with CATA, but 10 % higher *Cc* values compared with CETE.

As regards carbon consumption processes (Table 4.1), however, more heterogeneous results were observed. At CETA, statistically, significantly lower *Rn* and Rn/A_{net}, higher *Rd* and same R_{photo} values were shown at the early-vegetative stage, whereas at the vegetative stage the *Rn* and Rn/A_{net} were not modified, *Rd* was increased and R_{photo} was reduced; at anthesis, only the R_{photo} was altered (20 % lower values). Moreover, at CETE conditions and in the early-vegetative stage, a reduction of 18 % was recorded for *Rd*. Nevertheless, at the vegetative stage, *Rn* and Rn/A_{net} were increased about 30%, whilst *Rd* was kept unchanged and R_{photo} presented a reduction by 19 %. Lastly, at anthesis, CETE conditions increased *Rn* and Rn/A_{net} values but did not modify *Rd* and R_{photo}.

4.2.1.2 Drought treatments

Drought treatments caused a reduction in A_{net}, but its extent varied with the growth stage and environmental conditions. Under CATA conditions, the vegetative drought (VD) decreased A_{net} by 22 % (Fig. 4.2A) and anthesis drought (RD) by 56 % (Fig. 4.3A). Under CATE conditions, A_{net} was decreased by 18 % in VD treatment and by 35 % in RD treatment. In CETA conditions, the reduction of A_{net} in percentage was lower than in the other environmental conditions. As a matter of fact, under elevated CO_2, the reductions were 9 % and 21 % for VD and RD treatments, respectively. Finally, in CETE conditions, A_{net} was reduced by 15 % in VD treatment and by 32 % in RD treatment.

Besides, when plants suffer a period of water shortage, the first constraint concerning A_{net} performance occurs at the gas-exchange level, where both the carbon uptake and consumption processes use to be altered.

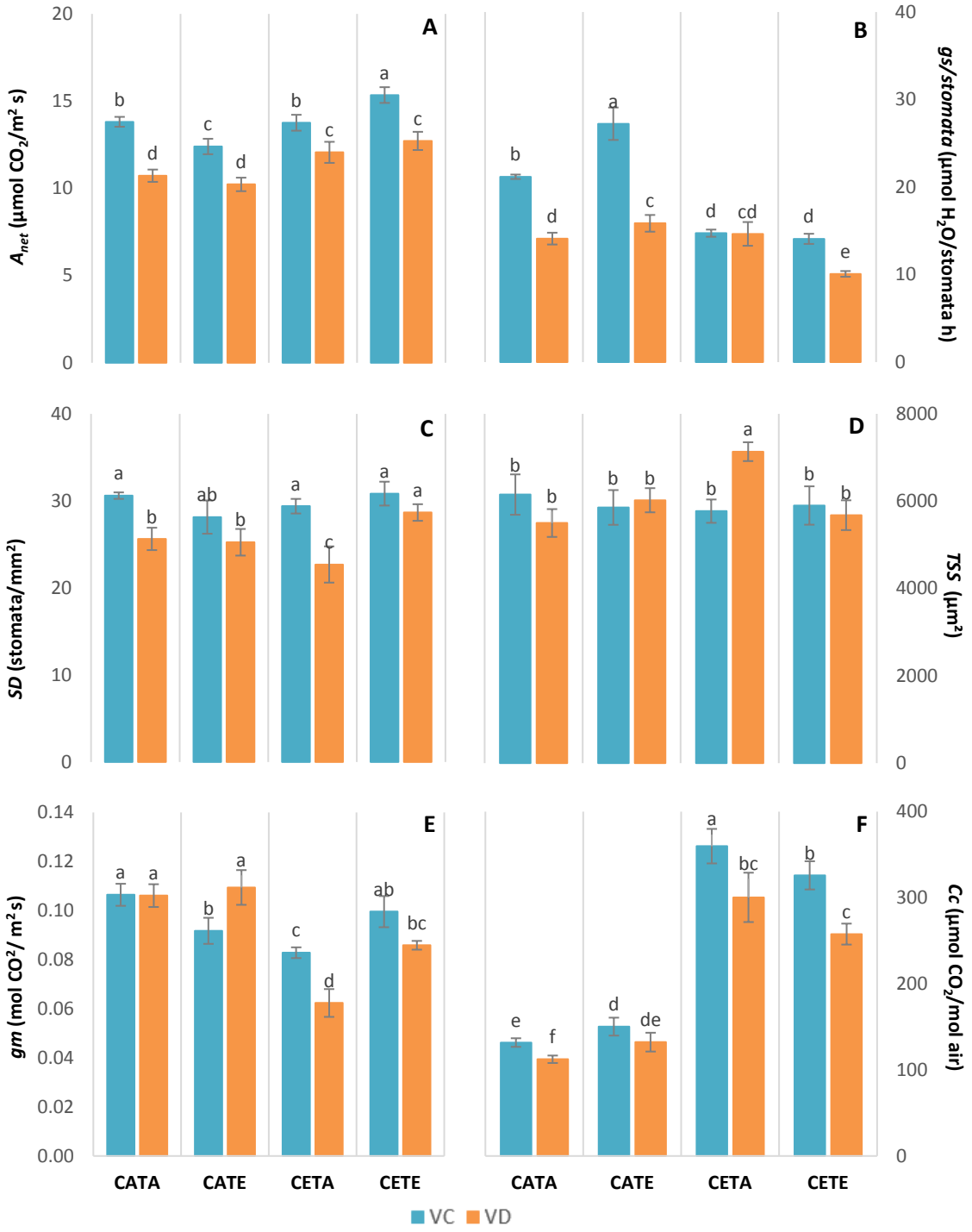

Fig. 4.2. Net photosynthetic rates (A_{net}; A), stomata aperture *(gs/stomata;* B), stomata density (*SD*; C), total stomata size (*TSS*; D), mesophyll conductance (*gm*; E) and chloroplast CO_2 concentration (*Cc*; F) at the vegetative stage Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.

At the vegetative stage, the VD treatment at CATA reduced both *gs/stomata* and *SD* values by 33 % and 16 %, respectively, whereas *TSS* and *gm* kept constant, but *Cc* decreased by 16 %. Nevertheless, either *Rd, Rn/*A_{net} or *Rd* parameters were increased by VD treatment, whilst

R_{photo} did not. At CATE conditions, however, a higher reduction on *gs/stomata* compared with its VC treatment was recorded for VD treatment (43 %), whilst an astonishing statistically significant increase on *gm* was shown, not altering *Cc*. In addition, it also presented statistically significant higher *Rn* and Rn/A_{net} values. Moreover, CETA VD treatment did not modify *gs/stomata,* but reduced *SD* and increased *TSS*, together with *gm* and *Cc* diminution. It is worth noting too that *Rn* was not altered, whereas Rn/A_{net} ratio was slightly increased and *Rd* presented 39 % higher rates, but it reduced R_{photo} by 17 %. Lastly, differently from the rest of the environmental conditions, VD treatment at CETE conditions reduced by 29 % gs*/stomata*, kept constant *SD* and *TSS*, but diminished *gm* and *Cc* about 16 %. Furthermore, it decreased by 40 % and 30 % *Rn* and Rn/A_{net}, respectively, whereas *Rd* was increased. R_{photo} was reduced as it happened for CETA VD treatment.

Moreover, at the anthesis stage, RD treatment exacerbated the observed alterations in VD treatments either for carbon uptake and carbon consumption parameters. In addition, some differences were recorded between environmental conditions.

At CATA, firstly, drought reduced *gs/stomata* by 85 % and did not modify *SD and TSS*, and secondly, drought decreased *gm* and *Cc* by 30 % and 40 %, respectively. Furthermore, it increased significantly *Rn*, Rn/A_{net} and *Rd*, whereas it reduced R_{photo}. At CATE conditions, the RD treatment, on one hand, reduced *gs/stomata* by 79 %, maintained *SD* and increased significantly *TSS* values. On the other hand, it did not alter *gm*, but it reduced *Cc* by 50 %. Similar values for carbon consumption parameters as the above-mentioned for CATA were observed at CATE RD treatment, except that R_{photo} kept constant. In the case of CETA conditions, the lowest reduction on *gs/stomata* by RD treatment compared with its RC treatments and a no alteration neither in *SD* nor in *TSS* were recorded, whereas *gm* and *Cc* reduced by 25 % and 35 %, respectively. Furthermore, the observed increases for the carbon consumption parameters were to a lesser extent than the ones at CATA. Lastly, the CETE RD treatment decreased lower *gs/stomata* compared with the same treatment at CATA, but it reduced significantly *SD*, and recorded lower *gm* values, being the reduction percentage for *Cc* the same as for CATA. The *Rn* and Rn/A_{net} values were also less increased, whereas *Rd* was not modified and R_{photo} was reduced too.

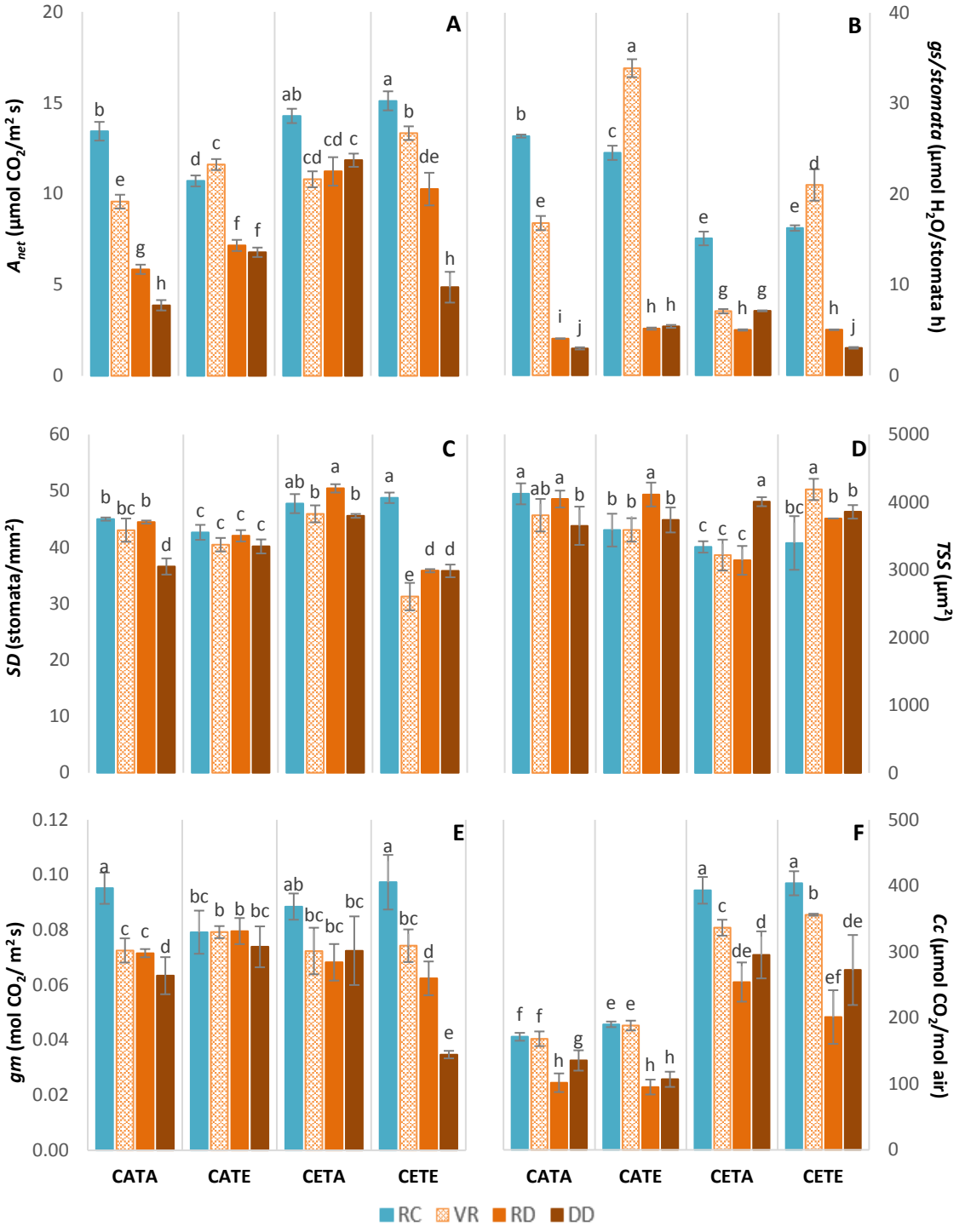

Fig. 4.3. Net photosynthetic rates (A_{net};A), stomata aperture (*gs/stomata*; B), stomata density (*SD*; C), total stomata size (*TSS*; D), mesophyll conductance (*gm*; E) and chloroplast CO_2 concentration (*Cc*; F) at the anthesis stage. Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.

4.2.1.3 Plant memory

Otherwise, when the plant memory effect on A_{net} was analysed, different trends were observed for the recovery (VR *vs.* RC) and priming (DD *vs.* RD) depending on the environmental conditions.

As concerns the recovery, under CATA and CETA conditions VR treatment recorded about 25 % lower A_{net} values compared with its RC treatments, whilst the reduction at CETE conditions for VR treatment was of a lesser extent (12 %). At CATE, statistically significant higher values were recorded. Regarding the possible priming effect, DD treatment at CATA presented 34 % lower A_{net} values compared with its RD treatment, whereas the observed reduction at CETE was higher (52 %), and no longer alterations were observed at CATE and CETA.

In addition, if data for the diffusional processes are analysed, interesting results are observed. On one hand, focussing on the recovery data, lower *gs/stomata* and *gm* values were shown for VR treatment at CATA and CETA, whilst *SD* and *TSS* were not altered, and *Cc* was decreased only at CETA. Moreover, higher Rn/A_{net} and lower R_{photo} values were observed at CATA VR treatment, and specially at CETA, together with a 100 % increase on *Rn*.

Differently to the latter environmental conditions, in the case of CETE VR treatment, higher *gs/stomata* and *TSS* but lower *SD*, *gm* and *Cc* values compared with its RC treatment were observed, whereas at CATE VR treatment higher *gs/stomata* and maintenance of the rest of the above-mentioned parameters was observed. At CATE statistically significant lower *Rd,* and at CETE higher R_{photo} values were observed for VR treatments compared with RC ones.

On the other hand, regarding the possible priming effect as concerns gas-exchange parameters, it is noticed that at CATA statistically significant lower *gs/stomata*, *SD*, *TSS* and *gm* values were recorded for DD treatment, whereas *Cc* and *Rd* were increased. In the case of CETE conditions, the same values for *gs/stomata* as CATA between DD and RD treatments were observed, although the percentage of decrease respect its RD treatment was higher. Furthermore, no differences were shown for *SD* and *TSS* values, whilst a surprisingly 50 % lower *gm* value was recorded at DD compared with RD treatment, together with a trend towards higher *Cc*, despite it was not significantly different. In addition, both *Rn* and R_{photo} registered 23 % and 36 % lower values at DD compared with RD treatment at CETE.

Table 4.1. Effects of environmental conditions and water regimens on night respiration (*Rn,* µmol CO_2/m^2 s), day respiration (*Rd,* µmol CO_2/m^2 s), night respiration related to net photosynthetic rates (Rn/A_{net}) and photorespiration (R_{photo}, µmol CO_2/m^2 s). Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1. EAR denotes the early-vegetative stage. The rest of the abbreviations are explained in Table 3.1.

GS	EC	WR	*Rn*	Rn/A_{net}	*Rd*	R_{photo}
EAR	**CATA**	**EVC**	0.69 ± 0.06 a	4.86 ± 0.15 a	1.78 ± 0.06 b	5.95 ± 0.40 a
	CATE	**EVC**	0.46 ± 0.02 c	3.25 ± 0.11 b	1.92 ± 0.07 b	6.42 ± 0.19 a
	CETA	**EVC**	0.53 ± 0.05 b	3.12 ± 0.08 c	2.42 ± 0.34 a	6.10 ± 0.07 a
	CETE	**EVC**	0.75 ± 0.04 a	4.70 ± 0.10 a	1.46 ± 0.13 c	5.83 ± 0.40 a
VEG	**CATA**	**VC**	0.63 ± 0.03 c	4.59 ± 0.09 c	1.50 ± 0.16 a	5.92 ± 0.33 a
		VD	0.82 ± 0.02 a	7.84 ± 0.29 a	1.96 ± 0.19 b	5.96 ± 0.34 a
	CATE	**VC**	0.48 ± 0.05 d	4.62 ± 0.17 c	1.20 ± 0.09 b	4.47 ± 0.24 b
		VD	0.75 ± 0.04 b	7.52 ± 0.30 a	1.46 ± 0.18 ab	4.92 ± 0.27 b
	CETA	**VC**	0.57 ± 0.04 c	4.13 ± 0.20 d	1.96 ± 0.12 b	4.03 ± 0.18 c
		VD	0.63 ± 0.03 c	5.00 ± 0.26 c	2.72 ± 0.32 a	3.34 ± 0.23 d
	CETE	**VC**	0.83 ± 0.04 a	5.83 ± 0.30 b	1.38 ± 0.11 ab	4.79 ± 0.26 b
		VD	0.51 ± 0.00 d	4.14 ± 0.16 d	1.98 ± 0.07 b	4.20 ± 0.19 c
ANTH	**CATA**	**RC**	0.29 ± 0.01 g	2.12 ± 0.06 i	1.17 ± 0.19 cd	5.75 ± 0.37 a
		VR	0.29 ± 0.02 g	3.02 ± 0.09 h	1.04 ± 0.09 d	4.15 ± 0.30 ab
		RD	0.42 ± 0.03 e	7.18 ± 0.21 c	2.30 ± 0.27 b	4.29 ± 0.30 h
		DD	0.36 ± 0.03 f	8.12 ± 0.39 b	3.11 ± 0.38 a	4.06 ± 0.54 h
	CATE	**RC**	0.45 ± 0.02 de	4.21 ± 0.09 f	1.05 ± 0.05 d	4.19 ± 0.39 c
		VR	0.49 ± 0.01 d	4.18 ± 0.08 f	0.73 ± 0.07 e	4.40 ± 0.18 de
		RD	0.67 ± 0.02 b	8.79 ± 0.20 a	2.04 ± 0.10 b	4.71 ± 0.32 e
		DD	0.55 ± 0.06 cd	7.77 ± 0.05 b	1.61 ± 0.24 c	4.30 ± 0.25 c
	CETA	**RC**	0.27 ± 0.02 h	1.89 ± 0.03 i	1.10 ± 0.12 d	4.54 ± 0.26de
		VR	0.56 ± 0.03 c	5.21 ± 0.14 e	1.09 ± 0.13 d	3.30 ± 0.32cd
		RD	0.38 ± 0.02 f	3.55 ± 0.18 g	1.52 ± 0.09 c	3.35 ± 0.21d
		DD	0.74 ± 0.03 a	6.29 ± 0.12 d	1.30 ± 0.10 c	3.53 ± 0.17e
	CETE	**RC**	0.53 ± 0.04 cd	3.55 ± 0.09 g	1.54 ± 0.17 c	6.05 ± 0.27ª
		VR	0.51 ± 0.03 d	3.86 ± 0.06 g	1.22 ± 0.41 cd	4.10 ± 0.09 b
		RD	0.68 ± 0.05 b	6.97 ± 0.48 c	1.90 ± 0.22 bc	3.65 ± 0.33 f
		DD	0.51 ± 0.02 d	8.05 ± 0.40 b	1.87 ± 0.36 bc	2.32 ± 0.54 g

Lastly, although no differences were recorded for A_{net} between DD and RD treatments at CATE and CETA conditions, some differences in carbon uptake and consumption parameters were shown. Being this way, at CATE, statistically significant lower Rn/A_{net} and *Rd* values were recorded. At CETA, on one hand, higher *gs/stomata* and *TSS*, but lower *SD* were observed, and on the other hand, *Rn* and Rn/A_{net} increased by 96 % and 78 %, respectively.

4.2.1.4 AQPs as CO_2 transport protein channels

In the same way, as it was done for the water flow-related parameters in Table 3.2 in Chapter 3, to verify leaf *AQPs* correlation with CO_2 transport-related parameters, different correlation matrixes were carried out (Fig. S2) which are summarized at Table 4.2.

Table 4.2. Summary table for the main division correlations between gas-exchange related parameters (A_{net}, *gm* and *Cc)* and leaf aquaporin candidate isoforms that met the requirement of r> ±0.55 and P< 0.05. Abbreviations are explained in Table 3.2.

Divisions	*Leaf AQP*	P/N	Parameter
All	*HvPIP2;1*	P	A_{net}
	HvTIP1;1	P	A_{net}
Controls	-	-	-
Drought	*HvPIP2;1*	P	A_{net}
	HvPIP2;5	P	A_{net}, *Cc*
	HvTIP1;1	P	A_{net}, *Cc*
Veg	*HvPIP2;5*	P	A_{net}, *Cc*
	HvTIP1;1	P	A_{net}, *Cc*
Anthesis	*HvPIP1;3*	P	A_{net}, *gm, Cc*
	HvPIP2;1	P	A_{net}, *Cc*
	HvPIP2;2	N	A_{net}, *Cc*
CATA	*HvPIP1;3*	P	A_{net}, *Cc*
	HvPIP2;1	P	A_{net}
	HvPIP2;2	N	*Cc*
	HvTIP1;1	P	A_{net}, *Cc*
CETE	*HvPIP1;3*	P	*Cc*
	HvPIP2;1	P	*Cc*
Plant memory	*HvPIP2;1*	P	A_{net}, *gm*
	HvPIP2;2	N	A_{net}
Recovery	*HvPIP1;3*	N	A_{net}
	HvPIP2;1	P	A_{net}, *gm*
	HvTIP1;1	N	*Cc*
Priming	*HvPIP2;1*	P	*Cc*
	HvPIP2;5	N	A_{net}

The physiological parameters that most correlated with leaf *AQPs* genes were A_{net} and *Cc* specially with *HvPIP2;1* and *HvTIP1;1*. Moreover, *HvPIP1;3* and *HvPIP2;5* presented a more

specific correlation pattern. The former was positively correlated both with A_{net} and *gm* for anthesis treatments, whilst for the latter it has been paid attention their positive correlations at vegetative and drought main divisions with A_{net}.

4.2.2 Photochemistry

The chlorophyll fluorescence parameters provide an estimation of the status of the photosynthetic light reactions. In Table 4.3, data of photochemistry efficiency in dark-adapted leaves (*Fv/Fm*), actual quantum yield of PSII (*ΦPSII*), photochemistry efficiency in light-adapted leaves (*Fv'/Fm'*), photochemical quenching (*qP*), non-photochemical quenching (*NPQ*), electron transport rate (*ETR*) and electron transport rate related to net assimilation rate (ETR/A_{net}) at early-vegetative, vegetative and anthesis stages treatments are presented.

Concerning *Fv/Fm*, no differences were observed neither for the environmental conditions nor among water treatments. For the rest of the parameters presented in Table 4.3, to a greater or lesser extent, differences between environmental conditions and among treatments were shown.

4.2.2.1 Well-watered treatments

It should be highlighted that *ΦPSII* measured under light conditions depends on the *Fv'/Fm'* and *qP* parameters. Keeping this in mind, under well-watered conditions, elevated temperature (CATE) did not modify *ΦPSII* at early-vegetative and vegetative stages, while at anthesis it increased by 13 % due to *qP* higher values. Nevertheless, under elevated CO_2 alone (CETA), *ΦPSII* increased by 30 % at the early-vegetative stage because of the higher values of *qP*, but it was not altered along the rest of the plant life-cycle. Concerning future environmental conditions (CETE), *ΦPSII* increased by 16 % at the early-vegetative stage thanks to *Fv'/Fm'* higher values and decreased about 13 % at vegetative and anthesis stages due to *qP* lower values.

Table 4.3. Effects of environmental conditions and water regimens on photochemistry efficiency in dark-adapted leaves (*Fv/Fm*), actual quantum yield of PSII (*ΦPSII*), photochemistry efficiency in light-adapted leaves (*Fv'/Fm'*), photochemical *quenching* (*qP*), non-photochemical *quenching* (*NPQ*), electron transport rate (*ETR*) and electron transport rate related to net photosynthetic rates (ETR/A_{net}). Growth environmental conditions, water regime treatments and statistical analysis are explained in Table 4.1.

GS	EC	WR	*Fv/Fm*	*ΦPSII*	*qP*	*Fv´/Fm´*	*NPQ*	*ETR*	ETR/A_{net}
EAR	**CATA**	**EVC**	0.80 ± 0.00 a	0.33 ± 0.01 c	0.60 ± 0.01 a	0.57 ± 0.02 b	1.14 ± 0.10 a	58.42 ± 1.28 c	5.72 ± 0.12 c
	CATE	**EVC**	0.80 ± 0.00 a	0.35 ± 0.01 c	0.65 ± 0.01 a	0.54 ± 0.01 b	0.68 ± 0.03 b	61.80 ± 0.97 c	6.17 ± 0.16 c
	CETA	**EVC**	0.81 ± 0.00 a	0.43 ± 0.01 a	0.64 ± 0.00 a	0.68 ± 0.02 a	0.74 ± 0.05 b	75.05 ± 1.90 a	4.94 ± 0.13 a
	CETE	**EVC**	0.81 ± 0.01 a	0.38 ± 0.01 b	0.66 ± 0.01 a	0.59 ± 0.01 b	0.66 ± 0.04 b	66.96 ± 1.34 b	5.20 ± 0.09 b
VEG	**CATA**	**VC**	0.81 ± 0.00 a	0.35 ± 0.01 a	0.62 ± 0.01 a	0.57 ± 0.01 a	0.88 ± 0.04 b	61.62 ± 1.14 a	5.82 ± 0.09 c
		VD	0.81 ± 0.00 a	0.36 ± 0.01 a	0.62 ± 0.01 a	0.57 ± 0.02 a	1.03 ± 0.03 a	59.31 ± 2.89 a	6.82 ± 0.22 ab
	CATE	**VC**	0.79 ± 0.01 a	0.39 ± 0.02 a	0.64 ± 0.01 a	0.61 ± 0.03 a	0.66 ± 0.04 d	65.70 ± 4.15 a	6.47 ± 0.22 b
		VD	0.81 ± 0.00 a	0.37 ± 0.01 a	0.63 ± 0.01 a	0.59 ± 0.01 a	0.91 ± 0.05 b	64.25 ± 1.74 a	7.14 ± 0.20 a
	CETA	**VC**	0.81 ± 0.00 a	0.37 ± 0.01 a	0.65 ± 0.00 a	0.57 ± 0.01 a	0.64 ± 0.02 d	64.26 ± 1.37 a	5.68 ± 0.20 c
		VD	0.80 ± 0.00 a	0.35 ± 0.01 ab	0.63 ± 0.01 a	0.56 ± 0.02 a	0.86 ± 0.05 b	60.83 ± 1.97 a	5.74 ± 0.26 c
	CETE	**VC**	0.80 ± 0.00 a	0.30 ± 0.02 b	0.64 ± 0.02 a	0.46 ± 0.03 b	0.61 ± 0.04 d	57.66 ± 1.54 a	5.29 ± 0.13 d
		VD	0.81 ± 0.00 a	0.33 ± 0.00 b	0.65 ± 0.01 a	0.50 ± 0.00 b	0.78 ± 0.04 c	57.40 ± 1.13 a	5.81 ± 0.11 c
ANTH	**CATA**	**RC**	0.80 ± 0.00 a	0.32 ± 0.01 b	0.66 ± 0.01 a	0.48 ± 0.01 c	0.54 ± 0.04 d	55.48 ± 1.23 c	5.21 ± 0.13 ef
		VR	0.80 ± 0.00 a	0.27 ± 0.01 cd	0.64 ± 0.01 a	0.42 ± 0.01 d	0.67 ± 0.08 c	47.60 ± 1.99 d	5.89 ± 0.49 c
		RD	0.78 ± 0.02 a	0.25 ± 0.01 d	0.55 ± 0.02 c	0.43 ± 0.02 d	1.18 ± 0.10 a	43.14 ± 2.37 e	9.31 ± 0.61 b
		DD	0.80 ± 0.00 a	0.17 ± 0.01 e	0.61 ± 0.02 b	0.31 ± 0.03 e	0.91 ± 0.19 ab	29.18 ± 1.56 f	11.13 ± 0.55 a
	CATE	**RC**	0.81 ± 0.00 a	0.36 ± 0.01 ab	0.66 ± 0.01 a	0.54 ± 0.01 b	0.63 ± 0.05 c	62.62 ± 1.90 b	6.21 ± 0.27 d
		VR	0.81 ± 0.01 a	0.39 ± 0.02 a	0.66 ± 0.01 a	0.60 ± 0.03 a	0.64 ± 0.09 cd	62.47 ± 1.32 b	5.06 ± 0.27 f
		RD	0.81 ± 0.00 a	0.36 ± 0.01 ab	0.63 ± 0.02 ab	0.62 ± 0.06 a	0.78 ± 0.05 b	70.48 ± 4.29 a	7.66 ± 0.33 c
		DD	0.81 ± 0.00 a	0.30 ± 0.02 bc	0.61 ± 0.02 b	0.48 ± 0.03 c	0.86 ± 0.10 b	52.82 ± 4.76 cd	8.29 ± 0.57 bc
	CETA	**RC**	0.79 ± 0.01 a	0.34 ± 0.02 b	0.63 ± 0.01 ab	0.53 ± 0.02 bc	0.65 ± 0.03 c	58.68 ± 3.07 bc	4.52 ± 0.14 g
		VR	0.79 ± 0.01 a	0.29 ± 0.02 bc	0.65 ± 0.01 a	0.46 ± 0.03 c	0.70 ± 0.03 c	51.05 ± 3.32 cd	5.03 ± 0.05 f
		RD	0.78 ± 0.01 a	0.30 ± 0.01 bc	0.62 ± 0.01 b	0.50 ± 0.02 c	0.70 ± 0.03 c	52.00 ± 2.09 c	5.28 ± 0.21 ef
		DD	0.79 ± 0.01 a	0.31 ± 0.00 b	0.63 ± 0.01 ab	0.52 ± 0.00 c	0.75 ± 0.05 bc	54.27 ± 0.10 c	5.00 ± 0.15 f
	CETE	**RC**	0.81 ± 0.00 a	0.28 ± 0.01 c	0.61 ± 0.02 b	0.49 ± 0.03 c	0.71 ± 0.12 bc	48.26 ± 2.18 d	4.30 ± 0.09 h
		VR	0.79 ± 0.01 a	0.25 ± 0.02 d	0.60 ± 0.01 b	0.41 ± 0.02 d	0.91 ± 0.22 ab	43.30 ± 3.24 e	4.25 ± 0.09 h
		RD	0.80 ± 0.00 a	0.18 ± 0.01 e	0.60 ± 0.02 b	0.30 ± 0.01 e	0.96 ± 0.14 ab	30.97 ± 1.87 f	5.66 ± 0.21 e
		DD	0.80 ± 0.01 a	0.15 ± 0.03 e	0.60 ± 0.02 b	0.25 ± 0.05 e	0.80 ± 0.13 bc	26.20 ± 4.95 f	7.77 ± 0.57 c

Moreover, as concerns *NPQ*, all the environmental conditions decreased it about 30 % at the early and vegetative stage, whereas no differences were shown compared to CATA conditions at the anthesis stage.

Concerning *ETR*, similar trends as the ones observed for *ΦPSII* were recorded by the different environmental conditions and water treatments. Besides, ETR/A_{net} ratio, a parameter that explains how many electrons are needed to reduce a CO_2 molecule by the Rubisco activity (Table 4.3) was not altered by CATE at early-vegetative, while at vegetative and at anthesis stages increases of 11% and 19% were detected. Nevertheless, CETA decreased the ratio by about 13 % at early-vegetative and anthesis, not modifying it at the vegetative stage. At CETE the ETR/A_{net} ratio was reduced by 42 %, 9 % and 17 % at the early-vegetative, vegetative and anthesis stages, respectively.

Besides, another pivotal components of plants photosynthesis and, concretely, of light energy conversion to chemical energy, are the photosynthetic pigments; data for chlorophyll a (*Chl-a*), chlorophyll b (*Chl-b*), chlorophyll a to b ratio (*Chl a/b ratio*) and carotenoids (*Carot*) are depicted in Fig. 4.4.

Under well-watered regimens, CATE at the vegetative stage increased *Chl-a*, *Chl-b* and *Carot* concentration by 50 %, keeping without modification the *Chl a/b ratio*, while at anthesis no differences were shown for the photosynthetic pigments. Nevertheless, at CETA no differences were detected at the vegetative stage for *Chl-a*, but it increased by 44 % at anthesis, whereas *Chl-b* concentration was increased about 30 % at both growth stages and *Carot* levels by 121 % at anthesis. Furthermore, lower *Chl a/b ratio*s and higher ones at vegetative and anthesis, respectively, were observed. Lastly, CETE conditions raised *Chl-a* concentration by 34 % and 21 % both at vegetative and anthesis stages; it did the same for *Chl-b* at vegetative and *Chl a/b* ratio at anthesis, and *Carot* at both growth stages by 40 %.

4.2.2.2 Drought treatments

Analysing drought effect on *ΦPSII*, it is observed that VD treatment did not alter it compared to control values regardless of the environmental conditions. In addition, it should be pointed out that in general, drought treatments presented higher values for *NPQ* than control treatments. At CATA conditions, VD increased *NPQ* by 18 %, whereas at CATE, CETA and CETE conditions VD treatment increased it by about 30 %. Furthermore, VD at CATA increased the

ETR/A$_{net}$ by about 17 %, whilst at CATE and CETE it increased by 10 % and at CETA it was not altered.

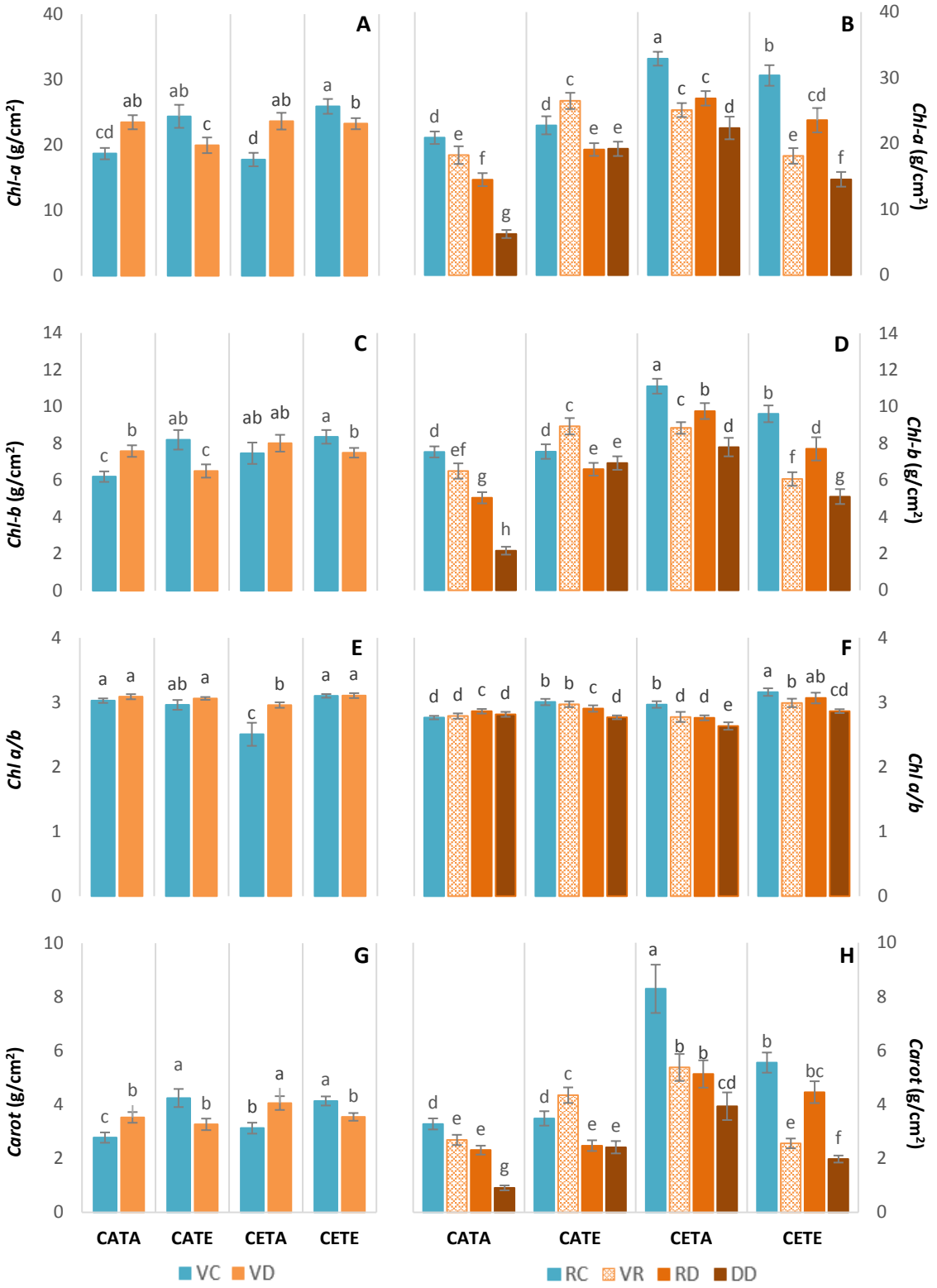

Fig. 4.4. Chlorophyll a (*Chl-a*; A-B), chlorophyll b (*Chl-b*; C-D), chlorophyll a to b ratio (*Chl a/b*; E-F) and carotenoids (*Carot;* G-H) at the vegetative (A, C, E, G) and the anthesis (C, D, F, H) stages. Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.

Regarding photosynthetic pigments, in general, drought treatments affected to a similar extent all of them, therefore not modifying the *Chl a/b ratio*. At CATA, an increase at VD treatment by 28 % was shown, whereas for CATE and CETE conditions reductions by 14 % and 11 % were observed; at CETA conditions, pigments contents were kept constant.

Otherwise, at RD treatment, different trends were observed for photochemical related parameters depending on the environmental conditions (Table 4.3). At CATA conditions, RD decreased the *ΦPSII* by 22 %. The reason for the lower values observed for the RD treatment was the reduced values registered for the *Fv'/Fm'*. At CATE and CETA conditions, no differences were registered for those parameters. Lastly, at CETE conditions, RD reduced the *ΦPSII* by 39 % owing to the reduction observed in *qP*.

As concerns *NPQ*, CATA RD increased it by 119 %, whilst for the rest of the environmental conditions, the RD treatment did not significantly alter it due to high errors. Moreover, ETR/A_{net} ratio was increased at RD treatment by 79 %, 23 %, 17 % and 32 % for CATA, CATE, CETA and CETE conditions, respectively.

Lastly, the RD treatment reduced about 30 % *Chl-a* concentration at all the environmental conditions. The trend observed for the *Chl-b* concentration was the same, although some differences were recorded, giving, as a result, a slightly higher *Chl a/b* ratio at CATA, and lower at CATE and CETA, respectively. *Carot* concentration was reduced too at RD treatment except for CATE conditions.

4.2.2.3 Plant memory

It is worth noting that in a general way, all the parameters related to the photochemistry recorded in Table 4.3 were recovered by VR treatment regardless of the environmental conditions, except *ΦPSII at* CATA that presented 14 % lower values owed to lower *qP.*

As concerns pigments contents, surprising values were registered. At CATA and CETA, the VR treatment recorded statistically significant lower *Chl-a, Chl-b and Carot* concentration values even though at VD treatment higher ones were observed. In addition, at CETE the VR treatment still presented lower values, whereas at CATE conditions they increased. Lastly, only at CETA conditions, lower *Chl a/b* ratio values were shown.

Attending to the possible priming effect at DD treatments, at CATA higher *ΦPSII* values were registered due to the increase on *qP* values, whereas at CATE 16 % lower values were shown. No longer differences were recorded neither at CETA nor at CETE conditions for those

parameters. Lastly, *NPQ* was maintained without change between both treatments irrespective of the environmental conditions, whereas ETR/A_{net} ratio was increased by DD treatment compared with RD at CATA (38 %) and CETE (17 %).

In the case of the content of the photosynthetic pigment, lower values at DD treatments compared with RD ones were recorded for all of them under CATA, CETA and CETE conditions, whereas at CATE conditions remained equal, although lower *Chl a/b* ratio values were shown.

4.2.3 Photosynthetic biochemistry

Lastly, once plants gas-exchange and photochemistry processes have been analysed, it remains to elucidate how the different environmental conditions and water regimens affected plant ability to fix CO_2 and to use the synthesized substrates (NADPH and ATP) through the Calvin-Benson cycle. In this regard, we analysed the maximum rate of carboxylation (Vc_{max}), the maximum rate of electron transport (J_{max}), the triose phosphate utilization (*TPU*), the leaf nitrogen percentage (*leaf N*) and the leaf density (*LD*).

4.2.3.1 Well-watered treatments

In the well-watered regimen, CATE conditions increased significantly Vc_{max} and J_{max} at the early-vegetative stage (Fig. 4.5A-B), not altering *TPU* (Fig. 4.5C) and *leaf N* (Fig. 4.5D). However, at the vegetative stage, CATE conditions decreased about 15 %, Vc_{max}, J_{max}, *TPU* (Fig. 4.6A-C) and *LD* (Fig. 4.8A), whereas at anthesis it reduced to a higher extent (25 %) Vc_{max}, J_{max}, *TPU*, but increased significantly *leaf N* (Fig. 4.7A-D).

On the other hand, CETA conditions at the early-vegetative stage did not alter Vc_{max}, whilst it increased J_{max} and *TPU*, but reduced *leaf N*. Nevertheless, at vegetative and anthesis stages it decreased the values of all the above-mentioned parameters, being of special relevance the registered 30 % lower of Vc_{max}, whilst *LD* remained unchanged.

Lastly, under CETE conditions generally not statistically significant alterations were observed in biochemistry parameters of photosynthesis, except that lower *leaf N* and *TPU* values were registered at early-vegetative and vegetative stages, respectively, and higher *LD* values at the vegetative stage.

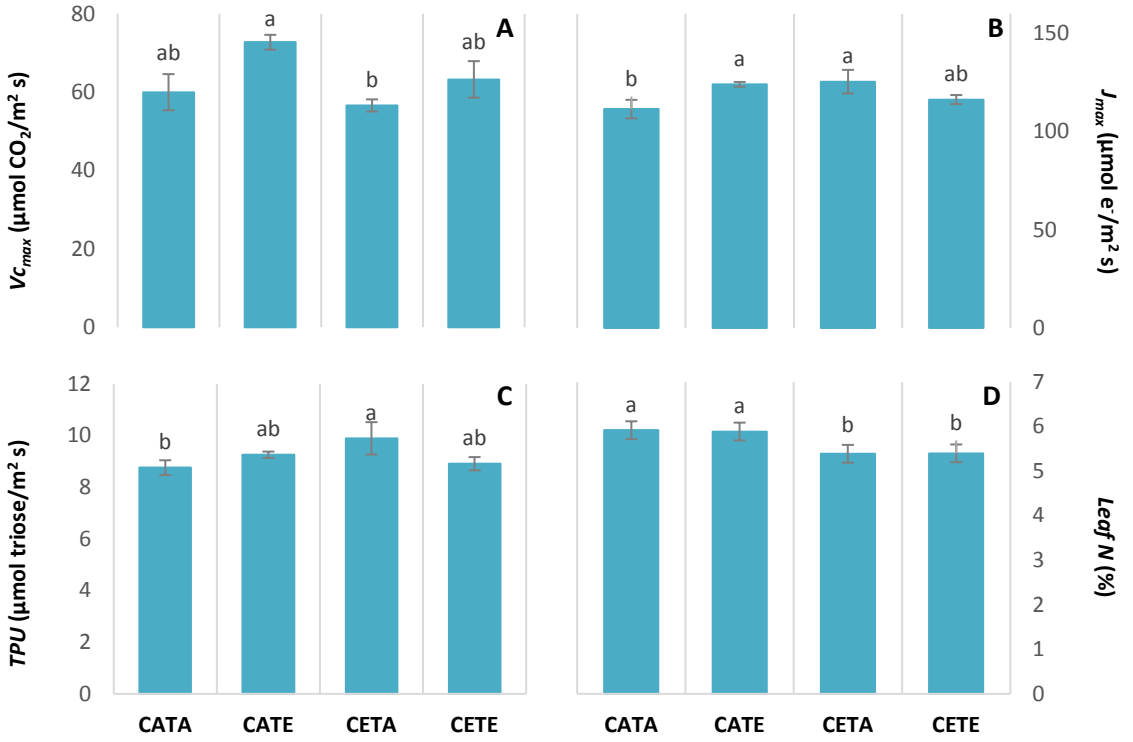

Fig. 4.5. The maximum rate of carboxylation (Vc_{max}; A), maximum rate of electron transport (J_{max}; B), the triose phosphate utilization (*TPU*; C) and leaf nitrogen percentage (*leaf N; D*) at the early-vegetative stage control treatment. Growth environmental conditions and statistical analysis are as described in Fig. 3.1.

4.2.3.2 Drought treatments

As concerns drought treatments, on one hand, the drought at anthesis (RD) caused higher effects on plant photosynthetic biochemistry compared with VD (Fig. 4.6 A-C and Fig. 4.7A-C) independently of the environmental conditions. On the other hand, it is also worth noting that at CATE conditions no longer alterations were registered neither for VD nor for RD treatments. In addition, regardless of the environmental conditions and growth stage, drought treatment reduced *leaf N* content.

VD treatment at CATA and CATE conditions reduced *leaf N*, concretely by 32 % and 16 %, respectively, whilst at CETA and CETE conditions –the ones at ECO_2– a statistically significant reduction on Vc_{max}, J_{max} and *TPU* were also recorded. In addition, it is worth noting that VD CETA treatment plants increased *LD* by 21 %.

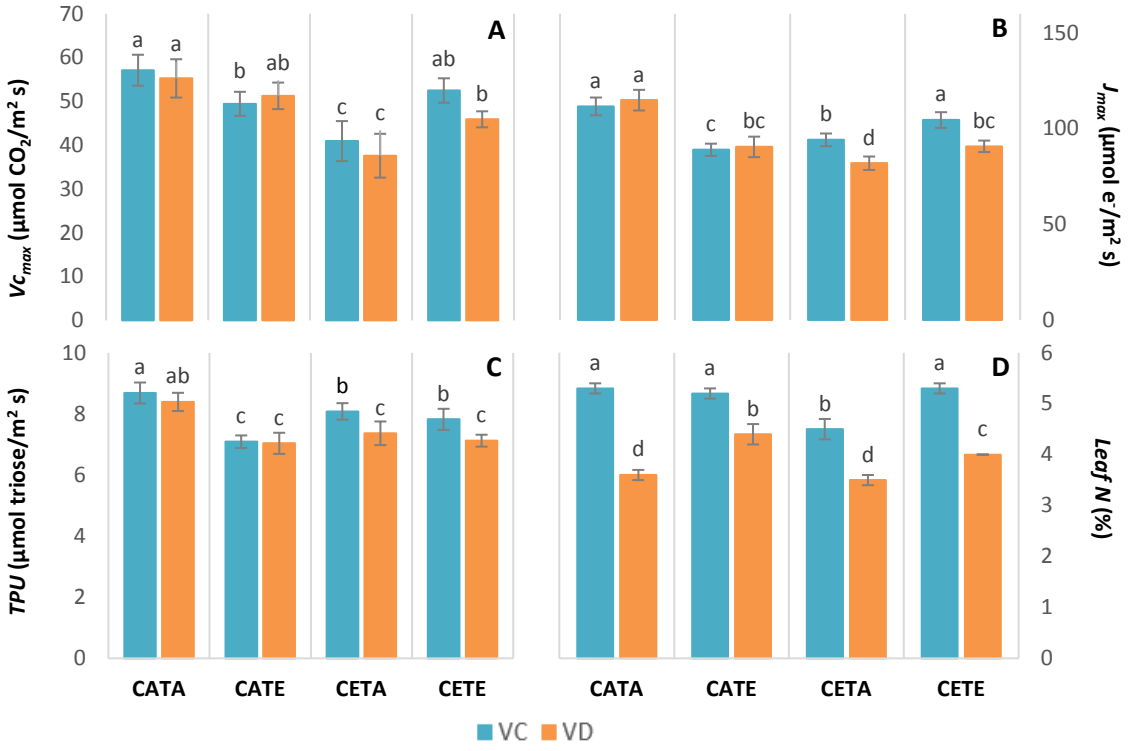

Fig. 4.6. The maximum rate of carboxylation (Vc_{max}; A), maximum rate of electron transport (J_{max}; B) use of triose phosphate (*TPU*; C) and leaf nitrogen percentage (*leaf N; D*) at the vegetative stage. Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.

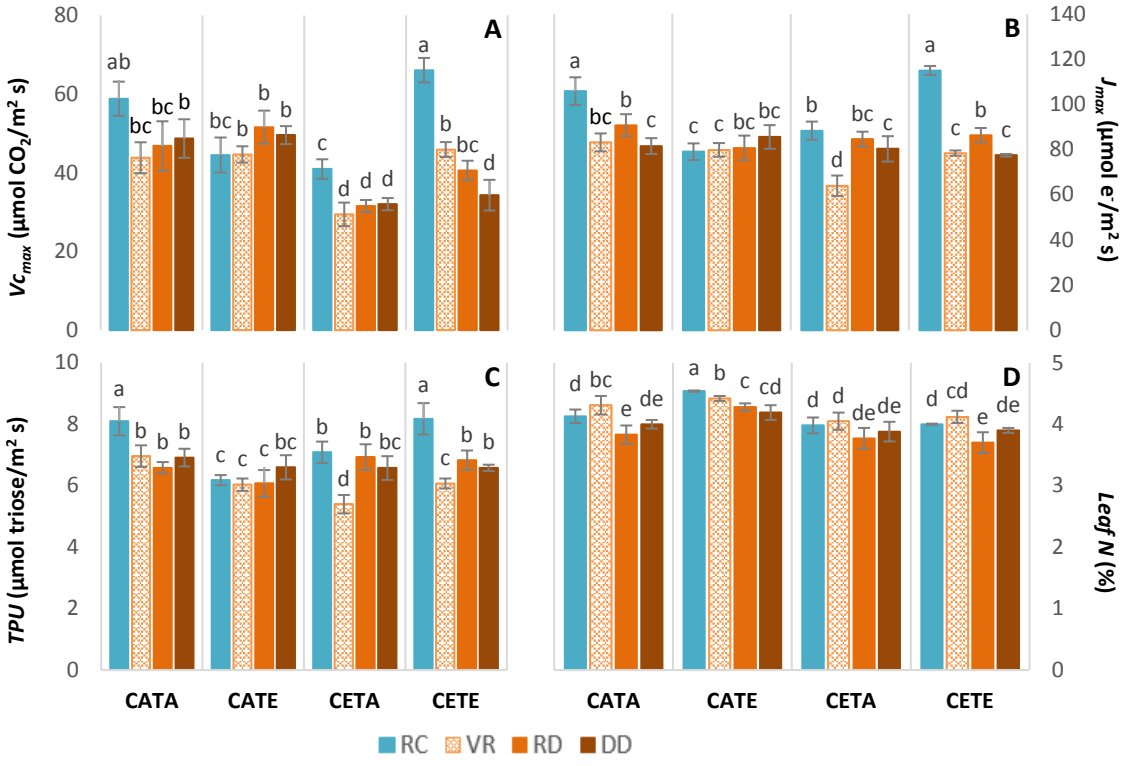

Fig. 4.7. The maximum rate of carboxylation (Vc_{max}; A), maximum rate of electron transport (J_{max}; B) use of triose phosphate (*TPU*; C) and leaf nitrogen percentage (*Leaf N; D*) at the anthesis stage. Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.

Besides, RD treatment at CATA reduced about 25 % the Vc_{max}, J_{max} and *TPU* and by 7 % the *leaf N*. At CATE only *leaf N* was reduced by 5 %. Moreover, in the case of CETA conditions, RD treatment reduced Vc_{max} and J_{max} about 23 % but did not modify *TPU* and *leaf N*, whereas at CETE conditions it diminished by 39 %, 25 % and 17% the V_{cmax}, J_{max} and *TPU* values, respectively, and by 8 % the *leaf N*.

As regards *LD* values for RD treatments, under CATE no alterations were recorded whilst under CATA, CETA and CETE increases of 29 %, 11 % and 34 %, respectively, were detected.

4.2.3.3 Plant memory

Concerning plant recovery capacity, lower values than RC treated plants were recorded in VR treatments for Vc_{max}, J_{max} and *TPU* under all the environmental conditions except for CATE, which were not altered. Concretely, at CATA and CETA conditions the reductions were about 20-25 %, whilst at CETE conditions were of a higher magnitude (30 %). *Leaf N* content and *LD* values in neither case were changed.

Lastly, regarding the possible priming effect by DD treatments, it is noticed that in general no differences were registered compared with RD treatment, although at CATA but especially at CETE conditions some differences were recorded as it occurred for gas-exchange and photochemistry parameters. In this sense, DD treatment reduced Vc_{max} by 15 % at CETE conditions and J_{max} by 10 % both at CETE and at CATA.

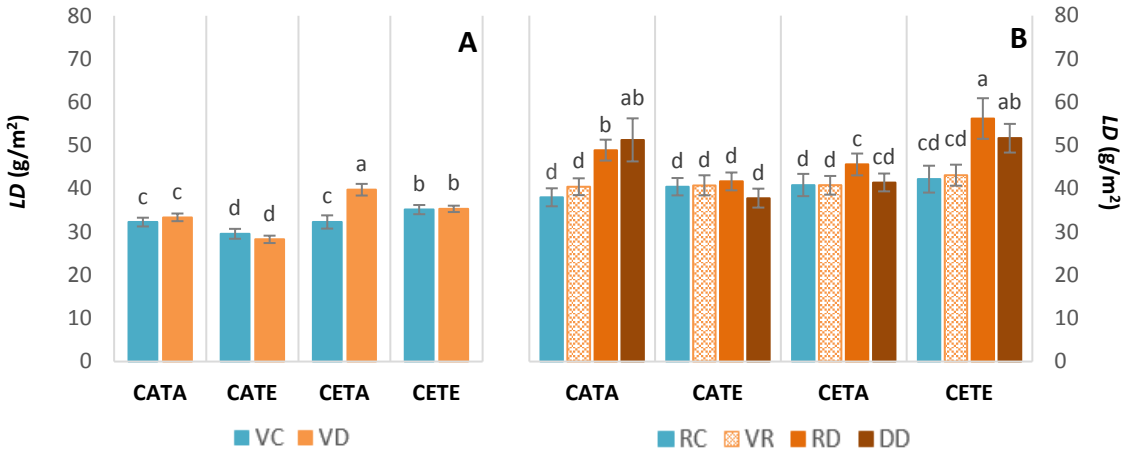

Fig. 4.8. Leaf density (*LD*) at the vegetative (A) and the anthesis (B) stages. Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.

4.3 Discussion

4.3.1 Photosynthetic metabolism of well-watered treatments

Barley plants, regardless of the environmental conditions, generally presented the highest A_{net} at the early-vegetative stage (Fig. 4.1A), decreasing A_{net} as ontogeny progressed (Fig. 4.2A and 4.3A). The higher carbon assimilation rates were associated with the greatest *qP* (Table 4.3).

Next, we will discuss the effects of the different environmental conditions, applied individually or in combination, in the photosynthetic metabolism. From now on, unless the opposite is stated, all the comparisons will refer to CATA control conditions for each growth stage as it has been done for the description of the results.

4.3.1.1 CATE conditions effects

At the early-vegetative stage, CATE conditions did not modify A_{net}, whereas at vegetative and anthesis stages the elevated temperature (ET) decreased it by 10 % and 20 %, respectively (Fig. 4.2A and 4.3A). Thus, CATE conditions became more stressful for the photosynthetic metabolism as the plant life cycle was going on.

Depending on the ET intensity, frequency and duration, plants are able to maintain or not leaf gas-exchange performance (**Wahid et al., 2007**). At the vegetative stage, CATE conditions triggered an increase in stomata aperture (*gs/stomata*; Fig. 4.2B) to lose the excess of the leaf heat (**Farooq et al., 2011**; **Liu et al., 2017**), whereas it reduced *gm*, giving as a result higher *Cc* (Fig. 4.2E). In addition, even though the photochemical parameters were neither modified (Table 4.3), the ETR/A_{net} ratio was increased. Therefore, it could suggest that a significant proportion of electrons were not consumed in the carboxylation reactions and were deviated to alternative processes such as photorespiration and Mehler reaction, increasing the risk of oxidative damage by reactive oxygen species (ROS) (**Salazar-Parra et al., 2012**). This fact, together with the decrease in the biochemical processes that will be exposed below, could explain the higher *Cc* levels, as a lesser proportion of CO_2 was fixed (**Sharma et al., 2015**).

Besides, Vc_{max} (Fig. 4.6A) and J_{max} (Fig. 4.6B) decreased by 14 % and 20 %, respectively, indicating that CATE conditions reduced the Rubisco activity, being this fact the possible main explanation for A_{net} impairment as **Crafts-Brandner and Salvucci** (**2000**) stated and **Wang et al.**

(**2015**) observed for wheat. This behaviour is characteristic of the C_3 plants, such as barley, which exhibit a low thermal optimum for photosynthesis (**Sage and Kubien, 2007**). The decrease in the carboxylation efficiency could be principally related to the thermo-sensitive of the Rubisco activase enzyme, being reduced by the elevated temperatures (**Sage et al., 2008; Dusenge et al., 2019**). We cannot forget that the Rubisco activase is responsible for facilitating the displacement of inhibitors from the catalytic site of Rubisco (**Sage et al., 2008; Slattery and Ort, 2019**). Accordingly**,** Rubisco activase is sensitive to moderate increase in temperature, decreasing its stability (**Bracher et al., 2017; Carmo-Silva et al., 2015**), which in turn limits the proportion of activated Rubisco (**Crafts-Brandner and Salvucci, 2000; Salvucci and Crafts-Brandner, 2004; Sage, et al., 2008**). In addition, the optimum vegetative temperature for wheat —a very close species of barley which can be taken as an example—, is 26 °C (**Hatfield et al., 2011**).

On the other hand, as concerns the processes of carbon consumption, both *Rn* and *Rd* were decreased due to a thermal-acclimation effect to balance it with the lower A_{net} (**Dusenge et al., 2019**, maintaining the Rn/A_{net} *ratio* constant (Table 4.1). Otherwise, R_{photo} was reduced at CATE conditions. Normally, at high temperatures, the photorespiration uses to be increased since the Rubisco specifies for the CO_2 decreases compared to one for the O_2, and because the solubility of the O_2 is less reduced than the solubility of the CO_2, being more probable the oxygenation by the Rubisco (**Dusenge et al., 2019 and references therein**). In this case, the most probable explanation for our results is that as the Rubisco activity was reduced –probably due to a lower activation state since *leaf N* remained unchanged (equivalent for Rubisco content)–, both the carboxylation and oxygenation were affected simultaneously, resulting in thermal acclimation of the photorespiration rates too as **Flexas et al.** (**2014**) suggested.

Moreover, CATE conditions affected to a higher extent A_{net} at anthesis, although it was not traduced in an accelerated senescence process (Fig. 4.5). In agreement with our results, different authors for wheat have also seen a similar trend (**Sharma et al., 2015; Abdelhakim et al., 2021**).

At this stage, a reduction in the *stomata aperture, density and size* (Fig. 4.3B-D) and *gm* was observed, whilst *Cc* (Fig. 4.3E) and *ETR* (Table 4.3) were increased. Altogether, it would indicate that a thermal-photosynthetic acclimation was developed at anthesis, although it was not enough to maintain A_{net} as **Way and Yamori (2014)** stated.

However, as it happened for the vegetative stage, at anthesis a decrease in both Vc_{max} (Fig. 4.7A) —in this case, it was slightly higher (20 %)—, and J_{max} (Fig. 4.7B) was observed. Thus,

A_{net} was reduced due to a reduction in the diffusional path and a reduction of the RuBP regeneration, and on the Rubisco activity. The latter was probably caused by the thermo-sensitivity of the Rubisco activase (**Sage et al., 2008**) as above has been mentioned, but also could be ascribed to lower Rubisco amount as the lower *leaf N* values could be indicating (Fig. 4.7D). Together with this, R_{photo} rates were reduced too (Table 4.1), evidencing again the negative impact on the Rubisco activity for both the carboxylation and the oxygenation.

Nevertheless, unlike the null effect observed on respiration balance in the vegetative period for CATE plants compared with CATA, at anthesis an increase on *Rn* was shown, thus increasing *Rn/A_{net} ratio* (Table 4.1). This fact, probably, enabled plants to obtain energy for use in defensive and antioxidant processes such as the synthesis of heat shock proteins (HSP) (**Slattery and Ort, 2019**) evidencing that the effect of the elevated temperature on plants was greater as the plant cycle was going on, being the reproductive stage the most sensitive developmental stage (**Slattery and Ort, 2019**).

4.3.1.2 CETA conditions effects

As regards our results of plants grown in CETA conditions and at the early-vegetative stage, a ≈20 % increase on A_{net} was observed (Fig. 4.1A), whilst at vegetative and anthesis stages a slight but no significant increases were shown (Fig. 4.2A and 4.3A), indicative of photosynthesis acclimation. Our results are in agreement with the published by other authors such as **Geiger et al.** (**1998**) and **Tausz-Posch et al.** (**2018**), who stated that the positive effect of the CO_2 was greater at the young stages of the growth development of the plants. The increase on A_{net} at the early-vegetative stage was given in part due to the increase on *Cc* (Fig. 4.1D) despite the *gs* was decreased (Fig. 4.1B) —a contrasting effect triggered by the elevated air CO_2 concentration (**Long et al., 2004**) which also can be shown for our data (Fig. 4.1B)—, while *gm* remained unchanged (Fig. 4.1C).

Together with the increase in *Cc* values, higher *qP* and *ETR* values were registered for CETA conditions at this stage, which could be triggered to cope with the probable increase of ATP and NADPH utilization (**Robredo et al., 2010**), what in turn, would also explain the greater J_{max} values observed (Fig. 4.5B).

On the other hand, respiration is a major process of carbon loss in plants. Regarding our data, at CETA conditions *Rn* was not altered, whereas *Rd* was increased (Table 4.1). Other authors as **Xu et al.** (**2015**) have also described different trends concerning plants respiration at

ECO_2 conditions. In this respect, it has been speculated that the respiration rates could increase due to the greater photoassimilates availability because of the higher A_{net}, avoiding their accumulation in leaves and therefore, the photosynthetic acclimation. On the other hand, frequently it has been seen that the respiration rates could decrease due to a N dilution effect, which would not need to produce so much energy for the turnover of proteins (**Xu et al., 2015**). Regarding our results, it seems that CETA plants at this early-vegetative stage had a trend towards the former, which will be supported by **Ainsworth et al.** (**2004**) work, where they stated that in this early stage of development plants have a high sink strength and are employing photoassimilates in respiration, using the energy obtained for the construction of new biomass. However, authors such as **Aranjuelo et al.** (**2011**) commented that the higher loss of the CO_2 through the light respiration could lead to the maintenance of the biomass, a fact observed by us that will be discussed in Chapter 5.

In addition, one of the beneficial effects of ECO_2 is the diminution of R_{photo} triggered by the higher CO_2/O_2 ratio at the carboxylation site of the Rubisco, which increases the carboxylation rate by this enzyme as **Xu et al.** (**2015**) reviewed. Observing our results, although decreases were not shown for R_{photo} at CETA conditions (Table 4.1), a lower R_{photo}/A_{net} ratio could support it, denoting that Rubisco enzyme at early-vegetative CETA drove in a greater extent carboxylation than oxygenation compared with CATA conditions.

Nevertheless, as plants were exposed for a longer time to elevated CO_2 air concentrations, the effect on the stomatal features was more appreciated. At the vegetative stage, *SD* and *TSS* were not modified (Fig. 4.2C-D), and only a stomata aperture reduction was registered (Fig. 4.2B). However, at anthesis, a lower stomata size was recorded at CETA conditions RC treatment (Fig. 4.3C). This fact would imply that as the life cycle of the plant was going on, ECO_2 could induce a persistent change in stomatal properties as other authors have stated (**Harrison et al., 2020; Li et al., 2020b**). Therefore, the stomatal resistance to gas-exchange should increase (**Ainsworth and Rogers, 2007**), and this could be of special relevance when plants faced drought stress.

Moreover, it must be borne in mind that although the lower stomatal conductance (and hence, higher resistance), the higher CO_2 diffusion due to the greater *Ca-Ci* gap, gave as a result an increase in *Cc* during all the life cycle of CETA plants (Fig. 4.1D, 4.2F and 4.3F). However, at vegetative and anthesis stages despite higher *Cc*, a photosynthetic acclimation was observed since a reduction on Vc_{max} (30-40 %) and J_{max} (20 %) was shown in well-watered CETA conditions plants, which would indicate a reduction in Rubisco activity and RuBP regeneration. This

photosynthesis acclimation results in a reduction of the photosynthesis capacity below its maximum potential (**Long et al., 2004; Leakey et al., 2009**). The reduction of V_{Cmax} –also observed by **Leakey et al.** (**2009**)–, could result either from a reduced Rubisco activation state (**Sage et al., 1988**) or from lower amounts of Rubisco (**Drake et al., 1997; Stitt and Krapp, 1999**).

In agreement with our results for different recent works carried out in crop species, **Torralbo et al.** (**2019**) in a low-sink barley cultivar and **Vicente et al.** (**2015 and 2016**) and **Abdelhakim et al.** (**2021**) in wheat, neither observed an increase in A_{net} for CETA conditions plants. The authors also attributed the obtained results to a down-regulation of the Rubisco activity caused by a photosynthetic acclimation due to a lack of sink-strength.

Until today, the causes for the photosynthetic acclimation are not clear. Mainly, three hypotheses are considered as responsible for the down-regulation of the Rubisco activity at elevated CO_2 concentration (**Xu et al., 2015; Vicente et al., 2019 and references therein**). The first one is that at elevated CO_2 plants are unable to balance ATP and NADPH production with the extra substrate (CO_2) availability. Consequently, the activation state of the Rubisco and/or the capacity to regenerate the RuBP diminishes. The second one is the decrease in the Rubisco quantity. This hypothesis could be triggered due to the dilution effect caused by the greater carbon gain and/or the diminution of the N assimilation, the latter being a consequence of the lower translocation or the lower NH_4^+ re-assimilation caused by the lower photorespiration rates. Finally, the last hypothesis would attribute the down-regulation of the Rubisco activity or quantity and photosynthesis-related genes to the incapacity to export CHs from leaves and/or to use the gained photoassimilates (*TPU*), therefore accumulating it and unbalancing the source/sink ratio due to a lack of sink strength.

If we try to inquire the possible causes that triggered the commented photosynthetic acclimation both at vegetative and anthesis stages, a not clear pattern is observed. The main causes could vary depending on the plant's growth stage. In this regard, at the vegetative stage lower *leaf N* (Fig. 4.6D) but same *ETR* values (Table 4.3) were registered in CETA. Regarding the use of the photoassimilates produced in the Calvin-Benson cycle, no differences were observed neither for respiration rates (Table 4.1) nor for *TPU* (Fig. 4.6C) and nor for *LD* values (Fig. 4.8A) in CETA vegetative plants. Thus, it seems that the lower Rubisco quantity could be ascribed to a lower NO_3^- reduction and assimilation, where the fewer recorded R_{photo} rates might be involved on it among other causes. This topic has been extensively studied by Bloom and collaborators mainly for wheat through different works (**Rachmilevitch et al., 2004**, **Bloom et al., 2002, 2010, 2014 and 2020**), although there is still no consensus about it (**Dier et al., 2018**). In this regard, it

should be of interest to measure the transcript and/or activity of the enzymes involved in the N inorganic reduction and assimilation into proteins, and on the other hand, the genes encoding the Rubisco different subunits and the light-harvesting proteins transcripts as **Vicente et al.** (**2015**) did for wheat and **Torralbo et al.** (**2019**) for barley. This approach would allow us to try to clarify the possible causes and processes involved in the putative Rubisco lower quantity at CETA conditions for this stage.

Nevertheless, it should be noticed that at the early-vegetative stage a lack of sink strength started to be developed (higher *LD* values) due to the none ability to burn the produced extra higher CHs as **Ainsworth et al.** (**2004**) first stated and also **Xu et al.** (**2015**) reviewed. Therefore, we cannot discard the accumulation of photoassimilates due to a lack of sink-strength as one of the possible main cause for photosynthetic acclimation at the vegetative stage. In this respect, the starch has been proposed as the main C compound to store the excess of CHs, whereas sucrose is believed to be the main C compound for CHs translocation to developing sinks (**Stitt et al., 2010**). Within this cotext, to keep inquiring into the possible causes of the photosynthetic acclimation, a possible next step to take would be to measure them.

In the case of anthesis, in general, CETA RC treatment did not record any difference for the above-mentioned parameters related to the sink strength compared with CATA conditions RC treatment. Furthermore, the *ETR* was kept constant, whereas J_{max} rates were reduced, developing divergence in the observed trend for reductant power production and RuBP putative regeneration. Lastly, *leaf N* was not altered in CETA conditions for the RC treatment. However, as **Feller** (**2016**) suggested, the none observed differences for *leaf N* at anthesis could be explained due to the ECO_2 effect on leaf senescence delay as the photosynthetic pigments remained higher (Fig. 4.4B, D, F). Therefore, taking everything commented into account, it is difficult to know what was the main cause that triggered photosynthetic acclimation at anthesis (Fig. 4.7A), although an insufficient demand for CHs from the recorded fewer developing C-sinks at this stage (spikes; Chapter 5) could have triggered it (**White et al., 2016**). It must be borne in mind that source activity (photosynthetic performance) depends on sink activity (tissue growth) as **Körner** (**2015**) pointed out.

Lastly, although a photosynthesis acclimation was developed at vegetative and anthesis stages and A_{net} rates were not increased compared with CATA, it should be noticed that the ECO_2 at CETA conditions increased the photosynthetic pigment contents (Fig. 4.4), the same trend as the one observed by **Xu et al.** (**2016**) for soybean, denoting a delay in the foliar senescence. As

it will be discussed in Chapter 5, this fact was of relevance in final plant growth and yield response.

4.3.1.3 CETE conditions effects

At CETE conditions, a 12 % increase on A_{net} along all the life cycle of plants was shown (Fig. 4.1A, 4.2A and 4.3A). As it occurred in CETA conditions at the early vegetative stage, an increase on *ETR* (Table 4.3) together with higher *Cc* levels (Fig. 4.1D, 4.2F and 4.3F) could have explained the increases on A_{net}, whereas at vegetative and anthesis was owed to the higher *Cc* values (Fig. 4.2F and 4.3F).

Stomata can be regulated by different environmental signals, where some of them as ECO_2 close it, and other such as ET open it (**Merilo et al., 2014**). Thus, stomatal regulation at CETE conditions should have to face a complexity of opposing signals (**Merilo et al., 2014; Urban et al., 2017**). Regarding our results, the *stomatal properties* (stomata density and size) were kept stable along the life cycle of plants (Figs 4.2C-D and 4.3C-D); therefore, the reduction of the stomatal conductance was only attributed to the stomata aperture reduction which seems that was governed by ECO_2 effect.

Moreover, *gm* stayed without a change although the lower *gs* –with what used to go together (**Gallé et al., 2009; Pérez-Martín et al., 2014**)–; this fact could be due to overexpression or a better activation state of aquaporins. In this regard, different authors have suggested that in barley, leaf *AQPs* could facilitate the CO_2 diffusion inside the mesophyll cells (**Hanba et al., 2004; Katsuhara and Hanba, 2008; Horie et al., 2011; Mori et al., 2014**). Regarding our data, and as it was commented in Chapter 3, leaf *HvPIP2;5* isoform of VC treatment at CETE conditions was higher (Fig. 3.7A). Furthermore, a positive correlation was shown between this isoform at the vegetative stage and A_{net} and *Cc* (Table 4.2); this fact was evidenced by **Mori et al.** (**2014**) who found its participation in the CO_2 transport in barley, and, consequently, it is reliable to think that it could participate in the performance of A_{net}. In addition, it is of interest to point out that the work carried out for tomato at CETA conditions by **Zhang et al.** (**2021b**) had observed that PIP sub-family *AQPs* can modify their main function depending on the CO_2 concentration within the plant. Concretely, they did not observe a CO_2 transport function at current conditions (CATA) for a *PIP1;2* isoform, whereas at ECO_2 seemed to have it, not affecting plants water loss. In line with this, we suggest that in our case leaf *HvPIP2;5* isoform could present a differential main solute transport function between CATA and CETE conditions, switching on for CO_2 transport at CETE owed to the higher CO_2 pressure concentration.

Besides, at anthesis, the lack of differences for leaf *HvPIP1;3*, *HvPIP2;1* and *HvPIP2;5* between both environmental conditions (Fig. 3.8A), together with the observed positive correlation with A_{net} and *gm* (Table 4.2), could suggest that these isoforms were the most involved in CO_2 transport. In this regard, it has been observed that in the mesophyll cells the *AQPs* had a direct effect (**Flexas et al., 2013b**), whereas, in the case of guard cells of stomata, the role of *AQPs* in the CO_2 transport seems to be an indirect effect on the water channel function (**Martínez-Ballesta et al., 2009**). Thus, the *AQPs* expression response is not only isoform-specific, their function could be cell type-specific too.

Coming back to plants physiological response, as regards the carbon consumption processes, *Rn* presented higher values compared with CATA and CETA conditions along with plants whole life span (Table 4.1), which in part allowed CETE plants to avoid the photosynthetic acclimation unlike for CETA conditions plants (higher sink strength). Same Vc_{max} rates to CATA conditions were registered (Fig. 4.5A, 4.6A and 4.7A). In agreement with our results, **Aranjuelo et al.** (**2006**) and **Erice et al.** (**2006a**) for alfalfa, or **Dias de Oliveira et al.** (**2013 and 2015a**) and **Abdelhakim et al.** (**2021**) for wheat along with different cultivars, also registered higher A_{net} values in CETE but not in CETA. In their words, such a different response between both conditions could lie in the higher sink strength for the former but not for the latter, consequently being able to avoid photosynthetic acclimation. In line with this, and going back to our results, no differences were observed for *leaf N* (Fig. 4.5D, 4.6D and 4.7D), another evidence that could explain the non-observed photosynthetic acclimation (**Ainsworth and Long, 2005**). In addition, photosynthetic pigments were increased (Fig. 4.4) conferring to CETE plants delayed senescence, also observed by **Abdelhakim et al. (2021)** by a cultivar-specific response on wheat.

Consequently, the higher A_{net} and greenest maintenance were traduced in higher plant biomass –as it will be seen in Chapter 5–, giving, as a result, an even higher sink strength. In this point, it is of interest to highlight that, if an acclimation effect had not been developed at CETA conditions plants, the photosynthetic rates could have been higher than in CETE conditions (Fig. 4.1A), indicating a higher potentiality. On the other hand, the higher A_{net} in CETE conditions control treatment plants due to the avoidance of Rubisco activity downregulation caused by ET, could also be ascribed to the possible effect that ECO_2 had in the thermal displacement of the optimum of the A_{net}, a concept defined by **Long** (**1991**). This fact could be explained by the kinetic properties of Rubisco and the better employment of photoassimilates in defensive system development. Plants would have acquired a thermo-tolerance (**Wang et al., 2008**) which protected the enzyme kinetics from the elevated temperature constraints (**Faria et al. 1996;**

Gutiérrez et al. 2009), increasing RuBP regeneration and avoiding the downregulation of the Rubisco activity (**Chavan et al., 2019**).

Overall, the different response of CETE plants compared with CATE and CETA ones at the well-watered regimen strengthens the importance that this kind of research has to elucidate plant photosynthetic metabolism response at future environmental conditions. Hence, plants in the future will not experience climate change factors individually (**Gray and Brady, 2016**), and the effects of climate change factors are going to be modulated by the presence of other factors (**Kizildeniz et al., 2018**).

4.3.2 Photosynthetic metabolism of drought treatments

The second specific objective of this Chapter was to compare the response of the photosynthetic metabolism of plants that have suffered a water stress period at the vegetative or anthesis stage, and to inquire if the activated mechanisms to cope with it will vary under future environmental conditions.

4.3.2.1 Vegetative drought (VD) treatment effects under current and future environmental conditions

In CETE conditions, the vegetative drought treatment (VD) decreased A_{net} by 17 % compared to its control (VC); whereas in CATA conditions, the reduction caused by the VD compared was higher: 22 % (Fig. 4.2A). Other authors and for different species (**Hamerlynck et al., 2000; Naudts et al., 2013; Song et al., 2014; AbdElgawad et al., 2015b**) have also observed this trend.

Regardless of the environmental conditions, the diminution of the A_{net} by the VD treatment was mainly driven by diffusional limitations (Fig. 4.2B-E), while little difference was observed for photochemical (Table 4.3) or biochemical (Fig. 4.6) parameters compared with VC treatments at both environmental conditions. This fact could be a consequence of the mild effect that VD treatment had on barley plants water status (**Abdelhakim et al., 2021**), as was concluded in Chapter 3 by the lack of alteration on *LRWC* (Fig. 3.1C). Thus, the reduction in *gs* was enough to avoid excess water loss.

Attempting the different parameters related to the stomatal properties that would explain part of the diffusional limitations at drought treatment, at CATA a stomata aperture diminution (Fig. 4.2B) and lower *SD* (Fig. 4.2C) were recorded. At the same time, under CETE

conditions the decrease was mainly due to a reduction in the stomata aperture, although lower *gm* values were recorded too. Therefore, the decrease at both conditions came by the reduction firstly in the internal CO_2 concentration and consequently in the decreases in the supply of CO_2 to the mesophyll cells (*Cc*; Fig. 4.2F). Furthermore, it should not be forgotten that CETE VD plants were grown under ECO_2 conditions, thus the *Cc* was less limiting (**Van der Kooi et al., 2016**).

Going on with the carbon uptake process and diffusional limitations, the observed different leaf *AQPs* expression pattern between both environmental conditions under VD treatments (Fig. 3.7A) could have something to say on A_{net}.

At CATA conditions, no differences were observed for *leaf HvPIP2;5* isoform expression values between VD and VC treatments. This fact, together with its higher expression values, could be indicative of its relevance as a buffer *AQP* in an attempt to maintain cell biochemical and metabolic status, as it has granted a key position in the water (**Besse et al., 2011**) and CO_2 (**Mori et al., 2014**) transport regulation within barley plant. However, leaf *HvPIP1;3* and *HvPIP2;1 AQPs* presented lower expression values, both of them expected to be involved in the CO_2 transport (**Hanba et al., 2004; Mori et al., 2014**). These lower values could be explained by their participation in the recorded diminution in *Cc* and A_{net} levels. Besides, in the case of CETE conditions, the opposite trend was shown, it is, leaf *HvPIP1;3* and *HvPIP2;1* remained unchanged and leaf *HvPIP2;5* presented lower values at VD treatment compared with RC. In addition, it was higher than for CATA VD treatment, which, on one hand, would indicate its role in diffusional regulation at CETE conditions, and on the other hand, might strengthen its possible switch on as CO_2 transport *AQP* at ECO_2 conditions, postulated previously for the VC treatment. Overall, it could be said that an isoform-specific *AQPs* expression pattern was developed by VD treatments, which varied between current (CATA) and future (CETE) environmental conditions, being involved in both cases in the diffusional limitations.

Moreover, apart from diffusional limitations, it can be observed that VD CATA treatment caused an increase in Rn/A_{net} *ratio* (Table 4.1), probably to produce more ATP and NADPH as **Robredo et al.** (**2010**) stated. These products are used as substrates for the maintenance of processes such as protein turnover and antioxidant molecule production to ROS scavenging destined to reduce the damages caused by drought stress **(AbdElgawad et al., 2015b)**. Conversely, this ratio was less under CETE conditions, which might also denote less significant stress by VD treatment at this environmental condition.

4.3.2.2 Anthesis drought (RD) treatment effects under current and future environmental conditions

When we analyse the damages that the anthesis drought treatment (RD) caused, regardless of CATA or CETE conditions, the effect on *SVWC* was greater than in VD (Fig. 3.1A-B). As a result of that soil water deficiency, *LRWC* was dropped (Fig. 3.1C-D) and Ψw_{md} became more negative. This observed trend was due to a higher leaf area, water demand and transpiration rates of plants in anthesis, as has been concluded in Chapter 3 and **Izanloo et al.** (**2008**) observed for wheat**.** Consequently, withholding water supply at this stage caused a higher reduction on A_{net} compared with the vegetative stage (Fig. 4.2A and 4.3A). In the same way, **Wang et al.** (**2015**) and **Liu et al.** (**2017**) for different wheat cultivars grown at current environmental conditions also observed a higher reduction in photosynthetic rates in response to drought at anthesis than at the vegetative stage.

A first consequence of the worse water status of RD treatment plants compared with VD was the higher diffusional limitations, since the stomata aperture decreased twice (Fig. 4.2B and 4.3B) and *gm* was also reduced at both environmental conditions and to a higher extent (Fig. 4.2E and 4.3E), leading to lower *Cc* values (Fig. 4.3F). In addition to the diffusional limitations, the carbon consumption processes were increased by RD treatment compared to RC treatment as can be observed for *Rn* and *Rd* (Table 4.1), acting as maintenance respiration to obtain energy for dealing with processes such as protein turnover or defensive system (**Xu et al., 2015**).

Otherwise, unlike at vegetative stage, the decrease in A_{net} caused by RD treatment at both environmental conditions was due not only to the diffusional limitations discussed above but also due to photochemical and biochemical limitations, triggered by the observed greater extent on *LRWC* and Ψw_{md} (Chapter 3) as **Lawlor and Cornic (2002)** stated too. The *ETR* of RD treatment plants was decreased by 20 % (Table 4.3). This lower transport of electrons from the reaction centre to the final acceptor could lead to lower production of NADPH and ATP (**Robredo et al., 2010**), but also might have allowed photochemistry apparatus protection (**Baker and Rosenqvist, 2004; Zivcak et al., 2013**). In turns, as **Lawlor and Tezara** (**2009**) stated, it could be derived in a lower activity of the Calvin-Benson cycle since they did not have the substrates necessary for the regeneration of RuBP (Fig. 4.7B). Thus, Rubisco activity was inhibited (Fig. 4.7A) triggering the higher A_{net} reduction compared with VD as **Wang et al.** (**2015**) also observed.

Furthermore, the observed lower photosynthetic pigments content (Fig. 4.4B, D, F) could have participated in the above-mentioned lower *ETR*. This reduction could be due to an increased chlorophyllase activity (**Majumdar et al., 1991; Loggini et al., 1999**) in an attempt to reduce the energy capture and as a mechanism to alleviate the oxidative damage (**Munné-Bosch and Alegre, 2000; Galmés et al., 2007; Elsheery and Cao, 2008; Pérez-López et al., 2012; Abdelhakim et al., 2021**). Besides, RD treatment plants increased *NPQ* (Table 4.3), a fact that it is also considered a tolerance mechanism to increase the protection of PSII (**Flexas and Medrano, 2002b**). Nevertheless, the fact that carotenoids concentration did not increase at RD treatments compared with RC treatments (*Carot/Chls*), could be due to a greater VAZ cycle activity as **Abadia et al.** (**1999**) and **Müller et al.** (**2001**) proposed. In this point, it is worth noting that *Fv/Fm* remained unchanged (Table 4.2), indicating that the RD treatment did not affect the structural integrity of the photosynthetic machinery (**Robredo et al., 2010**).

Conversely, the commented higher *SVWC* and *LRWC* values found in CETE RD plants owed probably to a more conservative strategy (isohydric), compared with CATA RD –which drove a more risk-taking behaviour (anisohydric)–, derived in some positive consequences as regards the carbon metabolism results as explained below. The direct one was that at CETE conditions A_{net} was decreased by 41 %, whilst at CATA conditions the decrease on A_{net} was much higher, by 56 % (Fig. 4.3A). In this regard, **Dias de Oliveira et al.** (**2015a, b**) in wheat analysed an anthesis drought effect in wheat –applying similar temperature and CO_2 treatments–, registering a similar trend as us about photosynthetic rates.

Attending to the causes of the lower reduction of A_{net} rates at CETE conditions, it could be due, in part, to its better stomatal control (**Ghannoum et al., 2003**). Specifically, in CATA RD plants, the *SD* was kept constant compared with RC treatments plants, whereas for CETE conditions, a reduction by 27 % was shown (Fig. 4.3C). Moreover, when stomata aperture was analysed (Fig. 4.3B), a decline in was observed at both environmental conditions for the RD treatments, but being at CATA conditions the diminution higher with respect its control treatment.

In this respect, a lower reduction on the *gs* at CETE RD was shown, denoting a better stomatal control compared to RD CATA plants. This fact could be, in part, the reason why RD CETE plants under this severe drought conditions responded better to water scarcity, presenting a lower *DH* and greater *LRWC* (Chapter 3) drove by an isohydric behaviour (**Domec et al., 2017**) and giving, as a result, a higher A_{net}.

Furthermore, the observed better stomatal control at CETE conditions could be supported through the results obtained by **Caine et al.** (**2019**) for transgenic rice. According to their work, transgenic rice plants with lower *SD* that suffered a drought period under CETE conditions were able to respond better to the water scarcity, translating into higher CHs production and better final yields.

Regarding the diffusional limitations, it is of interest to add that the larger effect caused by drought on A_{net} at the anthesis stage at both environmental conditions, might be also attributed to the reduction of *gm* (Fig. 4.3C), causing ultimately a higher decrease in *Cc.* The lower expression of *HvPIP1;3* and *HvPIP2;1* could be involved in it.

In the case of *HvPIP1;3*, a positive correlation with *gm* was recorded (Table 4.2), although similar expression values were recorded between CATA and CETE (Fig. 3.8A). Until this date, it does not exist any direct evidence (*in vitro* assays) of the role of the *HvPIP1* subfamily on CO_2 transport for barley. However, in the case of tobacco, the implication of the PIP1 subfamily in that function have been observed (**Uehlein et al., 2003; Flexas et al., 2006**). For that reason, the observed positive correlation in our work between *HvPIP1;3* and CO_2 transport-related parameters, could strengthen its possible role in barley as a dual protein channel too.

Nevertheless, in the case of *HvPIP2;1*, it should be remembered that higher leaf *HvPIP2;1* expression was observed at CETE RD treatment (Fig. 3.8A). This isoform might come to substitute, or at least contribute, to the commented role for *HvPIP2;5* at the vegetative stage, since also presented correlation patterns as a dual protein on H_2O and CO_2 movement within the plant (Table 3.2 and 4.2) (**Horie et al., 2011; Mori et al., 2014**) without compromising its water channel function. However, as **Moshelion et al.** (**2015**) stated, it is difficult to distinguish if *AQPs* role in photosynthesis performance belongs to a direct or indirect role due to its water channel function, and even more in our case where there was not recorded any correlation with *gm* for it, consequently data should be interpreted with care.

In line with the last fact, **Maurel et al.** (**2016**) reviewed the effects of aquaporin genetic manipulation on plant water relations and photosynthesis, showing that, when both parameters had been measured, a covariation of *gs* with *gm* existed in all studies that they have analysed. In addition, **Otto et al.** (**2010**) suggested that both *PIP1* and *PIP2* might form heterotetramers, which could modify *AQPs* membrane transport function. Thus, the molecular and physiological mechanism that possibly link the two parameters (*gs* and *gm*) and hence processes are still unknown (**Flexas et al., 2013b**), even being many aspects to be clarified concerning the CO_2 transport through *AQPs*, which it should be carefully taken into account when data are analysed.

As concerns the biochemical limitations, lower *NPQ* (Table 4.3) and higher photosynthetic pigments values (Figs. 4.4B, D, F) were observed at CETE conditions, probably indicative of a lower oxidative damage (**Zivcak et al., 2013; Abdelhakim et al., 2021**) as the higher A_{net} acted as a sink for the ATP and NADPH utilization (**Pan et al., 2018**). In addition to the biochemical limitations, the carbon consumption processes were less increased at CETE conditions, denoting that they did not have to employ such part of the photoassimilates in the maintenance respiration to obtain energy for dealing with processes such as protein turnover or defensive system (**Xu et al., 2015**).

Lastly, it should be noted that at both environmental conditions an increase in *LD* (Fig.4.8B) was shown for RD treatment compared to RC treatment, being even higher at CETE conditions. In this regard, **Wall et al.** (**2001**) also observed the same trend, stating that higher leaf density could suggest a higher leaf thickness or greater total non-structural carbohydrates (NSC) in leaves, which might confer an adaptation to stress. In this respect, it might be of interest to analyse more deeply the metabolic profile of leaves to be able to elucidate the participation of different compounds on plants response to RD between CATA and CETE.

4.3.2.3 The analysis of individual environmental conditions effects to better understand the observed behaviour at CETE

Once analysed the joint effect of elevated temperature and elevated CO_2 (CETE) on carbon assimilation, our next purpose was to elucidate the role that these two environmental stressors could have in these responses, separately.

As regards the elevated temperature and actual air CO_2 conditions (CATE), neither VD nor RD treatment magnified the reduction of A_{net} compared with CATA conditions as other researchers had also stated for alfalfa (**Aranjuelo et al., 2006**) and wheat (**Días de Oliveira et al., 2015a, b**). The absence of a greater effect on net assimilation (A_{net}) due to ET (CATE) than in CATA under drought was ought to the lower transpiration rates and the better plant water status maintenance discussed in Chapter 3. As we have commented, the transpiration area before the water stress imposition was stood out as one of the most important variables which would define drought extent. In this regard, it should be noticed that the lower total leaf area developed (*TLA;* Chapter 5) might be a consequence of the lower CHs produced due to the above-mentioned constraints of ET on A_{net}. Thus, the hastened effects on CHs production and leaf development by CATE conditions before plants suffered a drought period, defined plants response when faced with a water shortage.

Besides, under the actual temperature and elevated air CO_2 conditions (CETA), the lowest reduction by drought treatments on A_{net} was registered. This fact was mainly due to the greater water conservation triggered by the greatest stomatal control (**Robredo et al., 2007; Qaderi et al., 2019 and references therein**), which would be explained through the model commented by **Li et al.** (**2020b**) in Chapter 3. It should be noticed that both CETA and CETE drought plants were governed by this stomatal control, whereas the registered differences on plant transpiration –and therefore drought extent on A_{net}–, were principally due to the transpiration surface capacity before water stress was imposed, being highest at CETE (Fig. 3.4B and 3.4D).

In addition, apart from the better stomatal control triggered by ECO_2, the mechanisms behind the beneficial effect of the elevated CO_2 against the drought stress could be several. Hence, on one hand, ECO_2 levels probably allowed lesser oxidative stress since the lower R_{photo} rates (Table 4.1) could produce less ROS as many authors have stated (**Aranjuelo et al., 2008; Mishra et al., 2013; Zinta et al., 2014; AbdElgawad et al., 2015b**). On the other hand, thanks to the greater carbohydrate supply, a probably increased level of defence molecules such as proline or antioxidants, could also be important to cope with drought stress (**Zinta et al., 2014; Li et al., 2015**). In this regard, it should be of interest to analyse the metabolic profile of leaves in CETA RD treatment plants to test the above-mentioned suggestions and to shed more light to CETE RD treatment response pattern.

Therefore, as a summary for drought treatment effects, the VD treatment at both environmental conditions (CATA and CETE) reduced A_{net} mainly due to stomatal limitations, considering it as a mild drought (**Farooq et al., 2009**). Nevertheless, the reduction caused by the RD treatment on A_{net} was much higher, taking part in both the diffusional and non-diffusional limitations, and consequently be considered as a severe drought (**Flexas et al., 2004; Galmés et al., 2007; Hu et al., 2010**).

Furthermore, the fact that at CETE conditions the VD and RD plants were grown under elevated CO_2 concentration, allowed them to present a lower drought extent compared with CATA conditions due to better stomatal control, giving as a result higher A_{net}. Besides, these plants could also be driving more photoassimilates to ROS scavenging, to reduce oxidative damage. In agreement with our results, different studies carried out for grass species (**Hamerlynck et al., 2000; Naudts et al., 2013; Song et al., 2014; Xu et al., 2014; AbdElgawad et al., 2015b**) and wheat (**Dias de Oliveira et al., 2013 and 2015b**), have highlighted the beneficial effects of ECO_2 at CETE on plants photosynthetic metabolism to deal with drought stress.

Lastly, the higher *AQPs* isoform-specific expression pattern observed at CETE conditions –principally leaf *HvPIP2;1* and *HvPIP2;5* PIP2 isoforms–, which relevance varied depending on the growth stage, are postulated as a key component to face drought constraints. This task deserves more attention, and breeders could consider investigating it, to keep looking for traits that would confer crops adaptive responses to face drought at future environmental conditions.

4.3.3 Plant memory effects on barley photosynthetic metabolism

In Chapter 3, we set the basis for understanding of plants memory effects (1) on the recovery of the water status of plants that had suffered a drought period at vegetative and were subsequently re-watered, and (2) on the possible priming effect –due to having suffered a vegetative drought period–, when plants face a subsequent drought at anthesis. In both cases, the modulation of the different studied environmental conditions in such responses was also studied.

Thus, the third specific objective of this Chapter was to keep elucidating plants memory effect on the recovery capacity of photosynthetic metabolism, and the possible priming effect when DD plants faced a subsequent drought period at anthesis.

4.3.3.1 Photosynthetic metabolism of vegetative recovery treatments (VR)

As concerns the recovery capacity, different trends were observed depending on the environmental conditions in which plants were grown, being possibly the growth temperature a key factor as was commented in Chapter 3. At CATA and CETA conditions, —that is plants grown at current temperature—, the VR treatment, despite the leaf water status was recovered, did not show photosynthesis recovery from the VD treatment since A_{net} kept lower compared to RC treatment (Fig. 4.3A). In the case of CETE conditions, that is elevated temperature and elevated CO_2 conditions, A_{net} of VR treatment was neither recovered, but the reduction was lower than the ones for current temperature conditions.

Thus, under these environmental conditions, it seems that a maladaptive memory effect (no recovery) was developed for VR treatment photosynthetic metabolism. This behaviour might be owed to the allocation costs of developing a plant stress memory and not having suffered subsequent stress as **Crisp et al.** (**2016**) and **Martínez-Medina et al.** (**2016**) stated in their reviews for the topic. However, until today, although the investigation of adaptative traits

has received a great deal of attention, the mechanism behind the plant maladaptive memories is not well known.

Nevertheless, at elevated temperature and actual CO_2 conditions (CATE), VR treatment plants not only recovered A_{net}, but also presented higher values than RC treatment; consequently, they developed a cross-talking priming effect that allowed them to deal with the hastened effects of ET. **Wang et al.** (**2015**) and **Liu et al.** (**2017**), both in wheat, observed a similar trend for CATE VR treatment. It must be said that in our case, the elevated temperature stress was differently applied since it was imposed from the sowing, but regardless of this, the fact is that the same water stress treatment at the vegetative stage was carried out.

To inquire into the possible causes of the above-mentioned different responses on photosynthetic performance, both the diffusional and non-diffusional processes were analysed more thoroughly following the observed trends at the different environmental conditions.

4.3.3.1.1 Diffusional limitations

Regarding the diffusional limitations and focussing first on plants grown at ambient temperature (CATA and CETA), *gs* was lower for VR treatments. This fact was mainly due to the reduction of the stomata aperture (Fig. 4.3B) as **Wang et al.** (**2015**) commented, since *SD* (Fig. 4.3C) and *TSS* (Fig. 4.3D) remained unchanged. According to this, **Gallé et al.** (**2009**) in tobacco and **Pérez-Martín et al.** (**2014**) in olive, also observed that plants that had suffered a drought period, despite being re-watered, did not present a full recovery of A_{net} (or needed a very long period to do it), being the *gs* the parameter which took longer to recover. In line with this, **Mendhana et al.** (**2020**) for wheat also did not observe a recovery for the *gs* and A_{net}, being such response cultivar-specific. The reason behind the slow recovery of the *gs* is not well known (**Pérez-Martín et al., 2014**), although it is thought that could be related to both the hydraulic (**Brodribb and Cochard, 2009**) and chemical limitations (**Lovisolo et al., 2008**).

In our case, knowing that water status and stomatal properties of VR treatment plants was not affected and that they recovered the proper water uptake capacity seems that the more probable limitation was the chemical one. In this regard, several authors have stated that the accumulation of the ABA during the stress period —a common plant response to avoid the water loss through a water stress period—, could have triggered the stomata partial aperture reduction even when plants have recovered the water status (**Lovisolo et al., 2008**). For example, **Wang et al.** (**2015**) observed that wheat plants presented higher leaf ABA levels also

at the grain filling stage, correlating it with the lower *gs* values, and therefore lower A_{net} and *yield.*

Nevertheless, it is also worth noting that other authors have also seen that the *gs* was not recovered despite the ABA levels were restored to control values (**Pou et al., 2008**). Therefore, it is still not clear why the *gs* is not completely recovered after re-watering the plants.

Furthermore, at CETE conditions, the *gs* was significantly lower but to a lesser extent than for CATA and CETA (Fig. 3.5B), being developed a trade-off between the stomatal characteristics as proposed by **Harrison et al.** (**2020**). On one hand, a greater *stomata aperture* and *TSS* were observed, whereas the massive reduction on *SD* defined final stomatal conductance behaviour.

On the other hand, at CATE conditions –the one that presented a priming effect–, the *gs* was higher for VR treatment than for RC treatment (Fig. 3.5B), allowing to dissipate part of the heat throughout the higher transpiration (**Liu et al., 2017**). This better stomatal control could be related to a better ABA signalling from the roots to the leaves (**Wang et al., 2015**) for VR treatment plants at these elevated temperature conditions. In agreement with the latter, **Liu et al.** (**2017**) also stated that a well-documented mechanism —which is described by **Crisp et al.** (**2016 and references therein**)—, is the priming-induced ABA signal which "can induce sustained expression of microRNAs and transcription factors and sensitize light-triggered stomatal aperture, giving as a result higher *gs* values" (**Liu et al., 2017**). In addition, apart from the above-mentioned function for ABA, it has also been shown its involvement in protection against the heat-stress induced oxidative damage (**Larkindale and Knight, 2002**) and membrane lipid peroxidation (**Liu et al., 2017**).

Thus, although the ABA participation in stomatal control appeared to be contradictory between elevated temperature and current temperature conditions, its role could be different depending on the development stage (**Liu et al., 2005**) and the other stimuli that plants have to cope with, such as air temperature (**Wang et al., 2015**). In the present work, we did not measure leaf ABA levels, but knowing the importance that ABA could have in the stomatal control among others, it would be of interest to analyse in a near future. In addition, as microRNA metabolism begins to be known of relevance in key physiological processes regulation (**Crisp et al., 2016**), it would be also interesting to inquire about it.

Besides, apart from the *gs*, the other component that defines the diffusional limitation is the *gm* (**Gallé et al., 2009; Pérez-Martín et al., 2014**). Normally, the *gs* and *gm* act coordinated, in such a way that when the *gs* is decreased the *gm* uses to have the same trend, but showing

faster recovery tendency than the *gs* (**Gallé et al., 2009; Pérez-Martín et al., 2014**). In addition, this observed feature of the *gm* has granted its important role in the recovery of plant photosynthetic metabolism. In the present work, at CATA, CETA and CETE conditions, we also observed a reduction in *gm* for the VR, whilst at CATE conditions remained unchanged (Fig. 4.3E).

As in Chapter 3 is discussed and described, the expression of different aquaporin isoforms was analysed for CATA and CETE conditions treatments to elucidate their possible role in plants water status recovery. Thus, due to their possible implication as dual proteins in the CO_2 transport, we additionally analysed their expression pattern and carried out correlation matrixes for plant memory and recovery treatments with the physiological CO_2 transport-related parameters (Table 4.2 and Fig. S2).

In this regard, for VR treatment and compared with RC treatment, lower leaf *HvPIP2;1* expression level was registered for both environmental conditions (Fig. 3.8A), together with a positive correlation with A_{net} and g*m* (Table 4.2). Therefore, our results would match with the ones of **Pérez-Martín et al.** (**2014**), asserting that *PIP2;1* isoform might be a key aquaporin isoform modulating the *gm* under re-watering conditions, in which case its downregulation would be involved in the non-recovery (Fig. 4.3E).

Moreover, it must not be forgotten that at CATA higher *HvPIP1*;3 and at CETE *HvPIP2*;*5* expression values were recorded for VR treatment compared with RC treatment, together with a negative correlation with gas-exchange related data for the former (Table 4.2). This fact, on one hand, could be a residual effect of the water flow function of this isoform at recovery since its higher expression values correlated positively with the higher *K* commented in Chapter 3. On the other hand, another explanation for this trend would be one given by **Yepes-Molina et al. (2020)**, who suggested that when an activation state of an *AQP* is very low, or protein degradation rates are high, to compensate for it plants lead to an overexpression. In our case, besides, we neither rule out that it could be also a mechanism to try to deal with the developed higher CO_2 diffusional resistance.

Besides, for CATE conditions as it was not measured, we cannot have the certainty of how these aquaporin isoforms responded. However, knowing that the general pattern of aquaporin expression response at elevated temperature conditions is to increase, enabling a lower hydraulic resistance to match with the higher transpiration rates (**Martínez-Ballesta et al., 2009 and references therein**). Regarding our results (Fig. 3.5) we hypothesize that a similar trend could have happened.

4.3.3.1.2 Non-diffusional limitations

As concerns the non-diffusional processes, either the photochemical and biochemical were affected at VR treatment under CATA and CETA conditions. In addition, **Pérez-Martín et al.** (**2014**) for olive **and Wang et al.** (**2015**) and **Mendhana et al.** (**2020**) for wheat did register the same trend. In our case, at VR treatment plants the *ETR* (Table 4.3), and the Vc_{max} and J_{max} (Fig. 4.7A-B) were reduced. These facts would indicate that the carboxylation efficiency was lesser, and since the *ETR* was decreased, it is probable that the required amount of NADPH and ATP for RuBP regeneration was not produced adequately (**Flexas and Medrano 2002a; Lawlor 2002; Possell et al., 2010**). It must be said that differently to the latter's, at CETE conditions only a downregulation of Calvin-Benson cycle activity was shown for VR treatment compared with its RC treatment values.

Analysing our data, it is difficult to distinguish if the lower energy and reductant power production and its use in the carboxylation process was a consequence of the lower *Cc* levels at the Rubisco site due to the observed diffusional limitations or, instead, because a maladaptive or damage at the photochemical and biochemical apparatus occurred. Another explanation for our data, which also suggested **Wang et al.** (**2015**) and **Liu et al.** (**2017**) could be that the leaf senescence was accelerated in the VR treatment plants since the photosynthetic pigment contents were decreased (Fig. 4.4B, D, F). The trigger for the acceleration of the senescence could be related to the higher ageing of the VR treatment plants (**Niinemets et al., 2005**), as the VD treatment caused a delay in the life cycle development as will be seen in Chapter 5.

Besides, the photochemical and biochemical parameters at CATE VR treatment kept constant. The same trend was observed by **Wang et al.** (**2015**) and **Liu et al.** (**2017**), corroborating that the beneficial effect of having passed a VD at ET conditions came principally from the higher stomatal conductance, generated by greater stomata aperture.

Therefore, it is clear that VR treatment plants, mainly at CATA and CETA and to a lesser extent at CETE conditions, did not recover from the vegetative drought treatment, whereas at CATE conditions a priming effect was developed allowing them to face better ET impairments**.** The *gs* and *gm,* with the probable involvement of *leaf HvPIP2;1* expression regulation, are postulated as the key parameters in the recovery capacity establishment of the photosynthetic performance.

4.3.3.2 Photosynthetic metabolism of double drought treatments (DD)

As regards the DD treatment plants at CATA and CETE conditions, —the environmental conditions at which the anthesis drought more affected soil and plant water status (see Chapter 3)—, presented lower A_{net} values than the RD treatments ones. However, DD treatment at CATE and CETA conditions, —the environmental conditions where the anthesis drought was milder—, maintained A_{net} statistically equal to RD treatment (Fig. 4.3A). These results are not unusual since **Wang et al.** (**2015**) stated that probably the degree of the effect of the previous drought or another stress could be essential in the subsequent plant memory response, and at the same time, **Martínez-Medina et al.** (**2016**) added that also the extent of the subsequent stress would define plants final response.

Furthermore, the reduction percentage for A_{net} at CETE DD treatment to its RD treatment was much higher compared with the one at CATA conditions. This fact matched with the recorded reduction on *LRWC* and $\Psi w_{md,}$ observed in Chapter 3, being the lack of capacity to increase the *shoot/root ratio* –a possible consequence of ET effects on photoassimilates partitioning to leaves over roots–, a key component. Therefore, it seems that the extent of the anthesis drought effect on plants water relations, and the capacity that plants had to deal with other environmental conditions, defined plants photosynthetic performance when faced with a drought period at anthesis.

Newly, the maladaptive memory effect has not been extensively studied as **Crisp et al.** (**2016**) stated, only **Skiryzc and Inzé** (**2010**) reviewed it commenting that repeated stress may result in a photosynthesis weakness. In the last years, different works for wheat have been carried out analysing priming as a strategy to face drought impairments in photosynthetic stress abiotic alleviation (**Wang et al., 2015; Abid et al., 2016; Liu et al., 2017; Mendhana et al., 2020**), prevailing over maladaptive ones. Then, in the subsequent sections, an attempt to shed some light on this topic has been done, inquiring as for the recovery section, both in the diffusional and non-diffusional limitations.

4.3.3.2.1 <u>Diffusional limitations</u>

Firstly, it should be remembered that under CATE and CETA conditions, the same *gs* was recorded between DD and RD treatments (Fig. 3.5B), where no differences at the stomatal properties were observed for the former, whilst for the latter, a trade-off was shown (Fig. 4.3B-

D), not altering final conductance capacity. Nevertheless, at CATA and CETE conditions lower *gs* values were registered at DD treatments, especially at CETE conditions where a lower stomata aperture was shown.

Moreover, *gm* values were reduced in parallel with *gs* ones, where at CETE DD treatments a surprising reduction was shown. To better understand the obtained results for *gm*, we analyse leaf *AQPs* expression results at both CATA and CETE conditions, although the results were very inconsistent as explained in Chapter 3. Concretely, we observed that leaf *HvPIP2;5* expression values were overexpressed, especially at CATA (Fig. 3.8A). However, a negative correlation was registered with A_{net} (Table 4.2). A possible explanation for this trend would be the same given for the VR treatments, it is, that DD treatment plants overexpressed *HvPIP2;5* to try to deal with the developed higher CO_2 diffusional resistance by higher CO_2 transport protein levels formation.

Thus, it seems clear that the diffusional limitations were involved in the lower A_{net} recorded both at CATA and CETE DD treatments. Besides, an increase in *Cc* values was registered too, specially at CETE, which might evidence that not only the diffusional processes were involved in it (**Sharma et al., 2015**), adding that those DD treatment plants were not able to use the delivered lower CO_2 within the chloroplasts at same rates as RD treatment plants.

4.3.3.2.2 Non-diffusional limitations

In line with the latter fact, we observed that the photochemical and biochemical parameters for DD treatments at CATA and CETE were affected. For the former process, the ETR/A_{net} was increased –being the increase higher in CETE–, suggesting that a significant proportion of electrons were not consumed in the carboxylation reactions and were deviated to alternative processes such as the photorespiration and Mehler reaction, increasing the risk of oxidative damage by ROS (**Salazar-Parra et al., 2012**). In parallel with the later fact, J_{max} values were decreased at both environmental conditions, and Vc_{max} at CETE too, denoting possible damages at RuBP regeneration and Rubisco activity level which would give as a result the observed higher *Cc* values (**Pérez-Martín et al., 2014**). At this point, it should be borne in mind the above-mentioned worst water status of CETE DD plants compared with its RD treatment plants –probably owed to the lack of ability to drive CHs for root growth–, which was reflected in the higher biochemical constraints (**Lawlor and Cornic, 2002**).

Besides, at both environmental conditions, the photosynthetic pigment concentration was decreased at DD treatment plants compared with RD treatment ones (Fig. 4.4B, D, F). This

fact could denote accelerated senescence (**Gallé et al., 2009**). Moreover, one of the possible causes might be the same postulated for the recovery treatments, that is, an ageing effect (**Niinemets et al., 2005**) that could have been triggered by the delay in growth development by VD (Chapter 5).

Definitely, after having suffered a stress period, plants can develop a memory effect or get a resetting. In either case, the recovery period appears to be a key period in which the plant develops one of these qualities. The mechanism behind the priming effect of the plants, which sometimes triggers adaptive effects and in other cases maladaptive effects, is not well known. However, it has been observed that the RNA metabolism could be a key regulatory point for both adaptive and maladaptive responses (**Crips et al., 2016**) and that the ageing and the diffusional processes, being probably hydraulic or chemical signalling like ABA the responsible of it (**Pérez-Martín et al., 2014**) could be important.

This issue, therefore, deserves more attention and should be taken into account by breeders when considering crop management or strategies to face future environmental conditions impairments.

4.4 Conclusions

- Well-watered plants at CETE conditions presented higher net photosynthetic rates along their whole life span due to the fertilization effect of ECO_2. On one hand, an appropriate sink strength allowed to avoid a photosynthetic acclimation except in CETA conditions plants. On the other hand, a thermal displacement of the optimum of the net photosynthetic rates allowed them not to suffer a downregulation of the Rubisco activity.
- Vegetative drought treatment (VD) diminished net photosynthetic rates mainly due to diffusional limitations, while anthesis drought treatment (RD) –because of its worst water status–, reduced net photosynthetic rates to a higher extent both due to diffusional and non-diffusional limitations.
- Plants at CETE conditions when suffered a drought period, especially when it was severe, responded better to drought constraints due to both better stomatal control and lower oxidative damage triggered by ECO_2. Furthermore, an isoform-specific higher leaf *AQPs* expression response at CETE seems to be involved directly or indirectly in the higher net photosynthetic rates.
- Drought extent on photosynthetic metabolism was directly related to plants water status. The effect that the different environmental conditions developed on plants transpiration capacity before water stress was imposed, played an important role.
- Both under current or future conditions, plants that suffered a vegetative drought period and were re-watered (VR) did not recover net photosynthetic rates, mainly because of the triggered diffusional limitations by a maladaptive memory effect. The stimulus triggered by the ET, which increased stomatal conductance due to the need for heat loss at CATE conditions, provoked a priming effect reverting to a higher o lesser extent the diffusional limitations.
- Plants that suffered a double drought period (DD) did not develop a priming effect on net photosynthetic rates. Furthermore, the extent of plants water status that anthesis drought had, defined DD treatment behaviour, triggering maladaptive effects at CATA and CETE conditions mainly owed to diffusional limitations. The highest reduction in net photosynthetic rates was registered at CETE due to the worst water status triggered probably by ET effect on biomass lack partitioning towards the root.

CHAPTER 5

BARLEY'S GROWTH, DEVELOPMENT AND YIELD

5. BARLEY'S GROWTH, DEVELOPMENT AND YIELD

5. Barley's growth, development and yield

5.1 Introduction

Growth is defined as the increase in dry biomass, volume, length or area and, generally, involves division, expansion and differentiation of cells (**Lambers et al., 2008**). On one hand, foliar area increase depends on the cellular expansion that, in part, is conditioned by the synthesis of cell wall new compounds and the generated turgor potential, this latter is the force necessary for the extension and elongation of the primary cell wall (**Taiz et al., 2014**). In addition, turgor potential is influenced by relative water content and osmotic potential (Chapter 3). On the other hand, the increase in biomass implies the deposition of mass in the cells and, taking into account that between 85-95 % of the plant dry biomass consists of carbon-based compounds (**Poorter and Villar, 1997**), the largest part of this mass comes from photosynthesis (Chapter 4).

The different growth and development processes of the plant, which starts with the vegetative phase, followed by the reproductive organ development and ending with the grain-filling stage, dictates final grain yield. This yield is defined by two components. The first one is the number of grains per square meter, which encompasses (i) the spike number per square meter and (ii) the grain number per spike –being this component resultant of the reproductive success–, and the seed-set, defined at reproductive first phases and is genetically marked. The second one is the averaged individual grain weight, which is the product of the grain-filling rate and duration (**Araus et al., 2002**).

As regards the role of the studied environmental agents on crop plants growth, development and final grain yield and yield-trait components, it is worth noting that the observed effects are complex and they vary among species and cultivars, growth stage and the intensity and the way the treatment is applied (**Jagadish et al., 2014**).

It has been shown that elevated temperature (ET) affects plants growth and development along almost all the developmental stages of crops, where, if exceeds the critical threshold, it causes negative effects (**Hatfield et al., 2011**). One of the direct effects is photosynthesis impairment, which leads to fewer photoassimilates production and hence, lower

shoot biomass accumulation during the vegetative phase (**Barnabas et al., 2008**). In addition, the reproductive organ development stage is the most sensitive to ET effects on crops. Besides, the temperature threshold is lower than for vegetative tissue growth, which reduces spike and grain development, owing mainly to inadequate assimilates supply, floral abnormalities and pollen viability diminution. In addition, it also affects either grain filling rates or duration, leading to fewer both grain size and individual grain weight (**Prassad and Staggenborg, 2008**).

Regarding elevated CO_2 (ECO_2), other factor related to climate change, it has been demonstrated that the effects on growth and yield-related traits are not as uniform as with temperature (**Jagadish et al., 2014**). The most common response by plants to ECO_2 is to take advantage of the higher photosynthetic rates and to invest the extra gained assimilates in biomass construction. This is translated into an improvement in vegetative biomass, tiller formation and grain yield and yield-trait components, measured as higher spike and grain density, higher grain weight and higher harvest index. In addition, since ECO_2 decreases stomatal conductance and transpiration, water use efficiency (*WUE*) uses to be improved. However, it has also been registered null increments on plant growth and grain yield and yield-trait components, as was the case observed by **Ingvordsen et al.** (**2015**) and **Mitterbauer et al.** (**2017**). The capacity of partitioning the photoassimilates could play an important role (**Pritchard et al., 1999**) mainly at the reproductive and grain filling stage, together with the lack of plasticity to counteract yield-trait components compensation (**Alemayehu et al., 2014**).

During plants vegetative stage, because drought impairments on transpiration, water status and photosynthetic performance threatens shoot elongation, leaf area and tiller development, which could not permit reaching maximum potential growth (**Gray and Brady, 2016**). Furthermore, if drought occurs at preanthesis, depending on the duration and the intensity could alter flowering time, either by advancing or delaying it (**Foulkes et al., 2007; Cattivelli et al., 2008**). Moreover, at the anthesis, drought can reduce spike and grain formation due to its effects on pollen and grain abortion; at the grain-filling stage, drought uses to shorten its duration and to reduce the filling rate by accelerating senescence processes. Interestingly, to cope with drought or ET negative effects on reproductive growth organ impairment, crops such as barley and wheat can develop an indeterminate growth habit, by developing late tillers, in an attempt to produce more sinks once the drought period has passed (**Prassad and Staggenborg, 2008**).

Finally, it has to be taken into account that the aforementioned environmental factors may happen together, so crops in the near future should grow under combined conditions (**IPCC,**

2013); thus, it becomes mandatory to investigate the interaction between stresses if we want to maintain food security (**FAO, 2018**). In this respect, little information exists about crops (**Jagadish et al., 2014**), being the most recent of them carried out for wheat (**Dias de Oliveira et al., 2013 and 2015a, b; Li et al., 2019; Abdelhakim et al., 2021**). Moreover, in the case of barley, although it ranks fourth as to production refers, which is widely used for livestock feed, human diet and in the brewery (**Kebede et al., 2019**), still little is known.

Usually, when drought and ET are applied together, the negative effects on plant growth and yield are magnified, being the causes the same as the ones for individual stressors action as **Jagadish et al.** (**2014**) picked up in their review for different crops. In this regards, **Mahalingam and Bregitzer** (**2019**) concluded that the interactive effects of ET and drought were more deleterious for barley growth and final yield than the sum of individual acts of each stressor, adding that the ET effects were higher than the ones provoked by drought. However, in recent years, there have been cases where a vegetative drought develops a cross-talk resistance to ET negative effects on wheat, conferring a better heat loss (**Wang et al., 2015; Liu et al., 2017**).

Besides, when ET or drought take place together with ECO_2, the main response by crop plants is to notice an alleviating effect provoked by the latter. In the case of the combined action of ET and ECO_2 wide ranges of responses have been shown depending on the growth stage that it is manifested. Plants take advance of elevated CO_2 by vegetative tissue growth, but not by reproductive organs as ET governs mainly its development (**Jagadish et al., 2014**), although the ET extent (**Dias de Oliveira et al., 2013**) and genetic resources (**Dias de Oliveira et al., 2015a; Ingvordsen et al., 2015; Li et al., 2019**) could modulate final grain yield**.** The interaction effect between drought and ECO_2 has been more largely studied in cereals. The most observed response on final plant production is to develop a reduction in grain yield loss compared to drought applied at current conditions, mainly by the increase in spike fertility and grain number per spike caused by ECO_2 effect (**Jagadish et al., 2014**), but mainly developing late tillers (**Li et al., 2000**).

Lastly, if triple interaction effects on plant growth are analysed, only a few works are found. In this regard, **Aranjuelo et al.** (**2006**) and **Erice et al.** (**2006a**) for alfalfa and **Yu et al.** (**2012**) for tall fescue observed that ECO_2 mitigates drought and ET adverse effects on plant water relations and A_{net}, leading to higher biomass accumulation. More recently, **Li et al.** (**2019**) for wheat have observed that the response could be cultivar-specific too.

Besides, concerning the triple interaction effects on grain yield, mainly the works were carried out by **Dias de Oliveira et al.** (**2013 and 2015a, b**) for wheat at tunnel houses, and the

above-mentioned one by **Li et al.** (**2019**) exist. The former's were able to demonstrate that the reductions in biomass and grain yield caused by a drought applied at anthesis were partially ameliorated by the joint action of ET and ECO_2, as long as temperature increase did not exceed +2 °C from current conditions, being plant growth largely dominated by ECO_2 effect. The latter's, observed higher yields when plants face drought and ET at ECO_2, ascribing it to changes in grain number per spike and thousand-grain weight, being cultivar-dependent.

Apart from taking into account the diverse effects of drought, it is also important to study the effects of re-watering since it is crucial for plants future prevalence. The recovery period is presented as the key phase for developing stress memory, which would also dictate the plant's ability to recover from vegetative drought effects (**Crisp et al., 2016**). The recovery implies developing a new developmental stage, where if plants do not suffer another stress period, could lead to suffering the allocation costs (**Martínez-Medina et al., 2016**). On the other hand, it has also been shown that a mild drought period at the vegetative stage could prime plants against subsequent drought stress conferring them a stress memory. For both processes, different works have been carried out for wheat analysing final plant growth and grain yield (**Wang et al., 2015; Abid et al., 2016; Liu et al., 2017; Mendanha et al., 2020**). However, the recorded results by those authors were heterogeneous depending on (1) the extent of the first and/or subsequent stress, (2) the interaction with other environmental agents (temperature) and (3) the cultivar-specific response.

Therefore, it is evident that a lack of information exists as concerns plants biomass, growth and yield response to the combined future climate environmental conditions, where especially crops such as barley, would be of special relevance to fulfil cereal production demand and global food security (**Tester and Langride, 2010; Kebede et al., 2019**).

Given this background, the main objective of this Chapter has been to analyse barley growth development, biomass allocation and final grain yield and yield-trait components under future environmental conditions both at well-watered and drought regimen. This general objective has been divided into three specific objectives:

1. To analyse if the response of well-watered barley plants growth along different key developmental stages and final yield and its components is dependent on environmental conditions.
    - ✓ We hypothesize that ECO_2 will promote vegetative growth (by improving photosynthetic rates), but ET will govern reproductive development, so under combined conditions, the final yield will be determined by the capacity of barley to take advance of the elevated CO_2 at vegetative growth.

2. To study the effects caused by drought stress applied at vegetative or anthesis on barley plants growth along different strategic developmental stages and final yield and its components under CATA and CETE conditions.
    - ✓ We hypothesize that plants at anthesis would suffer more drastic drought effects than at the vegetative stage due to its higher water consumption and photosynthesis impairment, independently of the environmental conditions. Besides, plants at future environmental conditions would obtain greater biomass and yield due to the better water status and photosynthetic performance.

3. To analyse growth parameters of plants that have suffered a vegetative drought period, determining the recovery capacity and priming capacity to deal with a subsequent drought at anthesis. For both cases, the influence of the different environmental conditions on such responses was studied.

    Our hypotheses for this specific objective are:
    - ✓ Barley plants will not recover after having passed a vegetative drought despite having been re-watered for a long period (VR), mainly due to lower leaf final size formation and reduced photosynthetic rates provoked by diffusional limitations. ET might counteract diffusional limitations due to heat loss need.
    - ✓ Plants that had suffered a double drought period (DD) will not present greater biomass and yields compared with RD due to the lack of priming effect at the photosynthesis metabolism level. However, in the case of CATA, but mainly CETE conditions, where a maladaptive effect was developed in photosynthetic performance (Chapter 4), lower biomass and grain yield will be determined in DD treatments.

5.2 Results

5.2.1 Barley growth: from sowing until anthesis

From now on, unless the opposite is stated, for each growth stage and well-watered plants, all the comparisons will have as a reference CATA control conditions, whereas at drought regimen treatments, the comparisons will be made taking the control of each environmental condition as a reference. In addition, when the biomass of individual organs is analysed, only the organs in which a statistically significant alteration have been observed will be described.

5.2.1.1 Well-watered treatments

In Fig. 5.1, plant total dry weight (*TDW*) at early-vegetative (Fig. 5.1A), vegetative (Fig. 5.1B) and anthesis (Fig. 5.1C) are shown. Under the well-watered regimen, CATE conditions did not modify *TDW* at the early-vegetative stage, whereas the ET reduced it along the rest of the growth stages. Under CETA conditions, only a reduction by 9 % was recorded at anthesis, whilst CETE conditions increased it by 20 % at early-vegetative and vegetative stages, and by 8 % at anthesis.

Analysing organ-by-organ (see also Figs. S3-S6), CATE conditions increased *leaf DW* at the early-vegetative stage, but reduced it at anthesis, whereas it decreased *stem DW* both at vegetative and anthesis. It reduced the *root DW* along all the growth periods. Furthermore, the *spike DW* was the most affected organ, being reduced by 70 %. As regards CETA conditions, ECO_2 decreased *leaf DW* by 14 % only at anthesis; it did not alter *stem DW* at any growth stage; the *root DW* increased, decreased and remained unchanged at early-vegetative, vegetative and anthesis, respectively, whereas *spike DW* was reduced by 45 %. Lastly, CETE conditions increased *leaf DW* by 30 %, both at early-vegetative and vegetative stages, it did the same for *stem DW* increasing by 16 % and 11 % at vegetative and anthesis, respectively, whilst it increased *root DW* along all the growth stages. However, it did not alter *spike DW*.

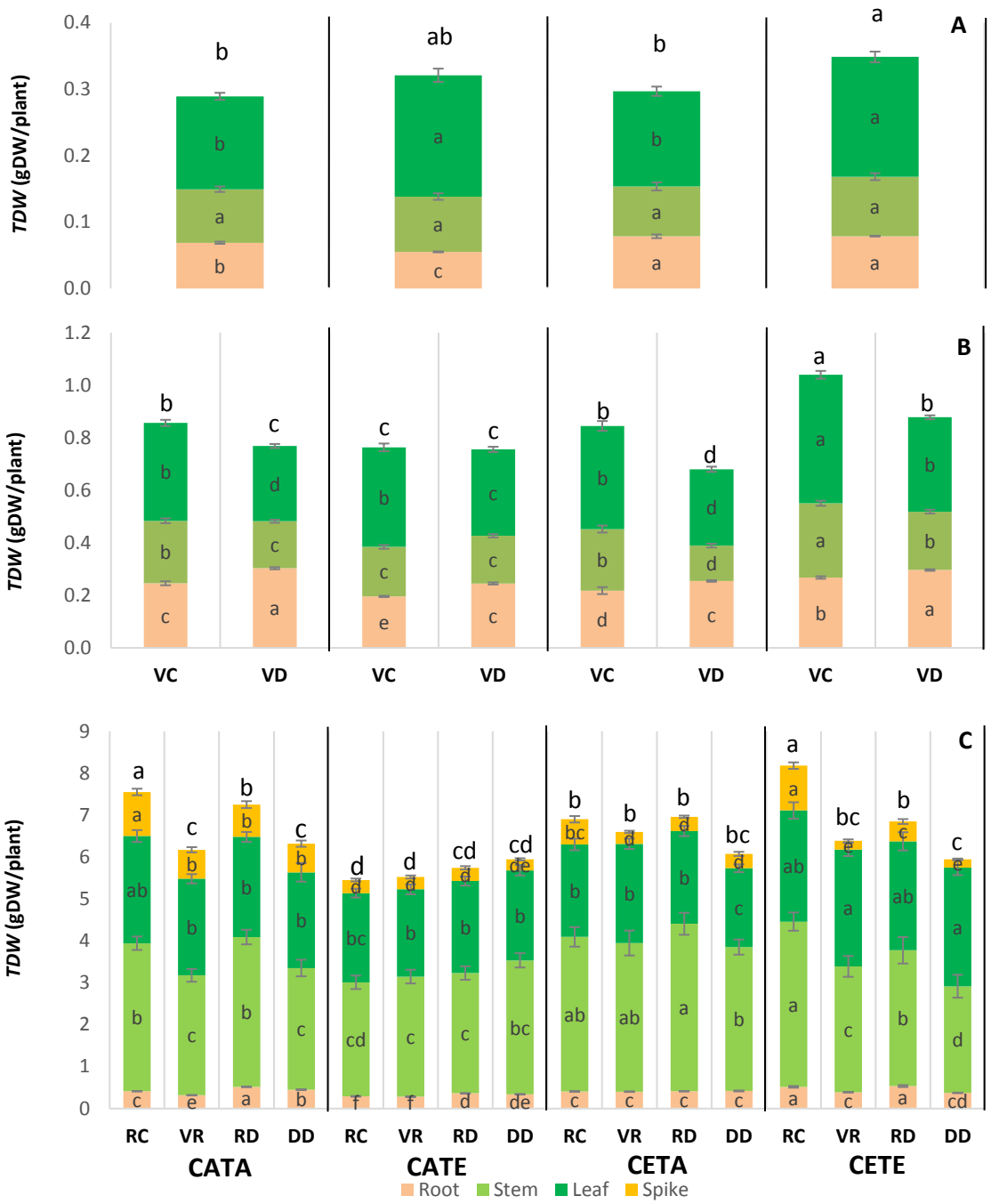

Fig. 5.1. Total dry weight (*TDW*) and organ by organ biomass at the early-vegetative (A), vegetative (B) and anthesis (C) stages. Growth environmental conditions, water regimen treatments and statistical analysis are as described in Fig. 3.1. Legend for organs is depicted in the figure.

Another important component of plant biomass is total leaf area (*TLA*). It was the same as green leaf area (*GLA*) in early-vegetative (Fig. 5.2A) and vegetative (Fig. 5.2B) stages, whereas, at anthesis, *GLA* (Fig. 3.4C-D) was always lower than *TLA* (Fig. 5.2C). For the well-watered regimen, CATE increased *TLA* at early vegetative, remained unchanged at vegetative, and it reduced at anthesis. The effect of CETA on *TLA* was variable across the whole life span of plants. In this way, at early-vegetative and anthesis a reduction of 19 % was shown, whereas it was not

modified at the vegetative stage. In the case of *TLA* at CETE conditions, results neither follow a clear pattern, since at early-vegetative and anthesis no differences were shown, but an increase by 21 % was recorded at the vegetative stage.

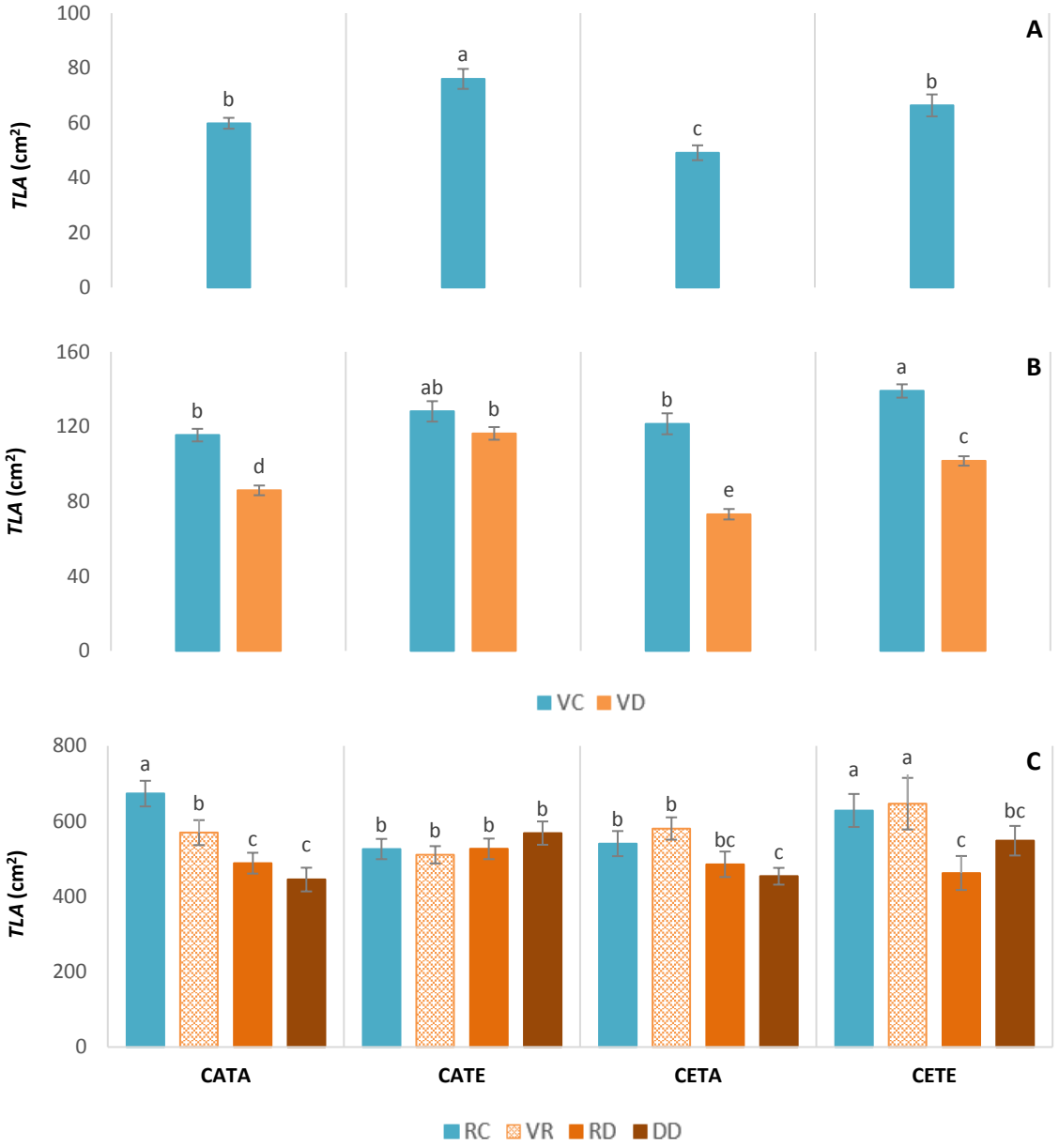

Fig. 5.2. Total leaf area (*TLA*) at the early-vegetative (A), vegetative (B) and anthesis (C) stages. Growth environmental conditions, water regimen treatments and statistical analysis are as described in Fig. 3.1.

In Table 5.1. different parameters related to growth at anthesis are shown. For control treatments, CATE did not alter the formed tiller number at anthesis *(ATN)*, whereas the spike number (*ASN*) was reduced by 60 %. However, the effect of the ET in the *phenology* was significant, since it increased in 31 days the time needed to reach anthesis. As concerns CETA conditions, ECO_2 increased *ATN* by 15 %. The effect on *ASN* was the opposite, reducing it by

25 %. At the same time, plant *phenology* remained unchanged by elevated ECO_2. Besides, the trend observed under CETE conditions on *ATN* was the same as for CETA conditions, whereas *ASN* kept constant. In addition, well-watered CETE plants needed 10 days more to reach anthesis than well-watered CATA ones.

5.2.1.2 Drought treatments

Regarding drought effects under the different temperature and CO_2 conditions, compared to each respective control treatment, it is shown that under CATA conditions, VD and RD treatments reduced *TDW* by 10 %. In the case of CATE conditions, drought treatment did not alter *TDW* in any growth stage. At CETA conditions, the reduction caused by VD treatment on *TDW* was around 20 %, whereas RD treatment did not modify it. However, different to the rest, under CETE environmental conditions, both VD and RD treatments reduced *TDW* by 15 %, although at the vegetative stage the recorded values were higher than the ones for CATA conditions.

In the same way, at CATA conditions VD treatment reduced *leaf and stem DW* by 20 % and 25 %, respectively, whereas both VD and RD treatments increased *root DW* by about 20-25 %. Furthermore, RD treatment decreased *spike DW* by 30 %. As concerns CATE conditions, VD treatment reduced by 13 % *leaf DW*, whilst either VD or RD treatments increased *root DW* by 25 %. Besides, at CETA conditions VD treatment reduced both *leaf and stem DW* by 25 % and 43 %, respectively, but increased *root DW* by 13 %. The RD treatment reduced *spike DW* by 43 %. In the case of CETE conditions, VD treatment reduced *leaf and stem DW* by about 25 %. However, the obtained DW values were higher than the ones for CATA conditions. In addition, VD treatment increased by 11 % *root DW*, whereas RD treatment decreased either *stem DW* or *spike DW* by 16 % and 54 %, respectively.

Drought treatments reduced *TLA* irrespective of the environmental conditions except for CATE conditions. At CATA, VD and RD treatments reduced *TLA* by 27 %. Nevertheless, the case for CETA conditions was different since VD treatment presented 40 % lower *TLA* values, but remained unchanged for RD treatment. Lastly, at CETE conditions, both VD and RD caused a decrease in TLA in the same order as for CATA. Absolute values for VD treatment were higher than at CATA.

Table 5.1. Effects of environmental conditions and water regimes on the tiller (*ATN*) and spike (*ASN*) number at anthesis stage and the days to reach the onset of anthesis after showing (*phenology*). Growth conditions, treatments and statistical analysis are as described in Fig. 3.1. MAT denotes the physiological maturity stage. The rest of the abbreviations are explained in Table 3.1.

GS	EC	WR	*ATN*	*ASN*	*Phenology-Anthesis*
MAT	**CATA**	**RC**	5.10 ± 0.25 b	4.19 ± 0.26 a	72.5 ± 1.55 d
		VR	4.23 ± 0.23 cd	2.90 ± 0.21 bc	84.5 ± 0.73 c
		RD	4.53 ± 0.19 c	3.13 ± 0.24 b	72.5 ± 1.55 d
		DD	3.94 ± 0.23 d	2.40 ± 0.29 c	84.5 ± 0.73 c
	CATE	**RC**	4.86 ± 0.36 bc	1.63 ± 0.13 d	105.1 ± 1.49 a
		VR	4.88 ± 0.34 bc	1.91 ± 0.24 cd	107.4 ± 1.08 a
		RD	5.00 ± 0.29 bc	1.59 ± 0.15 d	105.1 ± 1.49 a
		DD	5.09 ± 0.26 bc	1.42 ± 0.15 d	107.4 ± 1.08 a
	CETA	**RC**	6.52 ± 0.49 a	2.83 ± 0.26 bc	70.3 ± 1.87d
		VR	4.63 ± 0.34 cd	1.25 ± 0.21 d	93.7 ± 4.62 b
		RD	5.87 ± 0.48 ab	2.20 ± 0.18 c	70.3 ± 1.87 d
		DD	4.58 ± 0.25 c	1.50 ± 0.12 d	93.7 ± 4.62 b
	CETE	**RC**	6.36 ± 0.36 a	4.03 ± 0.32 a	82.7 ± 5.28 bc
		VR	4.13 ± 0.39 cd	0.82 ± 0.15 e	107.0 ± 2.61 a
		RD	5.25 ± 0.47 b	1.71 ± 0.33 d	82.7 ± 5.28 bc
		DD	3.97 ± 0.37 d	0.75 ± 0.13 e	107.0 ± 2.61a

Regarding RD treatment effects on the *ATN* and *ASN*, some differences were observed compared with RC treatments along with the different environmental conditions (Table 5.1). Thus, at CATA conditions, a reduction in *ATN* by 11 % was shown. For his part, *ASN* at such a growth stage was reduced by 25 %. Nevertheless, RD treatment under CATE conditions did not produce differences in either of these parameters. Besides, when the effect of RD treatment was analysed under CETA conditions, no differences were registered for *ATN*, whereas 30 % lower *ASN* was shown. Finally, RD treatment at CETE conditions decreased both *ATN and ASN*

by 16 % and 30 %, respectively, being significantly higher than for CATA RD for the former, but lower for the latter.

5.2.1.3 Plant memory

Ultimately, the effects of the vegetative drought on plant recovery (VR) and priming (DD) *TDW* biomass at anthesis were analysed. On one hand, as concerns VR treatment, 19 % lower values compared to their respective RC treatments were observed, but only under CATA and CETE conditions, presenting the same values at CATE and CETA. This lack of recovery was owed to the lower *spike, stem and root DW* values for both environmental conditions. On the other hand, when *TDW* values were compared between DD and RD treatments, a statistically significant reduction of about 13 % was recorded at CATA and CETE conditions. In the case of the former, it was owed to lower *stem DW* values, whilst for the second was due to both less *stem and spike DW*.

Otherwise, no differences were registered for *TLA* between VR and RC, irrespective of the environmental conditions, except for CATA (-15 %). Besides, regardless of the environmental conditions, DD treatment kept equal *TLA* values to the ones of RD treatment.

Lastly, those plants that had suffered a vegetative drought, to a higher o lesser extent, presented a delay in growth development to reach anthesis except, in CATE (Table 5.1). Under CATA conditions, a delay of 10 days was recorded, under CETA about 20 days and under CETE by 30 days. At the same time, except for CATE conditions, the VR and DD treatments presented lower *ATN* and *ASN* compared with RC and RD treatments, respectively.

5.2.2 Barley growth: from anthesis until physiological maturity

5.2.2.1 Well-watered treatments

Data of aboveground (*ADW*), *spike*, *stem* and *leaf DW* at final physiological maturity (GS99) are presented in Fig. 5.3 and Fig. S3-S6. For the well-watered regimen (RC), CATE reduced *ADW* by 20 %, whereas CETA and CETE increased it by 18 % and 22%, respectively.

Subsequently, and before the analysis of organ biomass and development behaviour is carried out, to better understand the final biomass accumulation is mandatory to inquire in the time to reach final physiological maturity from the end of anthesis *(phenology-maturity*; Table

5.2). CATE conditions increased it in 10 days, whilst CETA conditions did not alter it, and CETE conditions reduced it in 10 days.

Regarding organ-by-organ biomass and development, it was observed that the lower *ADW* biomass at CATE conditions was owed to the massive decrease in *spike DW*, concretely by 80 % (Fig. 5.3). Furthermore, not only decreased the latter, but it also diminished spike formation since it was reduced by 53 % the final spike number (*FSN)* at maturity (Table 5.2). Otherwise, at CETA conditions the biomass of all organs was increased about 20 %, together with the final tiller number (*FTN)* and *FSN* by 66 % and 75 %, respectively. Lastly, at CETE conditions, on one hand, the 36 % and 24 % higher *leaf and stem DW* values were responsible for the *ADW* increment (22 %), and on the other hand, *FTN and FSN* also increased by 75 % (Table 5.2).

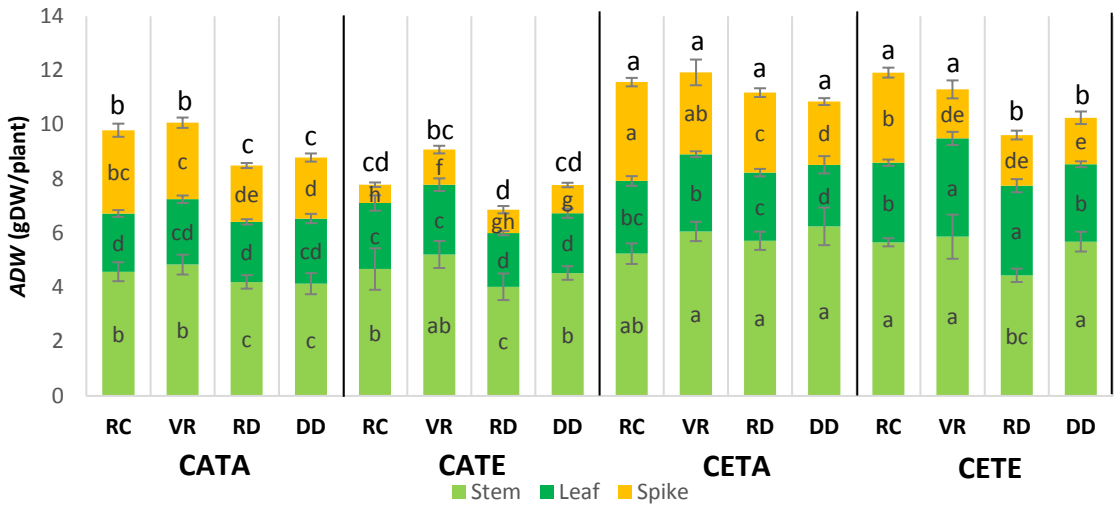

Fig. 5.3. Aboveground dry weight (*ADW*) and organ by organ biomass at final physiological maturity. Growth environmental conditions, water regimen treatments and statistical analysis are as described in Fig. 3.1.

Additionally, in Fig. 5.4 the *biomass partitioning* throughout the vegetative organs (leaf and stem), and reproductive ones (spikes) between anthesis and physiological maturity is depicted. Analysing the control treatments (RC), we observed that at CATA conditions the 80 % of the new biomass was driven to reproductive organ construction or grain filling. However, at CATE conditions, the opposite pattern was observed since 85 % of gained biomass was driven to vegetative organs. For his part, the control treatment at CETA conditions reduced the biomass driven to spikes formation by 20 %, but the total gained biomass was increased by 30 %. Besides, at CETE conditions, a near equal partitioning was shown between the vegetative and reproductive organs. In addition, it is also worth noting that the absolute gained biomass for

control treatments grown at CETE was much higher than that for CATA conditions, presenting the same pattern as the other elevated CO_2 condition (CETA).

5.2.2.2 Drought treatments

As regards anthesis drought (RD) effects on plant phenology, it is of highlight that irrespective of the environmental conditions, this treatment did not alter the time needed to fulfil the life cycle of plants compared with RC treatments (phenology-whole; Table 5.2). Nevertheless, RD triggered different effects on *ADW* values depending on the environmental conditions. At CATE and CETA it kept equal, whereas at CATA and CETE conditions was diminished by 13 % and 20 %, respectively. However, it is worth noting that although for CETE conditions a higher reduction was shown for RD treatment on *ADW* biomass compared with CATA conditions, its absolute value was higher.

Table 5.2. Effects of environmental conditions and water regimens on final tiller number (*FTN*) and spike number (*FSN*) per plant, and days to reach maturity from anthesis (*phenology-maturity*) and at whole life span from sowing (*phenology-whole*) at physiological maturity. Growth environmental conditions, water regimen treatments and statistical analysis are as described in Fig. 3.1. Abbreviations are explained in Table 5.1.

GS	EC	WR	*FTN*	*FSN*	*Phenology-maturity*	*Phenology-whole*
MAT	**CATA**	**RC**	6.64 ± 0.5 e	5.78 ± 0.3 c	63.5 ± 4.0 c	136.0 ± 8.6 d
		VR	7.20 ± 0.5 de	6.12 ± 0.4 c	70.5 ± 3.0 bc	155.2 ± 6.6 c
		RD	7.50 ± 0.3 d	4.94 ± 0.4 d	60.5 ± 5.0 c	133.0 ± 11.0 d
		DD	7.20 ± 0.4 de	5.80 ± 0.3 c	72.5 ± 3.0 b	157.0 ± 6.5 c
	CATE	**RC**	7.89 ± 0.6 d	2.69 ± 0.3 f	74.2 ± 1.5 b	179.1 ± 3.6 b
		VR	9.01 ± 0.4 c	4.82 ± 0.3 d	79.6 ± 1.1 a	186.4 ± 2.6 a
		RD	8.33 ± 0.6 cd	4.00 ± 0.2 e	79.0 ± 3.2 a	184.1 ± 7.5 a
		DD	6.29 ± 0.2 e	4.17 ± 0.3 de	76.1 ± 4.1 ab	183.4 ± 9.9 ab
	CETA	**RC**	11.00 ± 0.9 a	10.10 ± 0.6 a	65.4 ± 2.7 c	135.7 ± 8.8 d
		VR	11.67 ± 0.7 a	9.67 ± 0.3 a	70.3 ± 1.4 bc	164.2 ± 10.6 bc
		RD	12.55 ± 1.0 a	10.18 ± 0.9 a	67.7 ± 2.1 c	138.3 ± 3.3 d
		DD	12.00 ± 1.0 a	7.00 ± 1.0 bc	71.3 ± 1.0 b	165.9 ± 5.9 c
	CETE	**RC**	11.62 ± 0.5 a	10.07 ± 0.5 a	53.0 ± 2.0 d	135.7 ± 4.6 d
		VR	11.38 ± 1.3 a	7.75 ± 0.8 b	60.3 ± 3.5 c	167.3 ± 8.8 b
		RD	11.10 ± 0.5 a	7.28 ± 0.7 b	51.0 ± 4.0 d	133.7 ± 9.4 d
		DD	9.83 ± 0.2 b	4.67 ± 0.3 d	51.3 ± 5.3 d	158.3 ± 14.6 cd

In addition, the lower *ADW* values at CATA conditions came from the 30 % lower *spike DW* values. However, the *FTN* was increased, whereas *FSN* was decreased. In the case of CETA conditions, a trade-off between higher *shoot DW* biomass and lower *spike DW* was developed. It is worth noting that the *FTN* and *FSN* of CETA RD plants were greater than for the same treatment at CATA conditions. Besides, the observed reduction at CETE conditions was due to the 20 % and 45 % lower *stem and spike DW* biomass, respectively, whilst the *FSN* was also reduced by 28 %. Otherwise, as it has been previously commented for *ADW* biomass, the *FSN* of RD treatment at CETE conditions was higher than for the same treatment at CATA conditions (7.28 *vs.* 4.94).

If *biomass partitioning* is analysed (Fig. 5.4), under all the environmental conditions except in CATE —where the biomass driven to spikes was increased by 20 % compared with RC treatment—, RD treatments reduced the *DW biomass* destined to *spike*s. Nonetheless, as it has been commented for control treatments, the absolute biomass gained at CETA and CETE conditions was much higher (by 40-50 %) compared with CATA.

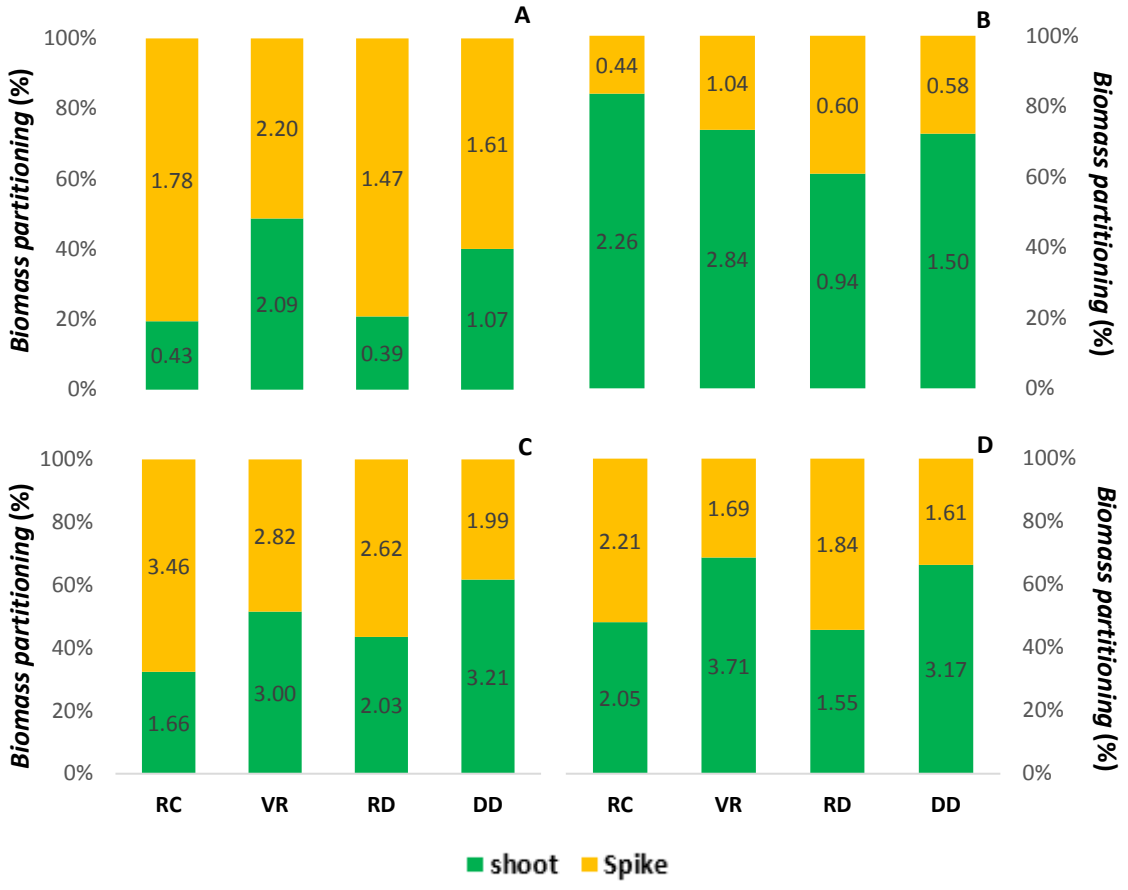

Fig. 5.4. *Biomass partitioning* between anthesis and physiological maturity at CATA (A), CATE (B), CETA (C) and CETE (D) conditions. Numbers inside the bars mean gained biomass in grams (g). Growth environmental conditions and water regimen treatments are as described in Fig. 3.1.

5.2.2.3 Plant memory

In general, it is worth noting that the treatments that had suffered a vegetative drought (VR and DD), needed more time to fulfil the reproductive growth either the full cycle (Table 5.2), compared to the ones that were well-irrigated at the vegetative stage (RC and RD). However, no differences were registered between both VR and RC nor DD and RD treatments for *ADW* biomass (Fig. 5.3). Besides, it is remarkable that at CATE conditions, the VR treatment presented higher *spike DW* values than RC, together with higher *FTN* and *FSN*. In the case of CETA and CETE conditions, the VR treatments recorded higher *leaf* and *stem DW* values but lower *spike DW* values than their respective controls (RC), developing, a trade-off between organs to give similar final biomass. In addition, at CETA conditions DD treatment presented statistically significant lower *FSN* compared with RD treatment, whilst at CETE both *FTN* and *FSN* were lower at VR and DD compared with RC and RD treatments, respectively.

In line with this, interesting results were recorded for treatments that had undergone a vegetative drought when *biomass partitioning* was analysed (Fig. 5.4). Concretely, for all the environmental conditions except for CATE, both VR and DD treatments showed a 30 % higher gained absolute biomass compared with RC and RD treatments. Furthermore, they also presented higher biomass partitioning to the vegetative organs.

5.2.3 Barley yield and yield-trait components at physiological maturity

As concerns the grain *yield (*Fig. 5.5*)*, it is defined by different yield-trait components: *FSN*, *TGNS*, grain filled percentage (*GF)* and individual grain weight (*IGW)* (Table 5.2 and Table 5.3). In addition, another interesting parameter that gives information related to yield is the water use efficiency for grain production (*WUEg*), which relates to how much water has been used to produce 1 g of grain yield. In this respect, regardless of the environmental conditions or water regimen, it is worth noting that the obtained values were defined by grain *yield*. This fact would come to say that the registered accumulated transpiration values along the whole life span of plants (Fig. S7) did not vary (or very little) in comparison with the effects triggered on grain *yield*.

5.2.3.1 Well-watered treatments

The well-watered plants grown under current environmental conditions (CATA) recorded a *grain yield* of 1.93 g/plant. CATE, massively reduced grain *yield* (95 %) which was owed to the lesser *FSN* (Table 5.2) and *TGNS*, *GF* and *IGW* (Table 5.3). When plants grew at CETA, the *yield* was not modified due to a trade-off between yield components. Firstly, *IGW* was not modified. Secondly, although *TGNS* was equal to CATA conditions, the *FSN* was greatly increased, giving as a result higher total grain formed per plant. However, a reduction in *GF* was developed leading to the aforementioned trade-off. Moreover, at CETE a reduction in grain *yield* compared with CATA conditions was also observed (14 %). However, the causes of this reduction in *yield* were different from those observed in CATE conditions. Thus, the *IGW*, *TGNS* and *GF* at CETE conditions were statistically reduced by 9 %, 28 % and 29 %, respectively. Nevertheless, as it occurred under CETA conditions, *TGNS* was increased —recording the highest values at these environmental conditions—, which allowed the partial relief of the observed reductions.

In addition, despite the lower *yield* performance at CETE compared with CATA conditions, it is worth noting that its reduction was lesser than for CATE. The higher *yield* was owed to a better performance of all the yield components, —except *IGW* that was maintained equal—, highlighting especially the higher *TGNS*.

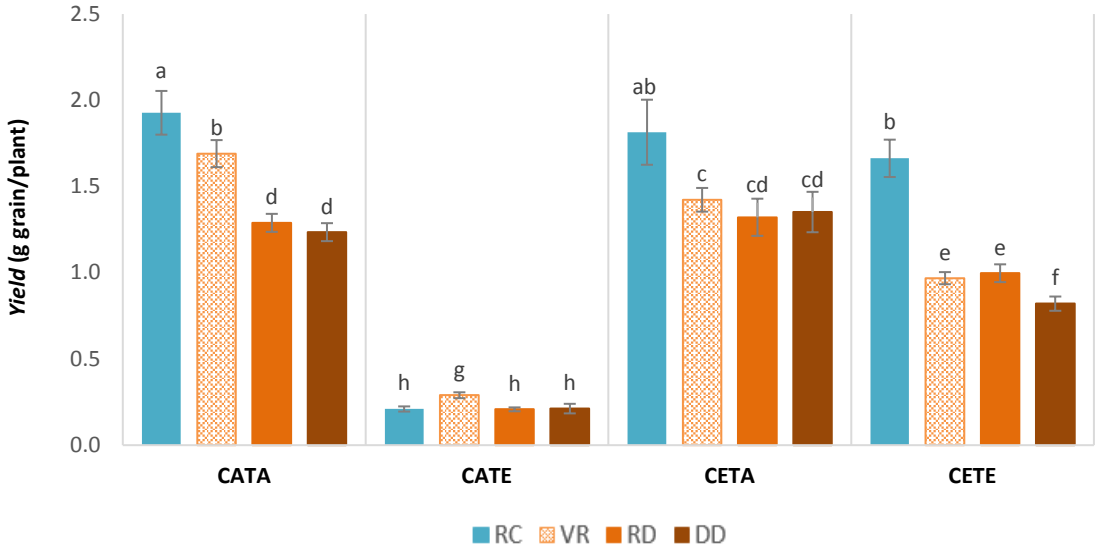

Fig. 5.5. Grain yield (*yield*) at final physiological maturity (GS99). Growth environmental conditions, water regimen treatments and statistical analysis are as described in Fig. 3.1.

5.2.3.2 Drought treatments

Drought, in general, reduced grain *yield* except for CATE conditions. However, different trends were observed about the yield components response that depended on the environmental conditions. Thus, at CATA, RD treatment reduced by 33 % grain *yield*. Such reduction was mainly due to the reduced *FSN* and *TGNS*. As regards CETA conditions, the observed 29 % lower *yield* value was principally attributed to the 12 % lesser *TGNS*. Moreover, at CETE conditions a 40 % less grain *yield* was recorded for RD treatment, but in this case, apart from the lower *FSN* and *TGNS*, a reduction in *IGW* was also recorded. Besides, the absence of reduction at CATE conditions was due to a trade-off between the lower *GF* and the higher *FSN*.

5.2.3.3 Plant memory

The analysis of the VR treatments on grain *yield* showed a reduction of this parameter compared with RC treatments under all environmental conditions, except in CATE in which it was increased. Therefore, although plants were well-watered along the rest of the life span, they did not achieve similar grain *yield* values to those of their respective controls. More specifically, VR treatments presented values of grain *yield* that were 12 %, 20 % and 42 % lower than their RC treatments, at CATA, CETA and CETE conditions, respectively. On the contrary, this parameter was increased by 38 % at CATE conditions after re-watering. At CATA, the non-recovery was owed to a reduction on *GF*, at CETA to a decrease on *TGNS*, whereas at CETE conditions it was attributed to both lower *FSN* and *GF*. Conversely, the increase at CATE conditions was reached due to the higher *FSN* and *TGNS*, alleviating the observed lower *GF* at RC treatment caused by ET.

On the other hand, it is of highlight that except for CETE conditions, the grain *yield* of DD treatments was maintained to RD treatments. The observed reduction in CETE was a trade-off between the lower *FSN* and the higher *TGNS,* having more importance the first trait in the final result.

Table 5.3. Effects of environmental conditions and water regimen on yield components at physiological maturity (GS99) stage. Specifically; total grain number per spike (*TGNS,* grain number), grain-filled percentage (*GF,* %), individual grain weight (*IGW*, mg), and grain water use efficiency (*WUEg*, g grain/kg H_2O). Growth environmental conditions, water regimen treatments and statistical analysis are as described in Fig. 3.1. Abbreviations are explained in Table 5.1.

GS	EC	WR	*TGNS*	*GF*	*IGW*	*WUEg*
MAT	**CATA**	**RC**	19.3 ± 0.77 a	35.8 ± 2.30 a	53.2 ± 1.05 b	0.71 ± 0.05 ab
		VR	19.3 ± 0.47 a	28.3 ± 1.35 bc	55.5 ± 0.68 a	0.67 ± 0.04 b
		RD	16.8 ± 0.71 c	36.3 ± 2.33 ab	50.3 ± 1.40 bc	0.49 ± 0.02 d
		DD	18.2 ± 0.44 b	31.8 ± 2.15 b	43.2 ± 0.92 e	0.51 ± 0.02 d
	CATE	**RC**	8.2 ± 0.94 f	21.1 ± 1.84 d	46.6 ± 1.20 d	0.06 ± 0.00 g
		VR	11.2 ± 0.33 e	12.2 ± 0.79 e	48.1 ± 0.74 c	0.08 ± 0.00 g
		RD	7.9 ± 0.49 f	16.5 ± 1.08 d	45.3 ± 1.85 e	0.06 ± 0.00 g
		DD	12.0 ± 0.42 e	12.6 ± 1.96 e	52.0 ± 1.07 b	0.07 ± 0.01 g
	CETA	**RC**	17.6 ± 1.20 ab	20.1 ± 3.17 d	52.9 ± 2.50 ab	0.76 ± 0.02 a
		VR	13.4 ± 0.20 d	24.3 ± 0.05 c	49.2 ± 1.20 c	0.57 ± 0.01 c
		RD	15.5 ± 0.70 c	16.5 ± 0.92 d	52.6 ± 1.42 b	0.50 ± 0.02 d
		DD	15.1 ± 1.10 cd	20.9 ± 3.67 cd	53.3 ± 1.82 ab	0.51 ± 0.05 cd
	CETE	**RC**	13.9 ± 0.45 d	25.4 ± 1.22 c	48.6 ± 1.79 cd	0.57 ± 0.04 c
		VR	14.8 ± 0.55 cd	17.9 ± 1.23 d	47.4 ± 2.49 cd	0.29 ± 0.01 f
		RD	11.0 ± 0.45 e	29.0 ± 1.85 bc	44.4 ± 1.41 de	0.41 ± 0.03 e
		DD	16.2 ± 0.44 c	25.6 ± 2.06 c	45.2 ± 1.68 de	0.27 ± 0.01 f

5.3 Discussion

5.3.1 Growth, development and yield of well-watered treatments

5.3.1.1 CATE conditions effects

5.3.1.1.1 From sowing until anthesis

The negative effects of the ET on plant development and growth were higher as the exposure time was increased (Fig. 5.1A-C), and this was principally related to the decline in photosynthetic rates (Chapter 4). At the early-vegetative growth stage, it seems that ET triggered an increase in leaf development either of biomass accumulation (Fig. 5.1A) or of leaf area (Fig. 5.2A), whereas as the life-cycle continued, it had a negative influence on biomass and leaf and stem growth (Fig. 5.1B-C and Fig. 5.2B-C).

In addition, CATE conditions also decreased *root DW biomass*, but in this case, the decrease was given along the whole life span of the plants (Fig. 5.1A-C). First of all, it must be borne in mind that soil temperature is closely related to air temperature (**Zheng et al., 1993**). Consequently, root development may be affected directly by elevated soil temperatures, or instead, by the alteration in the resource acquisition by the shoot. The combination of both could have also caused the reduction of root biomass (**Gray and Brady, 2016**). With our results, we can not distinguish which was the principal cause, although we suggest that the registered lower A_{net} rates (Figs. 4.1A, 4.2A, 4.3A) had a significant influence on the observed trend.

Moreover, apart from the impairment on biomass accumulation, CATE conditions massively delayed growth development, since plant´s needed 31 more days than CATA to reach the onset of the anthesis (Table 5.1). In this respect, we observed that when plants reached the jointing or booting stage (GS41), their development was stopped. This stage coincides with the phase of growth shift from early-reproductive to late-reproductive, a key period for spikes and spikelets (floret enclosed by two glumes; potentially grain) formation (**Alqudah and Schnurbusch, 2017 and references therein**). In this regard, and contrary to what was observed by us, it has been reported that numerous crop species use to present the opposite trend as temperature raises; that is, they progress more rapidly from vegetative to reproductive development as the temperature rises. Nevertheless, this fact is associated with the species-specific temperature optimum, which once is overcome, growth and development is slowed

down and could also be stopped as **Hatfield et al.** (**2011**) commented in their review, matching with the pattern that we observed (Table 5.1). Furthermore, **Jacott and Boden** (**2020**) for wheat and barley concluded that the maximum temperature for floral transition is 20-25 °C and for spike formation is 20 °C. These authors stated that temperatures that overcome those threshold values trigger unrecoverable negative effects on reproductive organ development, which could be the case of our temperature treatment.

Moreover, it should also be considered the high night temperature (HNT) effects that could have on plants growth, and not only the increment in diurnal temperature. In this regard, **Prasad et al.** (**2008**) in a work carried out for wheat in controlled environmental conditions, concluded that HNT above 20 °C –the same as in our case–, hastened net photosynthesis, and therefore, plant growth and grain formation. To date, the molecular processes that affect HNT are unknown, although it is believed that the increase in night respiration (*Rn*) could participate in it by reducing the photoassimilates available for plant growth and grain filling, jeopardizing photosynthesis dependent processes (**Jacott and Boden, 2020 and references therein**).

Besides, the registered higher constrains in spike formation at anthesis (Fig. 5.1C and Table 5.1) would be explained by the lower optimum threshold temperature on reproductive organ development (**Alemayehu et al., 2014; Jacott and Boden, 2020**) and probably the above-mentioned effects of HNT. To deal with the hastened effects –mainly on reproductive organ development–, plants should drive most of the photoassimilates to respiratory maintenance resources (**Slattery and Ort, 2019**), which could be supported by the increase in *Rn* and Rn/A_{net} (Table 4.1). Therefore, although the vegetative and reproductive growth period was increased due to the delay on 31 days to anthesis onset, there was not translated in higher vegetative biomass accumulation at anthesis due to the observed photosynthesis impairments and probably the metabolic changes triggered by ET, as **Maestri et al.** (**2002**) stated.

5.3.1.1.2 From anthesis until physiological maturity

As occurred to reach anthesis, CATE conditions also impose the need for more days to fulfil the whole plant cycle (Table 5.2) due to the above-mentioned slow-down effect on growth caused by ET (**Hatfield et al., 2011**).

CATE treated plants presented at final physiological maturity similar *shoot biomass* (leaf + stem) as CATA grown plants due to late tillering effect (**Bányai et al., 2014**). However, because the number of spikes (*FSN*; Table 2) and *spike DW* (Fig. 5.1C) were reduced, the final aboveground biomass (*ADW*; leaf + shoot+ spike) was lower (Fig. 5.3). In this regard,

Mahalingam and Bregitzer (**2019**) in a study carried out in barley through a heat shock treatment (HT) applied at vegetative or reproductive stage, observed a cultivar-specific and growth development-specific response. In the specific case of the Bowman cultivar, they obtained similar results as ours in terms of *shoot DW*. Furthermore, **Clausen et al.** (**2011**) and **Ingvordsen et al.** (**2015**) also observed lower final *ADW* for the overall ET treatment barley plants.

In our case, in response to ET, plants continue driving more resources to vegetative organs (Fig. 5.4A-B), which can lead to categorizing its growth pattern as indeterminate under these conditions (**Prasad and Staggenborg, 2008**; **Bourgault et al., 2017**). This trend was also partially shown by **Chavan et al.** (**2019**) in wheat, stating that when grain development is stopped or slowed down by certain stress conditions as ET, the crop develops (or at least tries to do it) new grains by producing additional late tillers. This behaviour is considered as an acclimation response by plants to deal with ET or other stress such as drought, by developing new sinks. Nevertheless, this trait was not translated in a recovery of final *yield*, as the ET could still affect grain filling or even cause grain abortion (**Chavan et al., 2019 and references therein**). This might have happened in our case, as we will see below when analysing the data of *yield* (Fig. 5.5) and its components (Tables 5.2 and 5.3).

5.3.1.1.3 Yield and yield-trait components

At final maturity, together with the commented lower *FSN*, grain *yield* was massively diminished by 95 % (Fig. 5.5); at the same time, all yield components also presented lower values (Table 5.3).

In agreement with our results, different authors have also shown lower *yields* for barley exposed to ET. Thus, **Clausen et al.** (**2011**) and **Alemayehu et al.** (**2014**), through different barley cultivars and mimicking a constant elevated day/night temperature, observed lower *yields* attributing it to reduced *FSN* and grain formation (*TGNS*) and grain filled percentage (*GF*), but same individual grain weight (*IGW*). In addition, **Ingvordsen et al.** (**2015**) studying 138 spring barley cultivars, observed an overall 55.8 % lower *yield* under CATE conditions compared with CATA. In that study, *FSN* was increased, whereas *TGNS* decreased. Coming back to our results, the water use efficiency of grain yield (*WUEg)* was massively decreased, which was defined by the large effect that ET had on grain *yield* (Table 5.3). It is worth noting, however, that in our experiment we imposed higher day/night temperatures (26/20 °C), in comparison with 24/17 °C

imposed by **Ingvordsen et al.** (**2015**) experiments, which could explain the observed extra damages on reproductive organ development compared to us.

Therefore, our results are not surprising since, on one hand, barley is not well adapted to ET effects (**Mahalingam and Bregitzer, 2019**) as other temperate crops such as wheat are (**Barnabas et al., 2008; Chavan et al., 2019**). On the other hand, the impact of elevated temperature on final *yield* or reproductive fitness is closely related to the developmental stage in which it occurs (**Gray and Brady, 2016**), that for our case, encompassed plants whole life cycle. In this respect, the above-mentioned HNT should also participate in the observed hastened *yield*. Furthermore, **García et al.** (**2015**) either for wheat or for barley, observed that HNT accelerated reproductive development and shortened the critical period for grain-filling, translating in lower *yields*. Thus, in our case, CATE conditions should have affected barley plants whole reproductive organ development, that is, both spike and kernel (grain) formation and grain filling periods, both due to the overcome of the optimum daytime temperature and HNT effects.

At floral initiation (anthesis), ET affects principally grain number due to lack of starch availability for developing florets (**Barnabas et al., 2008 and references therein**). Specifically, in the case of barley, pollen development and hence, fertilization and grain formation, is hypersensitive to ET (**Abiko et al., 2005**). Furthermore, it has been proposed that the sensibility of the pollen development to ET could be explained by the inability to synthesize all the HSP (**Mascarenhas and Crone, 1996**).

Going back to our results, the observed lower A_{net} values at anthesis (Fig. 4.3A) could lead to the lack of sugars and starch needed for florets development. Moreover, the ET effects themselves could have developed floral abnormalities conducting to spikelet sterility, which, for example in rice, represents a significant problem (**Takeoka et al., 1991**), being, therefore, another possible cause for the registered lower grain number formed per spike (Table 5.3).

Moreover, once barley grains have been formed and the spikes headed, anthesis is finished and the post-anthesis phase begins (**Alqudah and Schnurbusch, 2017**), where fertilized ovaries develop into caryopses by grain filling (**Barnabas et al., 2008**). Thus, if plants have managed to develop the spikes and the grains, after that, they have to deal with its filling (**Gray and Brady, 2016 and references therein**). The effect of ET on this stage is mainly ascribed to starch reduction accumulation, as in general more than 65 % of the *grain DW* biomass of cereals is accounted for it. If the stress is imposed at the early periods of grain filling, the lower *grain DW* can mainly be attributed to the lower number of endosperm cells formation. Nevertheless,

if the stress happens at later stages, the starch synthesis is impaired (**Ugarte et al., 2007; Rajala et al., 2011**), which could be due to the limited supply of assimilates for the grain (**Blum, 1998**) and/or the direct effects on the synthetic processes in the grain (**Yang et al., 2004**).

Thus, the reduction on A_{net} (Fig. 4.3A) could participate negatively in all the reproductive organ development, leading to the possible damages caused at the organ structural level too. Furthermore, the indeterminate growth habit of CATE plants was not traduced in an advantageous mechanism for the plant, being reflected by the lower grain *yield*.

5.3.1.2 CETA conditions effects

5.3.1.2.1 From sowing until anthesis

The results obtained for CETA plants as concerns biomass accumulation varied with the developmental stage. On this matter, at the early-vegetative stage, CETA plants presented an increase on A_{net} (Fig. 4.1A), but as the total leaf area (*TLA*) was decreased (Fig. 5.2A) —although *leaf DW* was kept constant—, a trade-off between both parameters occurred and final *TDW* biomass remained statistically unchanged (Fig. 5.1A). The lack of biomass partitioning to leaf expansion could be explained by the fact that at this stage, the relevance of root as a sink organ was increased compared with CATA conditions (Fig. 5.1A), a response described in a review by **Pritchard et al.** (**1999**). Moreover, as *leaf DW* was maintained and *TLA* was diminished (Fig. 5.2A), the leaf density was increased, which possibly meant that an accumulation of sugars in leaves was happening (**Poorter, 1993**). Thus, despite leaf was kept as the main sink organ in terms of *biomass DW* values, and A_{net} was increased, it is clear that CETA plants at the early-vegetative stage did not drive the photoassimilates to leaf expansion as CATA plants did it, but rather to root growth. This allocation pattern towards root at ECO_2 conditions has been observed as a general trend along other crop species (**Madhu and Hatfield, 2013**). In addition, other researchers at different growth conditions also showed the absence of a positive effect of elevated CO_2 (ECO_2) on leaf growth as registered by us at this stage (**Mitterbauer et al., 2017 and references therein**).

One hypothesis to explain the lack of leaf expansion is that the developed increase in root biomass was not enough to avoid CHs accumulation on leaves, denoting a lack of sink-strength. Therefore, CETA plants at the early-vegetative stage did not expand even more leaf area since the CHs production would even be greater and plants would not be able to use it

(**Kirschbaum, 2011**). The observed and commented photosynthetic acclimation 9 days after the early-vegetative stage (Fig. 4.6A) should strengthen our hypothesis for the none leaf expansion.

At the vegetative stage (Fig. 5.1B), CETA plants also presented the same *TDW* as CATA plants. However, the developmental pattern of each organ varied compared to the previous growth stage. Thus, *TLA* was kept equal and *root DW* was reduced compared with CATA, which it should mean that the leaf expansion rates along the 9 days between early-vegetative and vegetative periods were increased at CETA conditions, being the leaf the principal active sink organ. This fact could be ascribed to the developmental stage effects on biomass partitioning switch, since it matched with the onset of the tillering phase as **Seneweera et al.** (**1994**) suggested for rice, being the faster growth moment. Nevertheless, plants would still be sink-limited due to the accumulated CHs on leaves at the previous stage, which at this stage led to the commented photosynthetic downregulation (Fig.4.6A), giving, as a result, the same A_{net} as for CATA (Fig. 4.2A). In agreement with our results, **Bunce et al.** (**2014**) for soybean, and **Aranjuelo et al.** (**2006**) and **Erice et al.** (**2006a**) for alfalfa, also observed a photosynthetic acclimation on plants grown at elevated CO_2 conditions (CETA), and therefore, none gained biomass.

Lastly, different to both vegetative stages, 9 days after the onset of anthesis CETA plants presented lower *TDW*. This fact was owed to a combination of the same A_{net} –triggered by the developed photosynthetic acclimation (Fig. 4.7A)–, and lower leaf development (Fig. 5.1C and 5.2C). On the contrary to leaf development pattern at this stage, higher tiller formation (*ATN*; Table 5.1) and a greater percentage of biomass was driven to *stem DW* (Fig. 5.1C), which might be explained by the fact that at this stage stem becomes the higher sink organ (**Seneweera et al., 1994**). Therefore, fewer CHs were driving to the rest of the organs, and it should explain, perhaps, the observed reduction in leaf development. In line with the latter, another effect of the observed biomass partitioning pattern was the registered slow-down development of the reproductive organ compared with CATA (Fig. 5.1C and Table 5.1), a fact that also picked up **Castro et al.** (**2009**) for soybean.

Nevertheless, it must be highlighted that despite the reduction in *TDW* and the decreased spike development at anthesis, CETA RC plants recorded the same *GLA* (Fig. 3.4D) as for CATA. This fact could mean that the senescence process was slowed down (**Gray and Brady, 2016 and references therein**), which would also be supported by the registered higher photosynthetic pigment content (Fig. 4.4), defining in part the higher final *ADW* biomass (Fig. 5.3) as it will be seen later.

5.3.1.2.2 From anthesis until physiological maturity

As above-mentioned, at physiological maturity, CETA plants presented higher *ADW* than CATA plants (Fig. 5.3), whereas there was not recorded difference in the required time to reach final maturity (Table 5.2), a trend also commented by **Xu et al.** (**2013**) in their review. The gained biomass at this period was greater than for CATA due to the allocation pattern towards more biomass allocation at vegetative tissue (Fig. 5.4), together with the delayed senescence and photosynthetic rates maintenance along this period that are deduced from the above-mentioned greater leaf greenness (Fig. 3.4D and 5.2C). Our result are in agreement with the ones observed by **Schmid et al.** (**2016**) in barley. In fact, the commented trend was due to the indeterminate growth habit pattern that triggered ECO_2 increasing the vegetative biomass accumulation from flowering to maturity and providing vegetative sinks together with reproductive sinks (**Ingvordsen et al., 2015 and 2018**). Similar results were recorded in our case (Table 5.2), as *FTN* and *FSN* were increased by ECO_2, as other authors have observed for wheat too (**Ziska and Bunce, 2007; Thilakarathne et al., 2013; Dias de Oliveira et al., 2015a**).

Interestingly, the registered increase in tiller formation could be ascribed to elevated CO_2 alteration in shoot architecture (**Pritchard et al., 1999**), which firstly was described for wheat and rice by promoted axillary meristems (**Nicolas et al., 1993; Christ and Korner, 1995; Jitla et al., 1997; Slafer and Rawson, 1997**) or fertile tillers in wheat (**Dias de Oliveira et al., 2015a**). The molecular mechanisms behind this fact are still unknown, although, in the last year's works, **Morita et al.** (**2015**) for rice started shedding some light in this respect. They observed that an increase of starch content on leaf sheaths could alter the tillering angle, which should enable plants to develop more tillers, being this one possible hypothesis to explain such observed response.

5.3.1.2.3 Yield and yield-trait components

Lastly, as concerns grain *yield*, CETA did not alter it (Fig. 5.5). The most common response by plants grown at ECO_2 conditions is to present higher *yields* (**Long et al., 2006; Jagadish et al., 2014; Días de Oliveira et al., 2013 and 2015a**), and barley is not an exception (**Clausen et al., 2011; Alemaheyu et al., 2014; Ingvordsen et al., 2015; Schmid et al., 2016; Mitterbauer et al., 2017**). Nevertheless, **Ingvordsen et al.** (**2015**) in a pot experiment carried out in growth chambers for 138 spring barley cultivars recorded similar results in some cultivars as ours, whereas the causes are still unknown.

In our case, CETA conditions increased *FTN* and *FSN* (Table 5.2), remaining unchanged *TGNS* (Table 5.3) as **Högy et al.** (**2009**) observed too for wheat growing in a Free-air-CO_2-enrichment (FACE) facility. Furthermore, grains size was not altered *(IGW*; Table 5.3), which could be ascribed to its narrow plasticity to be increased (**Sadras, 2007**). Thus, the none increase registered for grain *yield* in our case was due to reductions in *GF* (Table 5.3), so that the *yield* of CETA plants was not limited by a lack of sink capacity, otherwise, it was due to a lack of allocates partitioning towards grains.

One possible explanation for our results is that our CETA plants exceeded the sink capacity due to the indeterminate growth development by ECO_2 increasing the vegetative tissue growth, which would be strengthened by the recorded greater biomass partitioning amount to vegetative tissue (Fig. 5.4A, C). On this point, **Ingvordsen et al.** (**2015**) for the overall of the studied barley varieties did also register a similar pattern. Focusing on our results, we suggest that the great increase in tiller, and subsequently spike formation at this period, consumed all the produced CHs, so plants exhaust the capacity to fulfil the formed grains. In agreement with our hypothesis**, Dias de Oliveira et al.** (**2015a**) for a wheat free-tillering line, also suggested that the increase of spikes — that is the sink capacity—, might exceed the ability for providing carbon supply, being finally, source limited. At the same time, this fact would also explain the observed decrease in *GF*, since the demand to fulfil the higher total grain number per plant was increased, negating the possible higher potentiality (**Dias de Oliveira et al., 2015a**).

Moreover, CETA plants did not increase the *WUEg* (Table 5.3). Contrary to our results, grain water use efficiency uses to be improved under ECO_2 (**Dias de Oliveira et al., 2013 and references therein**). In our case, the none increase could be ascribed both to the non-improvement of grain *yield*, and the same water use along the full life span of plants (Fig. S7). As concerns the latter possibility, it is worth noting that although the stomatal conductance (*gs*) was lower along all-growth stages (Chapter 3), the fact that CETA plants maintained leaf greenness and increased the vegetative tissue along the grain filling stage, led to the same water use as CATA plants. In consonance with our results, **Samarakoon et al.** (**1995**) and **Gray et al.** (**2016**) recorded similar trends for different species.

Therefore, the overall commented responses about grain *yield* would indicate that our CETA plants did not possess sufficient phenotypic plasticity neither the ability for fast genetic change necessary to take advantage of the positive effects triggered by ECO_2 in biomass accumulation (**Adams and Grafius, 1971; Alemayehu et al., 2014**), leading to a trade-off between yield-trait components. In line with this, considering the large registered response

between different barley cultivars to ECO_2 (**Ingvordsen et al., 2015**; **Mitterbauer et al., 2017**), it is important to indicate that the traditional plant breeding has not considered –as it should–, plant responsiveness to ECO_2, as already **Ainsworth et al.** (**2008**) stated. Thus, breeders should make efforts to break the yield-trait components compensation by limiting the sink capacity of cultivars with greater response to ECO_2, achieving semi-determinate cultivars, with enough sink capacity but also with the ability to drive the assimilates to grain filling instead of continuing developing vegetative tissue.

5.3.1.3 CETE conditions effects

5.3.1.3.1 From sowing until anthesis

Our plants at early-vegetative and vegetative stages presented the highest *TDW* biomass owed to both great *shoot* and *root DW* values (Fig. 5.1A-B). This fact was due to the registered higher A_{net} (Fig. 4.1A and 4.2A) and the active employment of gained assimilates in leaf area increase (Fig. 5.2A-B).

Moreover, the higher developed sink-strength at CETE plants is postulated as the key trait that enabled them to avoid photosynthetic acclimation (Fig. 4.5A and 4.6A), and hence, invest the gained extra assimilates in active growth. In agreement with our results, **Aranjuelo et al.** (**2006**) and **Erice et al.** (**2006a**) for alfalfa and **Yu et al.** (**2012**) for tall fescue, respectively, also observed an increase in biomass accumulation as a result of higher A_{net} on CETE plants but not in CETA plants, stating too that the higher sink strength was the key trait. Furthermore, at the vegetative stage, specifically, leaf *HvPIP2;5* seemed to be involved in the better photosynthetic performance –without water loss alteration– (Fig. 3.7A and Table 4.2). This fact as previously has been commented, was probably triggered by a switch on in its main transport function towards CO_2 transport to cope with the increased atmospheric CO_2 levels as **Zhang et al.** (**2021b**) postulated in tomato for a *PIP1 AQP*, an area that deserves further analysis.

In addition, CETE plants not only achieved the capacity of photosynthetic acclimation avoidance, but probably they were also able to trigger a thermal displacement of the optimum of the A_{net}, a concept defined by **Long** (**1991**). This concept suggests that plants acquired a thermo-tolerance (**Wang et al., 2008**) which protected the enzyme kinetics from the elevated temperature (**Faria et al. 1996; Gutiérrez et al. 2009; Chavan et al., 2019**) avoiding the downregulation of the Rubisco activity. As we have not observed lower Rubisco activity unlike at CATE conditions plants, our results would point in that direction.

Regarding the anthesis stage, our plants needed 10 more days to reach this stage (Table 5.1). This result could be ascribed either to the general delay effect that ECO_2 had on reproductive growth development by the maintenance of vegetative tissue growth (**Gray and Brady, 2016**) or due to a negative effect originated by ET (**Hatfield et al., 2011**). On one hand, the observed higher tiller development (Table 5.1) matched with the CETA conditions trend, although the *spike DW* and *ASN* were the same as for CATA. On the other hand, the lower tiller fertility at this stage could be due to ET effects on reproductive organ development.

Besides, differently to vegetative stage, at anthesis the recorded higher *TDW* biomass was only due to higher A_{net} (Fig. 4.3A) since *leaf DW* (Fig. 5.1C), *TLA* (Fig. 5.2C) and *GLA* (Fig. 3.4D) were not modified compared with CATA. Concretely, the higher *stem* and *root DW* values contributed to it (Fig. 5.1C). Thus, as it has been commented for CETA plants, at this stage the main sink organ was the stem, which the ECO_2 effect promoted (**Seneweera et al., 1994**). In agreement with our results, **Dias de Oliveira et al.** (**2013**) for a vigorous wheat variety, observed that at anthesis the increase above +2 °C negated the positive effect of ECO_2 on leaf area and leaf mass, although they registered higher final biomass due to higher A_{net} and the gained assimilated allocation in tiller formation.

Therefore, until this stage, which covers mainly the vegetative growth period and almost all the reproductive growth, the environmental agent that governed the response of CETE plants would be ECO_2, taking advance of it from the vegetative tissue (**Jagadish et al., 2014**). Nevertheless, the constraining effect of ET on reproductive organ development explained in the CATE section (5.3.1.1.3) started to play a key role as we will see in the next section.

5.3.1.3.2 From anthesis until physiological maturity

At physiological maturity, CETE plants did also present higher *ADW* biomass, as was observed under CETA conditions. It was owed to the higher *stem DW* since *spike DW* remained unchanged (Fig. 5.3 and Fig. S3-S6). Furthermore, our CETE plants also presented the highest *FTN* and *FSN*. In agreement with our results, **Dias de Oliveira et al.** (**2013**) for a high yielding wheat line recorded similar results, but only for treatments that did not exceed +2 °C. In addition, , **Ingvordsen et al.** (**2015**) observed a cultivar specific-response for the studied 138 barley cultivars, presenting some cultivars with the identical trend as us.

Besides, the time needed to complete the life span of our plants was reduced (Table 5.2), a trend also found by **Dias de Oliveira et al.** (**2015a**). This fact would imply that the grain-filling period was lower, and it should affect grain filling rate or duration, as will be discussed

later. Despite the latter fact, it is also mandatory to consider the role that other organs, such as spikes, could have in CHs production, an issue that is being studied in recent years, which leads us to understand the relevance that has "the canopy photosynthesis" in final grain *yield* (**Araus et al., 2021**). The pivotal role of the spikes came from the fact that they are the last photosynthetic organs developed and the nearest one to the sinks (florets and subsequent potentially grains). In fact, the recent work that **Araus et al.** (**2021**) picked up in the review came to point out that spikes are the main organ (taking it as a whole, not individually) contributing to the total canopy photosynthesis at post anthesis. Therefore, knowing that A_{net} at anthesis was higher than at CATA conditions and that *GLA* (Fig. 3.4D) and the induction of leaf senescence (Fig. 4.5) were quite similar, but final *leaf DW* and mainly *spike DW* were increased, our results should explain the registered higher final *ADW* biomass.

In addition, as Fig. 5.4D shows, the gained biomass for the last growth period was increased and the gained biomass was equally distributed between vegetative and reproductive tissues. The former fact, at least in part, could be due to the indeterminate growth habit pattern triggered by ECO_2 (**Dias de Oliveira et al., 2015a; Bourgault et al., 2017**) and previously observed in CETA conditions (Fig. 5.4). The observed partitioning to vegetative tissue resembled the late tillering registered under CATE conditions, and should be an adaptive mechanism to deal with ET effects on reproductive organ development (**Prasad and Staggenborg, 2008; Chavan et al., 2019**), which would also explain the observed higher *leaf DW*. Overall, both environmental agents played their role in reproductive organ development, as it will be explained below.

5.3.1.3.3 Yield and yield-trait components

CETE conditions diminished the grain *yield* (Fig. 5.5). Compared with current environmental conditions (CATA), and attending to the commented higher *FSN* and lower time needed to fulfil reproductive growth (Table 5.2) and the yield-trait components (Table 5.3), it is observed that the most positive effect of ECO_2 was ascribed to spike number increase as **Jagadish et al.** (**2014**) picked up in their review. Nevertheless, ET diminished total grain formation by the same causes as previously commented for CATE conditions (**see the review by Barnabas et al., 2008**), giving as a result lower *TGNS* (Table 5.3). In agreement with our results, **Ingvordsen et al.** (**2015**) for the overall of the 138 barley cultivars, observed greater spikes but lower grains per spike as a general pattern.

In addition to the lower grain formed, a reduction in *GF* was also observed (Table 5.3). This fact could be owed to either the shorter time to fulfil the grains (Table 5.2) or the greater

biomass partitioning to vegetative development by the indeterminate growth habit (Fig. 5.4D), as **Dias de Oliveira et al.** (**2013**) found in wheat. Furthermore, it was reflected in the lower *WUEg* (Table 5.3), as the grain *yield* was reduced and the water use along the full life span of plants was increased due to the same reasons explained in CETA conditions (Fig. S7); similar trend was also observed for rice by **Nakawaga et al.** (**1997**). Moreover, it has also to be borne in mind that stem non-structural carbohydrates (NSC) remobilization is also an important source of grain filling, mainly under stress situation (**Barnabas et al., 2008; Prasad and Staggenborg, 2008; Araus et al., 2021 and references therein**). Thus, observing the so high vegetative tissue biomass values at CETE RC, we also suggest that the shortened period to fill the grains might have disabled the capacity to translocate the overall accumulated NSC from stems to the grains. In that sense, the role of sucrose non-fermenting 1-related kinase 1 (SnRK1) and trehalose 6-phosphate (Tre6P) have been described as key players in the coordination of efficient reallocation of resources to grains (**Paul et al., 2018; Baena-González and Lunn, 2020**). It might be considered to inquire on, together with final NSC content, to try to better understand the observed results in our experiment.

Lastly, as **Baker** (**2004**) and **Tubiello et al. (2007**) stated in wheat, and **Jagadish et al.** (**2014**) in several crop species, it is worth noting that the beneficial increase in A_{net} and vegetative growth by ECO_2 was not enough to ameliorate ET effects on reproductive organ development. Hence, we conclude that in future conditions plants grown at the well-watered regimen will be governed by ECO_2 at vegetative tissue growth, whereas at reproductive tissue growth the ET will take the main role, being its negative effects more hazardous on final *yield* than the previous benefit of ECO_2.

Otherwise, it also must be highlighted that compared with CATE, higher *yields* were recorded at CETE conditions. This trend could be ascribed to the positive effect of ECO_2 either on photosynthetic rates increase (Fig. 4.3A) and/or on structural damage amelioration in both tiller and spike formation (Table 5.2) as **Dias de Oliveira et al.** (**2013**) stated**,** and on floret survival (**Dias de Oliveira et al., 2015b**) too. This fact could be due to a higher sucrose and hexose availability obtained by the higher photosynthetic rates (**Rollins et al., 2013**) and a better osmotic adjustment (**Wahid et al., 2007**), improving the tolerance to face ET constraints on reproductive organ development of plants (**Shanmugam et al., 2013**).

5.3.2 Growth, development and yield of drought treatments

Growth is determined by cell division, cell enlargement and differentiation, which are modulated by genetic, physiological, ecological and morphological events and their complex interactions (**Farooq et al., 2009**).

Irrespective of the developmental stage, when drought was applied either at current (CATA) or at future (CETE) environmental conditions, it reduced biomass accumulation and altered growth developmental processes. Nevertheless, depending on the interaction with the other environmental agents, the response to drought was accentuated or ameliorated. Even more, the response trend was different according to vegetative or reproductive tissues and growth stage (**Prasad and Staggenborg, 2008**).

Thus, in this section, plants growth, development and yield response to drought –under current and future environmental conditions, and at different developmental stages–, have been studied. Moreover, data obtained for CATE (current CO_2 and elevated temperature) and CETA (elevated CO_2 and current temperature) conditions –the individual environmental conditions that constitute future ones– had only been used to explain certain trends observed for CETE treatments. Furthermore, plants memory capacity was analysed along the different environmental conditions to elucidate the recovery capacity, and the possible priming effect of plants that had suffered a vegetative drought and subsequently suffered another one at anthesis.

For a better discussion of the results of drought treatments, we have divided them into (i) vegetative drought effects on plant growth and biomass accumulation, (ii) anthesis drought effects on plant growth and biomass accumulation, (iii) plant growth and development from anthesis until physiological maturity, (iv) *yield* and yield-trait components and (v) plant memory effects on plant growth, biomass accumulation and *yield* and yield-trait components.

5.3.2.1 Vegetative drought (VD) treatment effects in barley growth

As the *SVWC* was diminished by VD treatment (Fig. 3.1A), plants to keep ensuring water uptake, reduced not only their Ψw_{md} (Fig. 3.2A) but also their cell Ψt_{md} (Fig. 3.2E). On the other hand, to avoid massive water loss, plants reduced *gs* (Fig. 3.5A) and *GLA* (Fig. 3.4C). In consequence, they achieved to reduce water loss from leaves to the atmosphere either at the

leaf (Fig. 3.5C) and plant level (Fig. 3.4A). Nevertheless, at the same time, VD treated plants suffered a CO_2 diffusional limitation (Fig. 4.2F), performing lower A_{net} (Fig. 4.2A) and producing lower CHs. This is the typical response of plants when facing drought stress. As a result of the alteration of those processes and the water scarcity, firstly, cell elongation and expansion should be impaired due to Ψt_{md} diminution; secondly, the lower A_{net} might produce lower CHs that negated cell mitosis (**Nonami, 1998; Kaya et al., 2006; Hussain et al., 2008**). Overall, there was a reduction in *shoot growth* and *TDW* biomass (Fig. 5.1B).

At this stage, the leaves are young and more sensitive to the negative effects of drought. The cell division processes are of important relevance so that the plant's leaves can reach their final size, and if cell division is impaired at this early stage, they could not achieve their maximum size capacity (**Gray and Brady, 2016 and references therein**). In addition, a drought at the onset of the tillering stage can reduce tiller and spike number too. However, plants are more plastic in their stress responses at early stages due to their smaller leaf area, moderated rates of physiological activity and lower water demand; furthermore, they are more flexible to recover from stress damages in the subsequent re-watering phase (**Garg et al., 1984**). These facts should be borne in mind when plant recovery from VD and memory acquisition after DD will be discussed.

Nevertheless, as an opposite trend to *shoot biomass*, VD treated plants allocated more resources to increase *root DW* (Fig. 5.1B). This response is a common adaptive trait that plants switch on to deal with water scarcity as other authors have stated for different plant species (**Robredo et al., 2007; Markestejin and Poorter, 2009**).

As regards the different plant response to VD treatments between CATA and CETE conditions, greater biomass was registered at CETE conditions (Fig. 5.2A) due to the better water status (Chapter 3), which enabled them to maintain higher A_{net} and take advance from the increased C_C (Chapter 4). Specifically, water relations of CETE plants at this vegetative stage were governed by ECO_2 due to its effect on better stomatal control as **Li et al.** (**2020a**) proposed and previously was explained. In agreement with our results, **Aranjuelo et al.** (**2006**), **Dias de Oliveira et al.** (**2013**) and **Pastore et al.** (**2020**) for alfalfa, wheat and different grass species, respectively, did observe similar trends that they ascribed to the same causes.

Thus, at the end of the drought period, the water (*K*) and CO_2 (*gm*) conductance within CETE VD plants were higher which probably the higher expression of leaf *HvPIP2;5* and *HvTIP1;1 AQPs* enabled it (Fig. 3.7A, Chapter 3); that is, the *AQPs* –by an isoform-specific response– are presented as a key regulator on such modulation. The first (*K*) might have enabled the higher

observed Ψt_{md} as it has been commented in Chapter 3 and **Li et al.** (**2020b**) observed for cucumber seedlings grown at ECO_2, although the registered pattern was isoform and drought extent specific. The latter (improved *gm*) might have allowed the better flow of CO_2 within the plant, dealing with the higher CO_2 levels because of being growing in a CO_2 enrichment atmosphere (**Zhang et al., 2021b**), and achieving higher *Cc*, ultimately driving a lesser decrease of the A_{net}.

Therefore, at the vegetative stage, future environmental conditions will alleviate partially drought effects due to a lower stress pressure (**Zinta et al., 2014**), being less affected cell expansion and division processes, resulting in higher active biomass accumulation.

5.3.2.2 Anthesis drought (RD) treatment effects in barley growth, development and yield

5.3.2.2.1 Growth at anthesis

Drought treatment imposed at anthesis (RD) had a greater impact on plants water relations compared with VD treatment (Fig. 3.1C-D) and hence, the photosynthetic (Fig. 4.2A and 4.3A) and growth performance were more affected regardless of the environmental conditions (Fig. 5.1B-C). The main reason for the observed trend was that, as the plants were bigger at anthesis, the water amount that they needed to maintain their processes was greater (**Izanloo et al., 2008**). Consequently, in the course of the development of our plants the transpiration was larger due to the higher *TLA* and *GLA* area (Fig. 3.6), so plants water status decreased more (Fig. 3.4A-B). Therefore, not only higher diffusional limitations of photosynthesis were registered (Fig. 4.2 and 4.3), but also the photochemical (Table 4.3) and biochemical (Fig. 4.6 and 4.7) photosynthetic processes were impaired compared with VD treatment, presenting a similar trend as the observed in wheat by **Wang et al.** (**2015**) and **Liu et al.** (**2017**).

Nevertheless, despite this fact, the response on *TDW* biomass was not translated as directly as the data might suggest, which could be ascribed to a different strategy to deal with drought, being the response environmental-specific (**McDowell et al., 2008**).

Under CATA conditions, the RD treatment hardly affected Ψt_{md} (Fig. 3.2F). The mechanism behind this trend was the ability of those plants to make extremely elastic the cell wall (Table 3.1). Thus, despite the cell water volume was massively decreased by the large *DH*

effect, the cell wall could adjust to the new volume, allowing the vacuole to continue pushing and generating the turgor potential needed to cell expansion as **Pérez-López et al.** (**2009**) and **Miranda-Apodaca et al.** (**2018**) suggested for barley and two different grass species, respectively. On the other hand, we hypothesize that CATA RD plants tried to maintain the stomata opened with a minimum decline in photosynthetic rates until a threshold on the *SVWC* was reached, that it could be near to 25 %. At that moment an hydraulic failure was developed, triggering the massive stomata closure and plant hydraulic conductance slowed down. In this way, CATA RD plants could minimize growth reduction (Fig. 5.1C), but the biochemical and metabolic damages would be higher (**Serraj and Sinclair, 2002**).

Thus, CATA RD plants barely reduced their *TDW* biomass, except the *spike DW*, presenting a riskier management strategy (anisohydric behaviour), whereas, as we have shown below, the *stem DW* was also reduced in CETE RD plants, but achieving a better water status and taking then a more conservative strategy (isohydric behaviour). The differences in the behaviour between both strategies could be due to differences in the sensitivity of guard cells to a critical *Ψw* (**Sade et al., 2012**). In addition, the observed general decreases by RD treatments on *spike DW*, in comparison with vegetative tissue, reveals the greater sensitivity of reproductive processes in this period (**Kakumanu et al., 2012**).

In the case of CETE RD plants, as it was explained for VD treatment, it must be borne in mind that at the onset of the second drought period they had lower *gs* triggered by ECO_2 (Fig. 3.5B; **Li et al.,** (**2020a**), but same *TLA* and *GLA*. This fact enabled CETE RD plants to transpire less than CATA RD plants along vegetative and anthesis drought periods, being the observed differences only attributed to better stomatal control, not suffering so much stressful conditions at the end of the water stress period. Furthermore, CETE RD treatment reduced stomata density too (Fig. 4.3B), denoting a tight stomatal control as **Galmés et al.** (**2007**) stated. Thus, *LRWC* was higher at CETE, an indication of an isohydric behaviour (**Sade et al., 2012**), which was also achieved due to higher *K* at leaf level values, modulated by the greater *HvPIP2;1* expression, and the lower *root AQPs* expression, contributing to the slow-down of water loss.

Together with the better water loss control, these plants also carried out a more conservative strategy to deal with water uptake compared with CATA conditions, since they avoided a massive cell *DH* (Table 3.1), but at the expense of reducing the Ψt_{md} (Fig. 3.2F). Therefore, water consumption was slowed down, which could be one of the reasons why plants growth was lesser (the reduction respect its RC treatment was higher), although the cell metabolic and biochemical status was in a better status (**Merlaen et al., 2019**).

In addition, the registered lower reduction on A_{net} compared with the one observed at CATA at the end of the drought period was a consequence of the better water status. However, it must be considered that at this stage the reproductive organ development had started and that the massive decrease on *spike DW* at CETE RD treatment (Fig. 5.1C) could be due to the effects that ET had on its development as previously have been commented for the RC treatment (**Barnabas et al., 2008; Prasad and Staggenborg, 2008**). This feature could be a key factor, postulating as the second reason to explain our results, since plants might drive the extra gained CHs to deal with maintenance processes. In agreement with this idea, **Wahid et al.** (**2007**) stated that under elevated temperature stress (ET), plants allocate resources to cope with the stress, driving, less assimilates to reproductive organ growth.

5.3.2.2.2 Growth and development from anthesis until physiological maturity

Regardless of the environmental conditions, RD treated plants did not present any delay to reach maturity (Table 5.2), although they presented lower *ADW* biomass at the maturity stage, mainly due to the lesser *spike DW* biomass (Fig. 5.3). At CATA conditions, more tillers were recorded (Table 5.2), even though the biomass partitioning was maintained for the RC treatment (Fig. 5.4A). The increase in *FTN* was due to the late tillering effect, an adaptative mechanism of plants to deal with drought or temperature stress on reproductive organs by forming new source tissue (**Sadras, 2007)**. However, this does not have to be translated into the final same biomass (**Prasad and Staggenborg, 2008**). The maintenance of biomass partitioning would be ascribed to plants ability for translocating all the resources to reproductive organs when they suffer massive stress and want to develop rapid spike growth (**Bolaños and Edmeades, 1996; Zhang et al., 1998**). The latter was the case for RD treatment under CATA conditions, compared to the rest of environmental conditions, being an indicator of the higher *spike DW* at anthesis in current environmental situations (Fig. 5.1C).

At CETE conditions, as **Dias de Oliveira et al.** (**2013 and 2015a**) stated for wheat, a reduction in the *ADW* biomass with respect its control was also shown for RD treated plants. Nevertheless, and compared with RD treatment at CATA conditions, higher total biomass was recorded at this final maturity stage, basically due to the higher tillering caused by the ECO_2 (Table 5.2). In this case, the biomass partitioning was also maintained to its control treatment (Fig. 5.4D), but compared with CATA, higher partitioning was driven to vegetative tissue due to the commented late tillering effect, accentuated by ET effect too (**Chavan et al., 2019**). This fact

could also be corroborated regarding the CATE conditions trend. Otherwise, it must not be forgotten the fertilization effect that ECO_2 had on shoot development.

5.3.2.2.3 Yield and yield-trait components

Lastly, the commented impairment by RD treatments on the different processes such as the plant water relations, photosynthetic metabolism and plant growth and development, gave as a result lower grain *yields*, a well-studied consequence on barley (**Samarah, 2005**). However, depending on the environmental conditions, anthesis drought had a higher or lesser extent, varying the observed pattern for yield-trait components.

At CATA conditions, the lower *yields* were owed to less *FSN* and *TGNS*. As the RD treatment was imposed at barley anthesis —when the fertilization was occurring and the spike was also developing—, an inhibition in the complete spike development was occurred, leading to less fertile tillers (fewer tillers that developed spike) (**Prasad and Staggenborg, 2008**). At this point, is when the indeterminate growth habit played its role, allowing substantial reproductive compensation through great tiller formation (Table 5.2), and hence leaves, in the period that covered until physiological maturity. Nevertheless, it was not enough to achieve identical *yield* as RC treatment.

In addition, since drought stress during anthesis can lead to a fertilization failure by decreasing pollen or ovule function (**Barnabas et al., 2008 and references therein**), fewer *TGNS* were formed (Table 5.3). Besides, we cannot discern whether the reasons leading to less grain formation, were due to *Ψw* decrease at leaf level but also in the floral tissue, or otherwise, it was the result of the reduced CHs availability because of the photosynthesis reduction. However, the demand for assimilates by the embryos is low. On the other hand, the sink strength of these organs is much lower than during the vegetative tissue development, therefore the lack of photoassimilates seems not to be the main reason as **Prasad and Staggenborg** (**2008**) stated. In this regard, it has been suggested that elevated levels of ABA together with low levels of cytokines can be involved at whole-plant level as hormonal signals, which might participate in the early embryo abortion (**Cheikh and Jones, 1994**).

In addition to the above-mentioned processes, grain *yield* is also defined by plants ability to fill the grains (Table 5.3), which is determined by seed-filling rate and its duration. In our case, RD treatment did not alter the final *IGW* (Table 5.3), which enter within a common response taking into account that drought was imposed during anthesis and not at the grain filling stage (**Prasad and Staggenborg, 2008 and references therein**). In this respect, two were the main

reasons to explain why individual grain weight was not altered. The first one, and the most reasonable, was the maintenance of the grain filling period (Table 5.2). The second reason is supposed to be due to the ability to continue the seed-filling rate thanks to the massive CHs remobilization from stem to grain, that in the case of severe stress –as it was our case (Chapter 3)–, this partitioning could account for near the 70 % of grain yield (**Barnabas et al., 2008 and references therein**).

Besides, it has also been seen that at this post-anthesis period that spikes have an important role in CHs production as photosynthetic tissue for grain filling. Besides, recent works have granted a specially relevance under drought stress (**Vicente et al., 2018; Tambussi et al., 2021**). In this respect, we have not had the opportunity to measure it to know its putative relevance in grain filling, although it should be considered for future works.

Otherwise, under CETA conditions, unlike for other works carried out either in barley (**Schmid et al., 2016**) or wheat (**Dias de Oliveira et al., 2013 and 2015a, b**), our RD treated plants did not register higher grain *yield* compared with the RD treatment at CATA conditions. These results could be explained by the trade-off developed between the greater *FSN* and the lower *GF* (Table 5.3). Therefore, as it was commented for control treatments, our barley cultivar at CETA conditions had not enough plasticity to break the established compensation between yield traits and take advantage of the higher potentiality, possibly due to the strong genotype linkage (**Dias de Oliveira et al., 2015a and references therein**). The excessive sink-strength due to the indeterminate growth habit caused by ECO_2 effect, which triggered an exacerbated vegetative tissue development, could lead to a lack of enough source supply towards grains, not altering grain size, but reducing the overall grain filling.

As regards the observed trend at future environmental conditions (CETE), on one hand, *FSN* was more affected by RD treatment than at CATA, and on the other hand, *IGW* was also reduced unlike at CATA. We attributed both facts to the well-known ET effects on tiller fertility and grain filling rates and duration (**Asseng et al., 2011**). This fact, on one hand, gave as a result a higher decrease in respect to its RC treatment, and on the other hand, lower grain *yield* compared with RD treatment at CATA conditions. Besides, the positive effect that ECO_2 had on *FTN* and *FSN* formation, partially alleviated the ET negative effects as previously was also commented for the control treatment.

Moreover, in agreement with our result for RD treatment at CETE conditions, **Dias de Oliveira et al.** (**2013**) did also observe an increase in *FSN* together with lower *TGNS* in a non-vigorous variety, but in plants grown above +2 °C from its optimum temperature. Otherwise,

although the potential sink-strength and assimilated availability were enhanced by ECO_2, individual grain weight was diminished by ET inhibition on assimilates translocation.

It should be remembered that our temperature stress was imposed along the whole life span of plants and that the individual grain weight is defined by the stresses that occur after anthesis, during grain filling, showing a negative linear relationship to ET (**Hobbs and Sayre, 2001**). Nevertheless, **Dias de Oliveira et al.** (**2013**) did not record a lower *yield* for RD treatments at CETE conditions compared with the same treatment at CATA conditions. The difference in the final grain *yield* between their work and the present study could lie in the fact that at their work so high temperatures as the ones imposed in our experiment were not reached.

Therefore, as it was concluded for the well-watered regimen, plants vegetative tissue growth response at future environmental conditions when faced drought would respond better compared with current conditions due to ECO_2 effects on photosynthetic rates increases, better water loss control and biomass accumulation, the latter leading to same water use. However, the ET constraint effects on reproductive organ development would negate those ECO_2 positive effects on vegetative tissue development, together with that a great part of *biomass partitioning* was driven towards vegetative tissue enhancement at the final growth stage, giving, as a result, the recorded lower grain *yield* and, consequently, being reflected by the lower *WUEg* too (Table 5.3).

In conclusion, attending to the results obtained in our study, in the future, climate change would be more hazardous for barley grain production either at well-watered or drought regimen, threatening food security more than happens today.

5.3.2.3 Plant memory effects in barley growth, development and yield

Generally, drought accelerates plant development, but may also delay growth, retarding the spike initiation and/or causing the cessation of panicle development (**Prasad et al., 2006**). The latter is our case for those treatments that suffered a VD treatment, since, regardless of the environmental conditions, they needed more days to reach either anthesis (Table 5.1) or final maturity (Table 5.2). Hence, as other authors have stated (**Crisp et al., 2016; Martínez-Medina et al., 2016; Mendanha et al., 2020**), due to such delayed growth, those treatment plants presented a new developmental stage compared with the ones that did not pass a vegetative drought period.

5.3.2.3.1 Barley growth, development and yield of VR treatments

As it was concluded in Chapter 4 about VR treatments capacity to recover proper photosynthetic metabolism functioning, two main groups were also observed along with the studied environmental conditions for growth, biomass accumulation and grain *yield*. The first cluster encompassed the VR treatments that belonged to CATA, CETA and CETE conditions, always compared to its RC treatments. As concerns the recovery under those conditions, despite they required more days to perform vegetative growth, the lack of recovery of A_{net} (Fig. 4.3A) could explain the lower *TDW* at anthesis (Fig. 5.1C). Furthermore, we observed that the diffusional limitations were the main causes (Fig. 4.3; Chapter 4*)* to explain such lower A_{net}. The downregulation of Rubisco activity (Fig. 4.7A) and the reduction of the RuBP regeneration (Fig. 4.7B) would be owed to the limited substrate in chloroplasts, in line with what explained by **Flexas and Medrano** (**2002a**) and **Tezara et al.** (**1999**). Furthermore, **Pérez-Martín et al.** (**2014**) suggested that the absence of recovery of specific CO_2 transporter *AQPs* isoforms expression could participate in the lack of diffusional recovery. Our data showed a lower leaf *HvPIP2;1* isoform expression value, which would corroborate the above stated. Besides, the general lower *TLA* and *GLA* values found could also be ascribed to irreversible effects caused by VD on final *leaf size*, as previously have been commented (**Gray and Brady, 2016**). The reduction on both processes should result in lower CHs production, and, hence, minor *TDW* biomass at anthesis (Fig. 5.1C).

Another feature developed by VR plants was the pronounced delay in tillering formation along the last growth period, driving a substantial part of the gained biomass to vegetative tissue (Fig. 5.4), and hence, source tissue (**Prasad and Sttagenborg, 2008**). This fact, together with the greater time expended to fulfil such period, enabled VR treated plants to recover from a biomass accumulation point of view (Fig. 5.3). In agreement with this, **Mahalingam and Bregitzer** (**2019**) also analysed the recovery capacity from VD treatment of different barley plants at final maturity biomass, showing a cultivar-specific response.

However, VR plants were not able to achieve analogous grain *yields* as RC treated plants. **Wang et al.** (**2015**) and **Liu et al.** (**2017**) for wheat observed the same results as ours for grain *yield*, although they were not able to elucidate the causes that driven to such a trend. Firstly, in the case of current (CATA) VR treatment plants, a similar trend to the one observed by **Dias de Oliveira et al.** (**2015**) for wheat control plants grown at ECO_2 was shown. Concretely, lower *GF* was recorded, which could be due to the so large biomass investment on vegetative tissue growth (Fig. 5.4A), exceeding the sink capacity, and hence, diminishing the assimilates supply to

grain filling. Another possible cause that could explain our results is that VR treatment plants were not able to translocate the accumulated NSC on stems to grain filling when senescence processes governed plant behaviour since they spent a lot of time on vegetative tissue growth (**Prasad and Staggenborg, 2008**).

Secondly, for future (CETE) VR treatment plants, the same trend as the above-explained for CATA was developed, adding the fact that *FSN* formation was hastened, which led to even less grain *yield* compared with its RC treatment. Being one way or the other, it was also translated into a decrease in *WUEg*, mainly triggered by the massive delay to fulfil plant life span (Table 5.3). In this sense, it should be of interest to continue inquiring about stem NSC remobilization to grains.

Moreover, we supposed that the recorded diffusional limitations and lower A_{net} at anthesis probably were maintained along the rest of the plants life cycle. Therefore, as VR treated plants did not suffer another subsequent stress along the rest of their life span, they suffered the "costs of plant memory" (**Martínez-Medina et al., 2016**) leading to a "maladaptive plant stress memory" (**Crisp et al., 2016**), and were not able to recover properly from a grain *yield* point of view.

Nevertheless, differently to the above-explained trend for those conditions, at CATE the VR treatment presented a priming effect once passed the VD, which enabled them to face the ET effects in photosynthetic metabolism. Principally, it was achieved due to a higher *gs* (Fig. 3.5B), triggering a better heat loss and not needing to dissipate such energy compared with RC treatment (*NPQ;* Table 4.3) as **Wang et al.** (**2015**) and **Liu et al.** (**2017**) stated for wheat, translating into higher A_{net}. One possibility is that a higher basal level of stress-protective proteins and chaperones (such as HSP) –thanks to having passed a drought period at the vegetative stage– should probably be allowed them to cope better with ET negative effects, mainly at the grain formation stage (**Jagadish et al., 2014 and references therein**), which could be of interest to check it.

The result at CATE conditions, both at anthesis and maturity, was greater *final biomass* and *yield* values of VR plants compared with RC treatment plants. In this case, it was developed a "cross-talking memory acquisition", leading to a "priming" or "hardening" effect to face ET impairments as **Wang et al.** (**2015**) firstly commented, although the recorded values remained very distant from the obtained for CATA control ones.

5.3.2.3.2 Barley growth, development and yield of DD treatments

At the anthesis period, in line with the analysed for recovery, the observed results for *TDW* biomass matched with the recorded trend for the photosynthetic performance in Chapter 4. Thus, under CATA and CETE conditions, DD treatments presented lower *TDW* compared with RD treatments, whereas under CATE and CETA conditions statistically same values were registered (Fig. 5.1C), matching with drought extent registered at anthesis for each environmental condition (Fig. 3.1B, D). Thus, taking into account that either the first stress degree or the subsequent one could modulate DD treatment plants final behaviour (**Martínez-Medina et al., 2016**), in our case it could be said that the subsequent stress degree (anthesis drought) defined it.

If data for the environmental conditions where DD treatments presented lower biomass (compared with RD) are analysed, on one hand, it can be observed that diffusional limitations governed the developed lower A_{net} (Fig. 4.3A). On the other hand, the registered reduction at CETE was higher than at CATA, owed to the higher reduction on *gm* (Fig. 4.3E) and the altered Rubisco activity (Fig. 4.7A), both facts being translated in a higher decrease of A_{net} and *TDW* (Fig. 5.1C).

In an attempt to keep inquiring in the obtained results, we can notice that the more hastened effects on the photosynthetic metabolism and biomass accumulation of CETE DD treatment matched with the recorded lower *LRWC*, which we ascribed in Chapter 3 to the lower *shoot/root ratio* and more negative Ψw_{md}. To explain this fact, it is necessary to go more deeply into the biomass-partitioning pattern at anthesis and the possible effects that ET could have on it. In this regard, firstly, it has to be borne in mind that, root growth reduction by ET might be either due to direct effects on root development or due to less CHs production owed to lower A_{net} (**Gray and Brady, 2016**). Secondly, regarding our results for CETE conditions (Fig. 5.1C), it is observed that plants that suffered a VD treatment, along the recovery period, most of the produced CHs drove to shoot growth, probably in an attempt to increase heat loss (**Wang et al., 2015; Liu et al., 2017**).

In addition, differently to RD treatment, DD treatment plants at CETE when faced with anthesis drought did not invest resources on root growth development, with the subsequent effect on the worst water uptake capacity, leading to the registered lower Ψw_{md}, *LRWC and* A_{net}. Moreover, ET damage should be increased due to the lower capacity to carry out heat loss when faced drought, a common response that uses to be triggered when it acts in joint action with

drought as **Jagadish et al.** (**2014**) picked up for different crop species**,** being increased the energy dissipation processes (Table 4.2). Overall, either by lower photoassimilates production or more negative water potential (**Barnabas et al., 2008 and references therein**), it was translated in hastened effects on spikes formation (Fig. 5.1C and Table 5.2).

Going on with plants development, from anthesis until physiological maturity, irrespective of the environmental conditions, DD treatment plants developed an indeterminate growth habit, allocating a large part of the new biomass to vegetative organs (Fig. 5.4), thus increasing the source tissues (**Prasad and Staggenborg, 2008**). In addition to this fact, together with the delaying time required to fulfil the cycle (Table 5.2), it allowed them to achieve comparable *ADW* biomass as RD plants (Fig. 5.3).

However, unlike VR plants, DD treatment plants also presented equal grain *yield* compared with RD plants (Fig. 5.5), except at CETE conditions, where the previously commented negative effects on reproductive organ development at anthesis were maintained along with plants development –even accentuated by ET–, governing the observed response despite the beneficial effect of the late tillering (Table 5.3).

If we go to the literature to try to understand our results, **Mendanha et al.** (**2020**) working with two wheat cultivars grown at current environmental conditions neither observed any difference in grain *yield* between RD and DD treated plants. However, in their case, the reason was the better photosynthetic performance by the conferred priming status. Otherwise, **Liu et al.** (**2017**) despite having registered a similar priming effect on wheat photosynthetic metabolism as **Mendanha et al.** (**2020**), in their case those plants were able to translate the extra produced CHs to final higher grain *yields* compared with RD treatment. These different responses between our results and the commented ones could be owed to genetic variability that could exist between species, but also between cultivars, together with the imposed drought stress conditions both at vegetative (**Wang et al., 2015**) and anthesis (**Martínez-Medina et al., 2016; Mendanha et al., 2020**).

To sum up, taking for good the stated definition for "priming" by **Martínez-Medina et al.** (**2016**), as our plants did not record a "stress-tolerance" status (presented same grain *yield*), we cannot define as a priming effect the observed results for our DD treated plants. Nevertheless, the observed trend at CETE for DD treatment growth and *yield* could be catalogued as a ¨maladaptive stress memory¨ as it was commented too for recovery by VR treatment.

Lastly, what is clear is that the VD treatment triggered a new developmental stage on DD treatment plants, as happened in VR treatments, altering the time needed to fulfil their life span and triggering a "plant memory effect". Therefore, attending our results for barley, but also taking into account those commented for wheat by the other authors, to apply a mild vegetative drought with the aim to prime plants to better deal with subsequent stress could be a risky strategy, which final grain *yield* response might vary among different agents as genetic resources or environmental conditions. However, either under current or future environmental conditions, it is presented as a promising area that could lead to face better drought constraints on crops success.

5.4 Conclusions

- Under the well-watered regimen, future environmental conditions (CETE) diminished final grain yield compared with current (CATA) conditions. This response was governed by the hastened effects of elevated temperature (ET) on reproductive organ development, negating the positive effects triggered by the elevated CO_2 (ECO_2) on photosynthetic performance and vegetative tissue growth.
- Although CETE conditions plants yield was threatened, they presented a higher yield potential owing to the higher sink capacity triggered by ECO_2 effects on photosynthesis and vegetative and reproductive growth improvement.
- The effects of ET on barley growth were more detrimental than the ones caused by drought periods, principally on reproductive organ development, since ET stress affected plants along their whole life span. Our results are in agreement with bibliography data for the overall barley cultivars, which it becomes essential to make efforts to produce more thermo-tolerant cultivars.
- Although anthesis drought effects were more hazardous for plants water relations and photosynthetic metabolism compared with vegetative drought effects, it was not translated so directly to lower biomass accumulation and final grain yield.
- CETE conditions ameliorated drought stress effects along with vegetative growth and tissue development, whereas it exacerbated drought effects on final grain yield due to ET deleterious effects on reproductive organ development. In comparison with CATE, notable alleviation was recorded owed to ECO_2 positive effects on photosynthesis performance and vegetative and reproductive tissue development.
- Barley plants, except at CATE conditions, developed a maladaptive plant memory effect on photosynthetic metabolism and growth and yield after having passed a vegetative drought, compromising its ability to recover and face another drought period, developing a new developmental status and delaying growth. In an attempt to reduce the negative effects on reproductive organ development, barley plants presented an indeterminate growth habit by forming late tillers to improve sink capacity.
- At CATE, VD treatment primed VR treatment plants against ET. These plants were able to develop a priming effect on photosynthetic metabolism developing a better heat loss and allowing them to ameliorate ET adverse effects on grain yield. However, the ET effect on grain production remained very large.

- In the case of plants that suffered a doubled drought period (DD), they presented the same final biomass and grain yield as RD treatment plants, not developing a priming effect, whereas at CETE conditions maladaptive memory stress was developed.

CHAPTER 6

GENERAL CONCLUSIONS

6. General conclusions

The general objective of this PhD thesis has been to analyse future climatic conditions effects on barley physiological-biochemical and molecular processes and to inquire through which mechanisms affect growth, development and grain yield. In addition, both under current and future environmental conditions, plants recovery capacity after having suffered a vegetative mild drought, and the possible priming effect as a management strategy to deal with subsequent drought stress, has been studied.

To reach these goals, the two main plant physiological processes that could explain final growth and grain yield, namely, plants water relations and photosynthetic metabolism, have been deeply studied. In summary, the main conclusions that can be drawn from this work are:

1. Under future environmental conditions, barley plants faced better anthesis drought impairments due to an isohydric behaviour, leading to a more conservative strategy than under current conditions, driven by better stomatal control and an isoform-specific *AQPs* expression pattern. It allowed maintaining plant water relations in a better status and leaves greenness for longer.
2. Thanks to the better water status, future environmental conditions plants were able to maintain their photosynthetic performance to a higher extent than under current conditions plants. A possible switch on towards a CO_2 transport function by an isoform-specific *AQPs* response at future environmental conditions could lead to the higher photosynthetic performance.
3. Because of the above-mentioned, future climatic conditions plants that suffered an anthesis drought period presented higher biomass than current conditions plants. However, this was not translated into a higher yield, obtaining even lower values. The reasons were that, on one hand, future environmental conditions plants presented an indeterminate growth habit, allowing them to develop a greater source and sink organs, but driving more allocates to vegetative tissue growth rather than to grains. On the other hand, the future elevated temperature damaged grain formation and grain filling period, giving, as a result, a lower grain setting per spike, grain filling and individual grain weight. Therefore, future barley production could be more affected, jeopardizing, even more, food security.
4. Although future environmental conditions plants recorded lower yields, they presented a higher potentiality due to the greater sink formation. Breeders should exploit genetic

background to break the trade-off between yield-trait components and to control biomass partitioning between vegetative and reproductive organs to translate into greater grain production.

5. Under current or future conditions, plants that suffered a mild drought at the vegetative stage were not able to recover proper photosynthetic metabolism function owed to diffusional limitations triggered by a maladaptive memory effect, where the lower expression of the *HvPIP2;1 AQP* seemed to be involved. Those plants presented a new developmental stage triggered by a delay in growth. A late tillering formation enabled them to reach the same final biomass mainly constructing new vegetative/source tissue. Nevertheless, it was not enough to equalize grain yield compared with controls.
6. The elevated temperature (ET) is presented as the main climate change factor that compromises barley grain production, which geneticists, physiologists and breeders should pay more attention to it to ensure future global food security. Our results are consistent with the available information in the literature. One of the possible management strategies that could be considered to alleviate ET negative effects on grain production is the priming or hardening effect that plants can develop after having passed a mild vegetative drought period. This cross-talk effect might allow barley plants to develop a better heat loss, leading to greater photosynthetic performance, carbohydrates production, plant growth and grain production. There is still a long way to go.
7. Plants that had suffered a mild vegetative drought period at the vegetative stage did not develop a priming effect when suffered subsequent drought stress at anthesis. Even more, under current and future conditions, double drought treatments plants recorded a greater extent on photosynthetic reduction mainly due to diffusional limitations, which the response matched with drought extent.
8. In line with the previous conclusion, the higher reduction was not translated into lower yield under current conditions due to the late tillering effect. At future environmental conditions and double drought, however, less grain yield was recorded. This fact, was probably due to the lack of capacity of these plants to driven allocates to root development, triggering the worst water status and hence, photosynthetic performance and carbohydrates production, which together with the hastened effects of ET on spikes and grain development, compromised more final yield.

CHAPTER 7

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7. References

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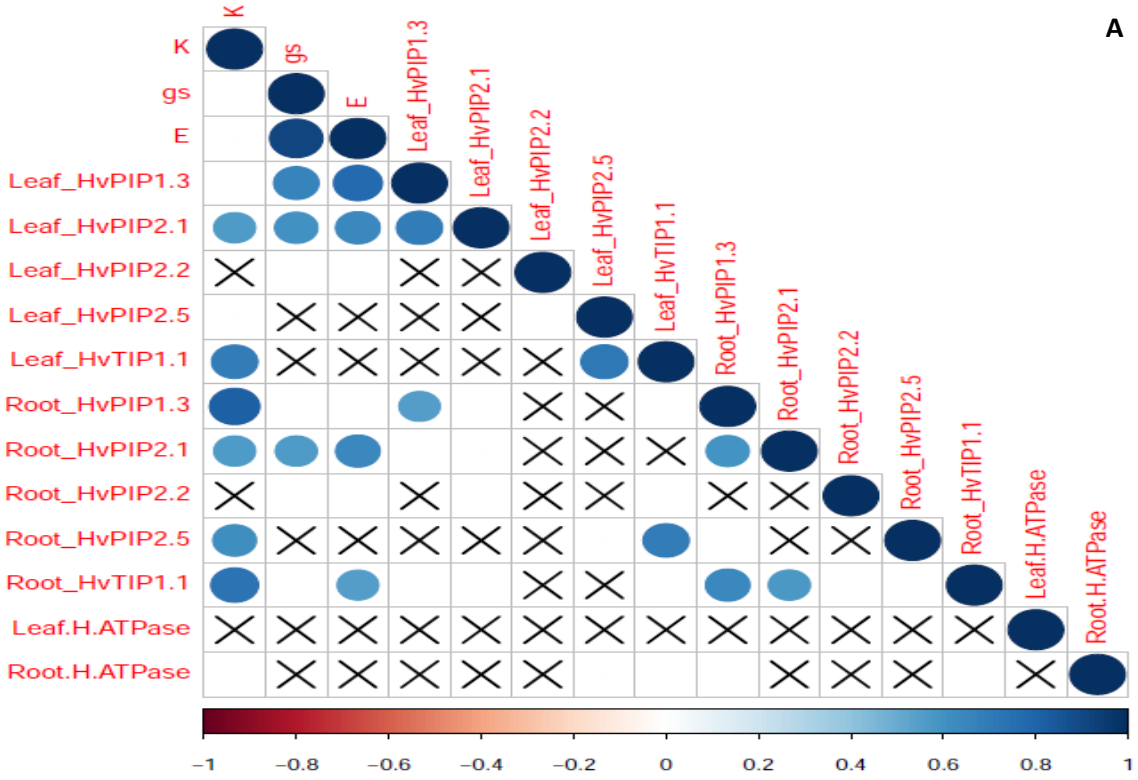
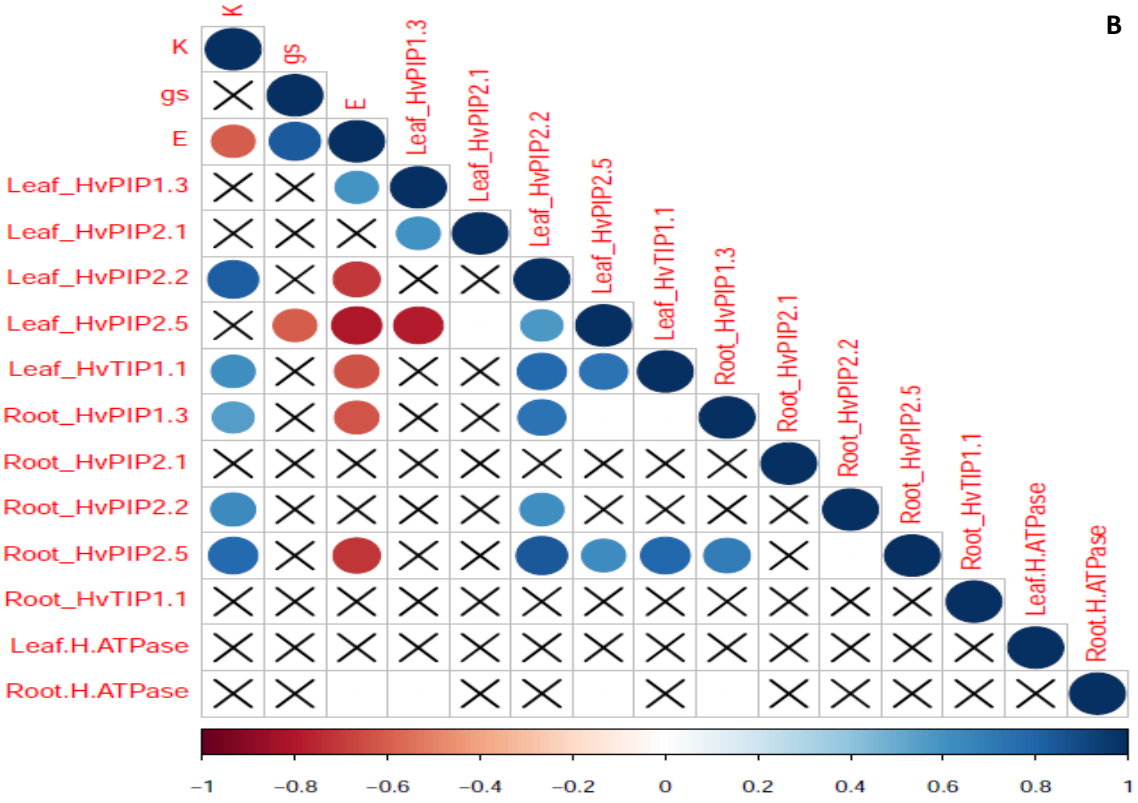
CHAPTER 8

SUPPLEMENTARY FIGURES

8. Supplementary figures





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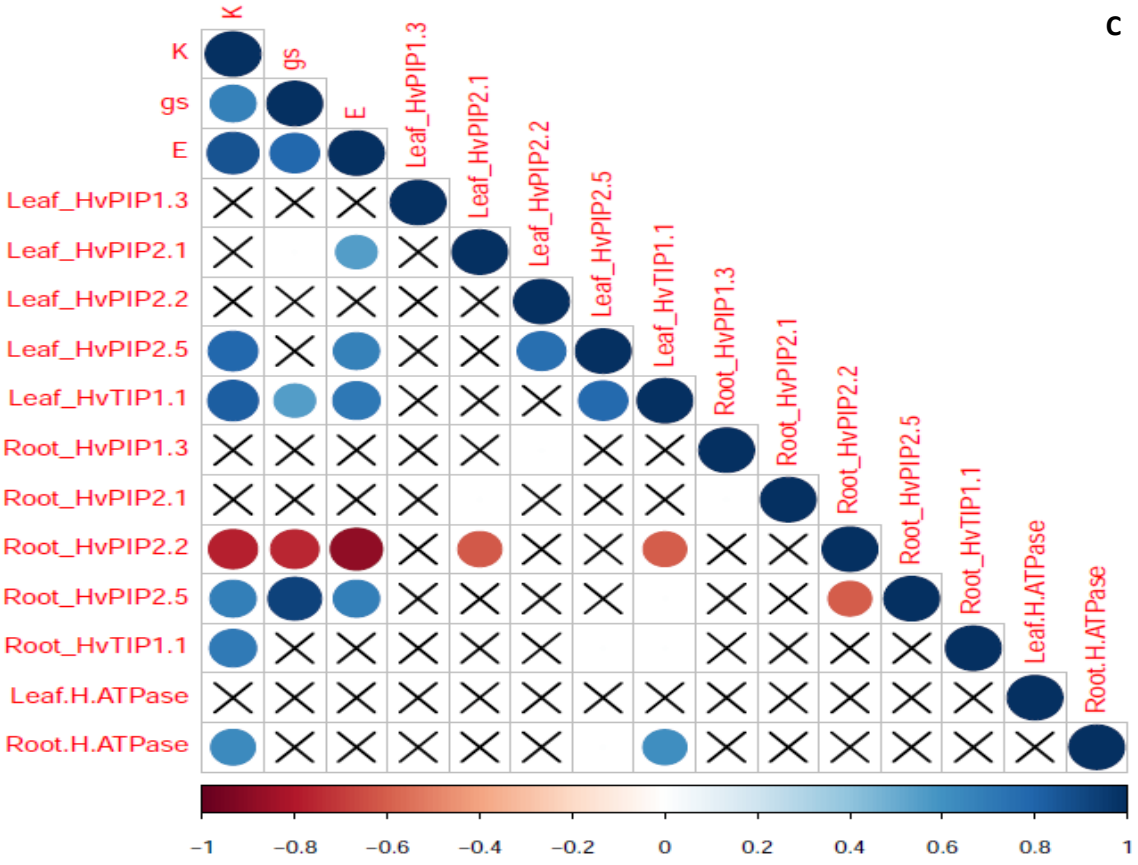
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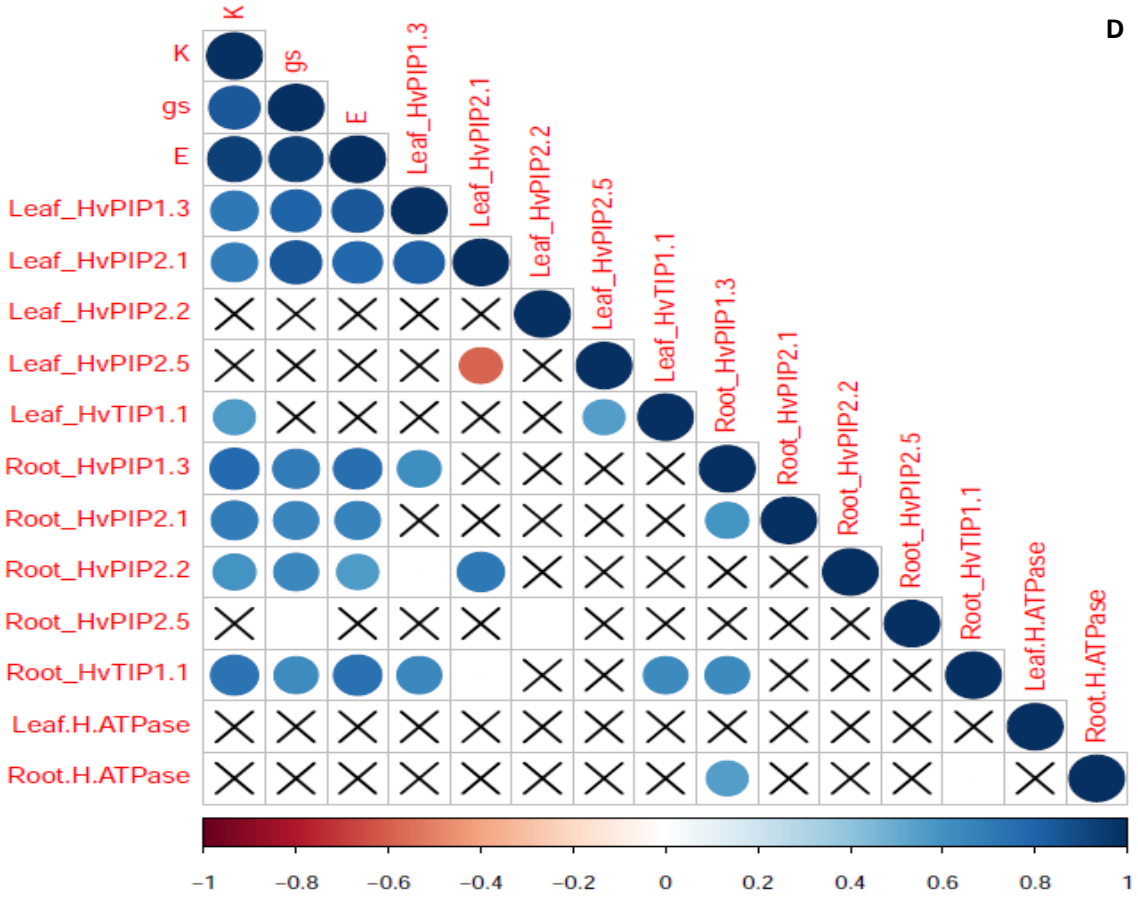
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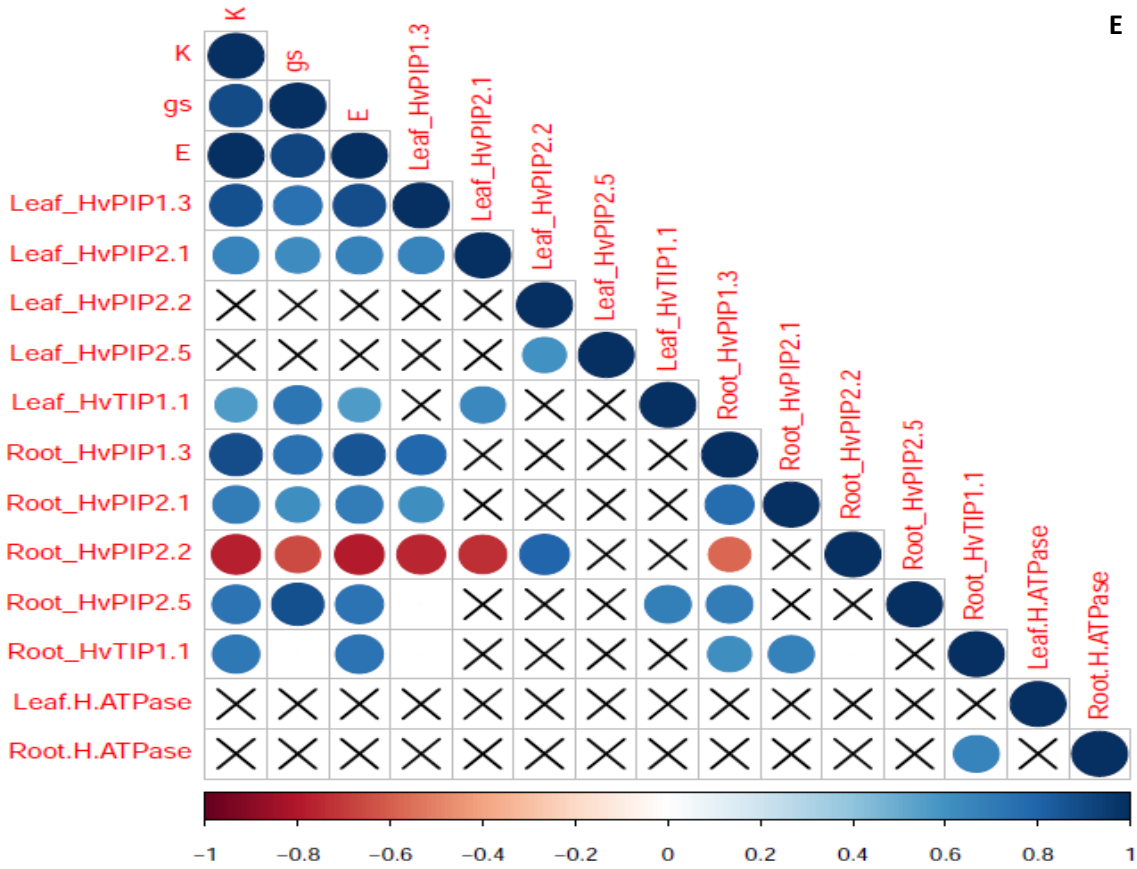
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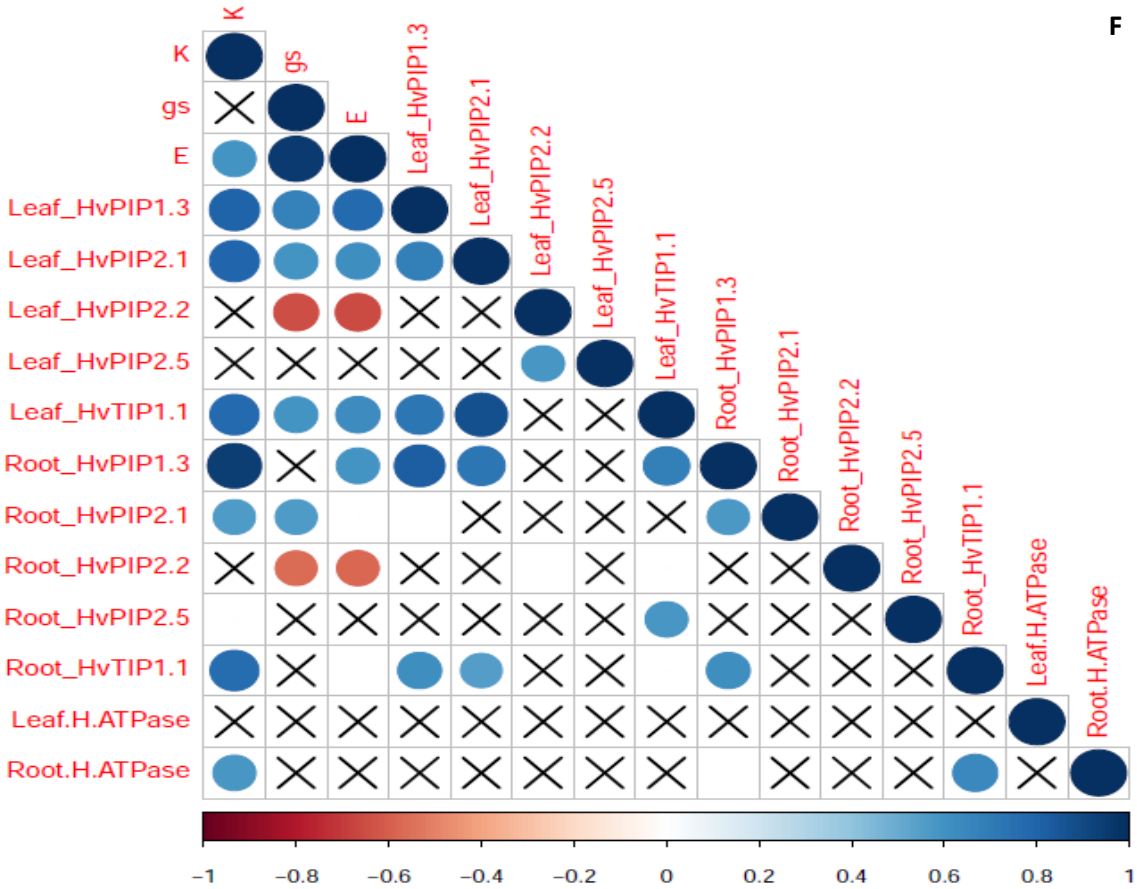
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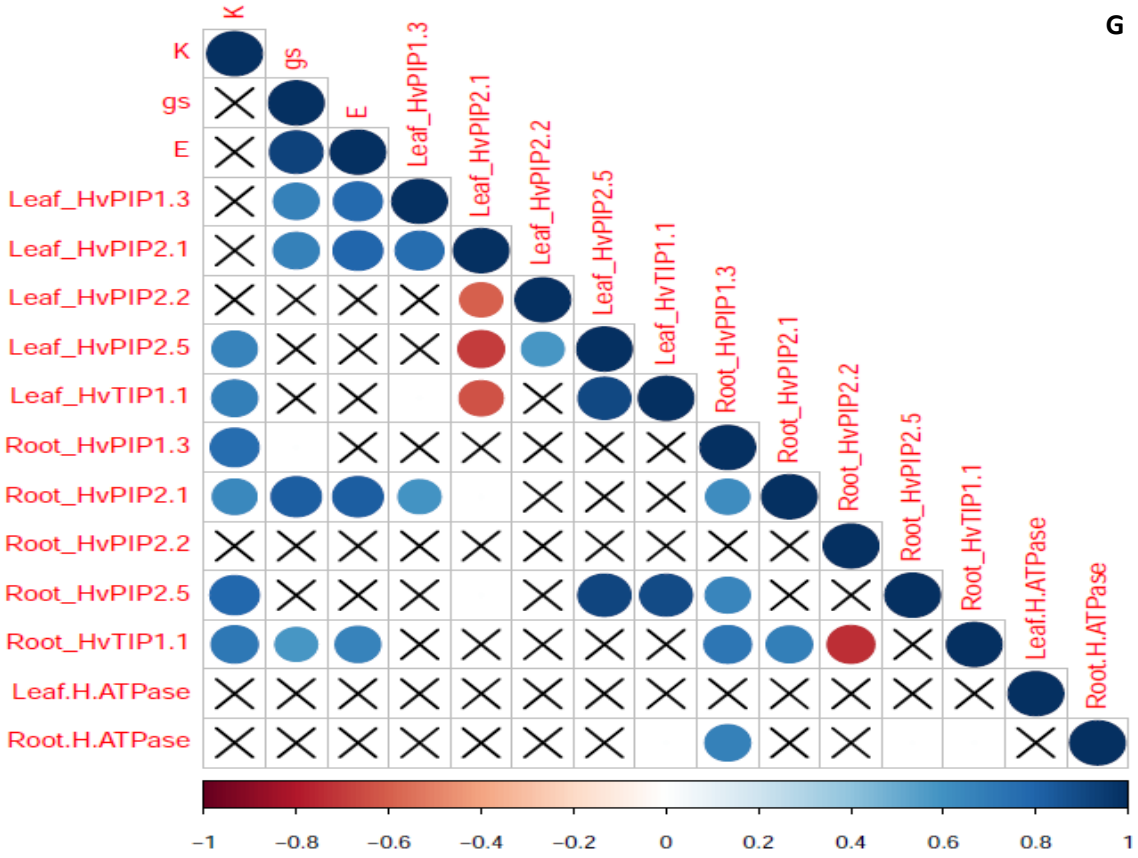
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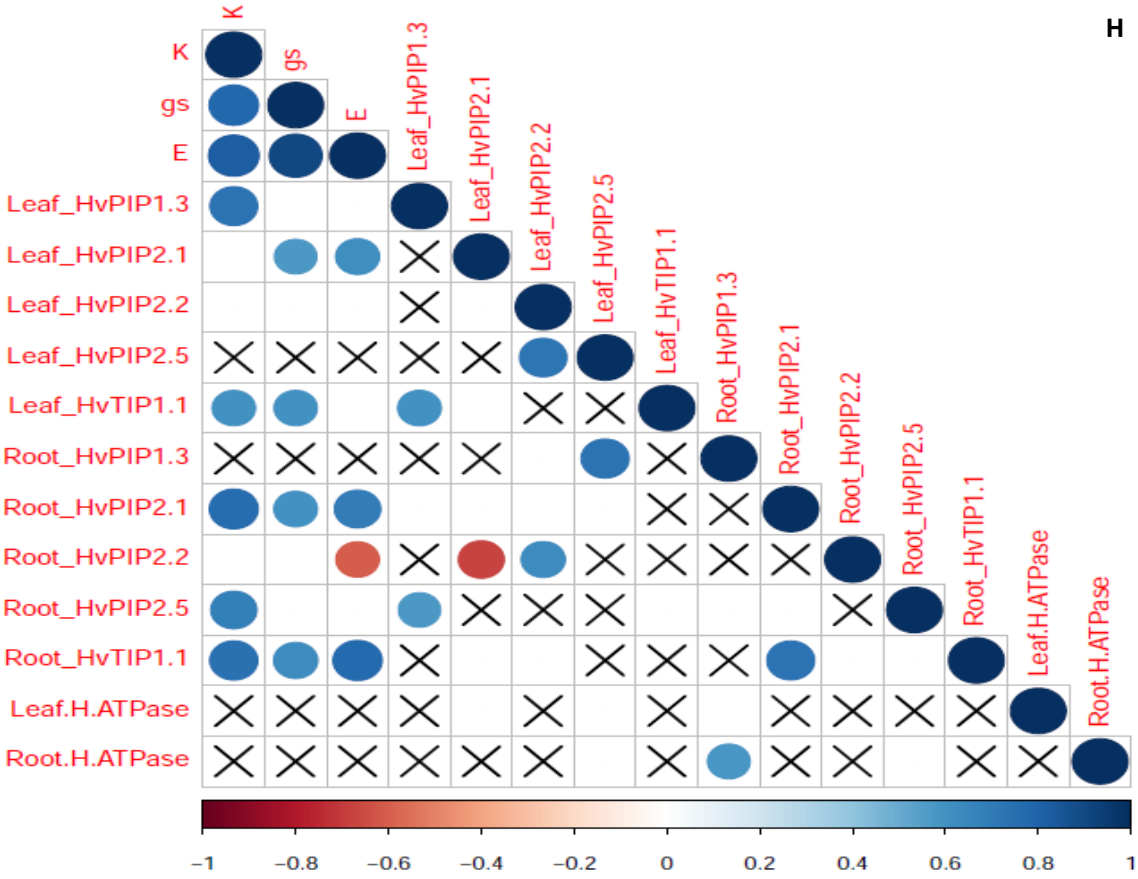
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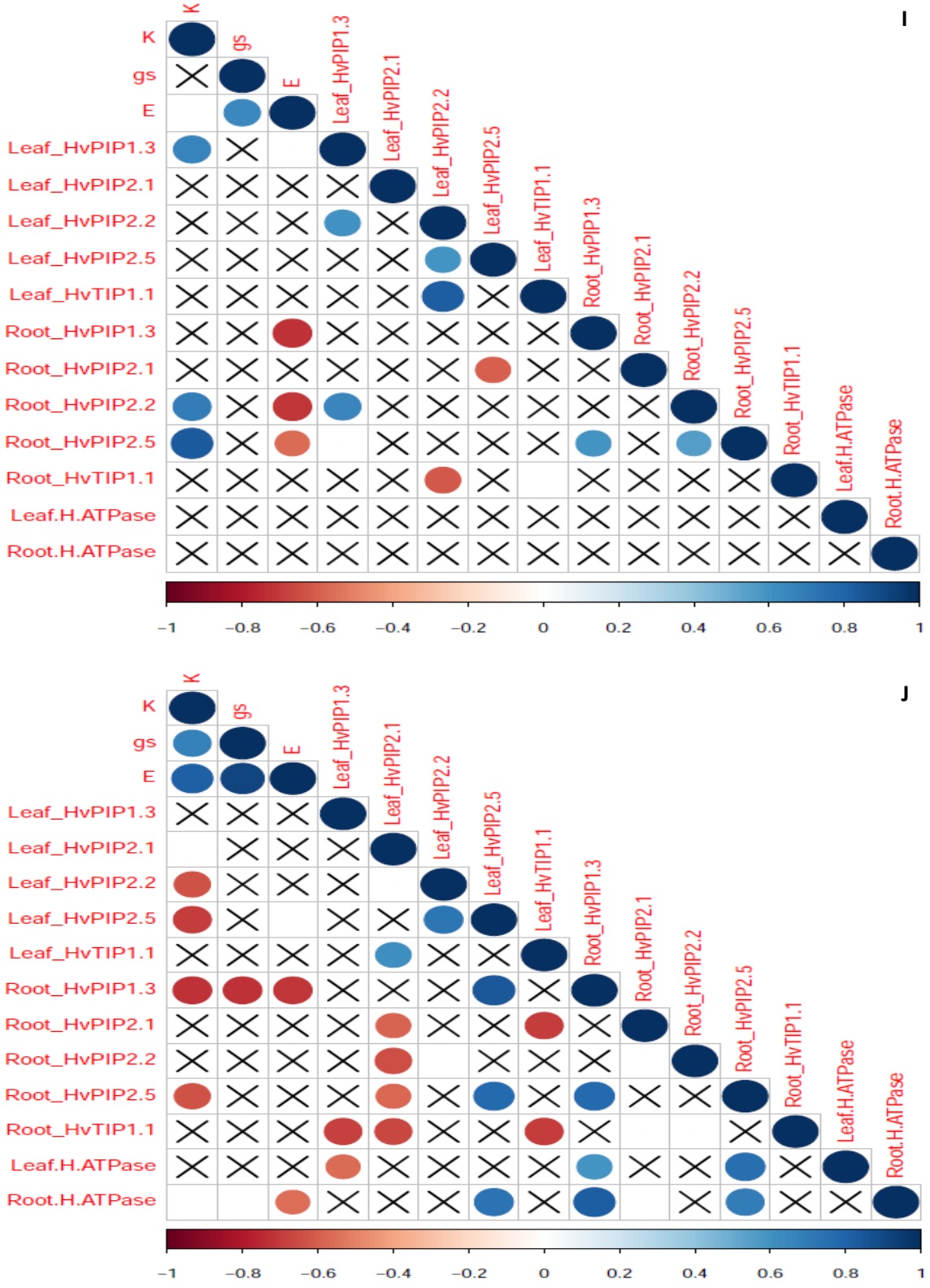
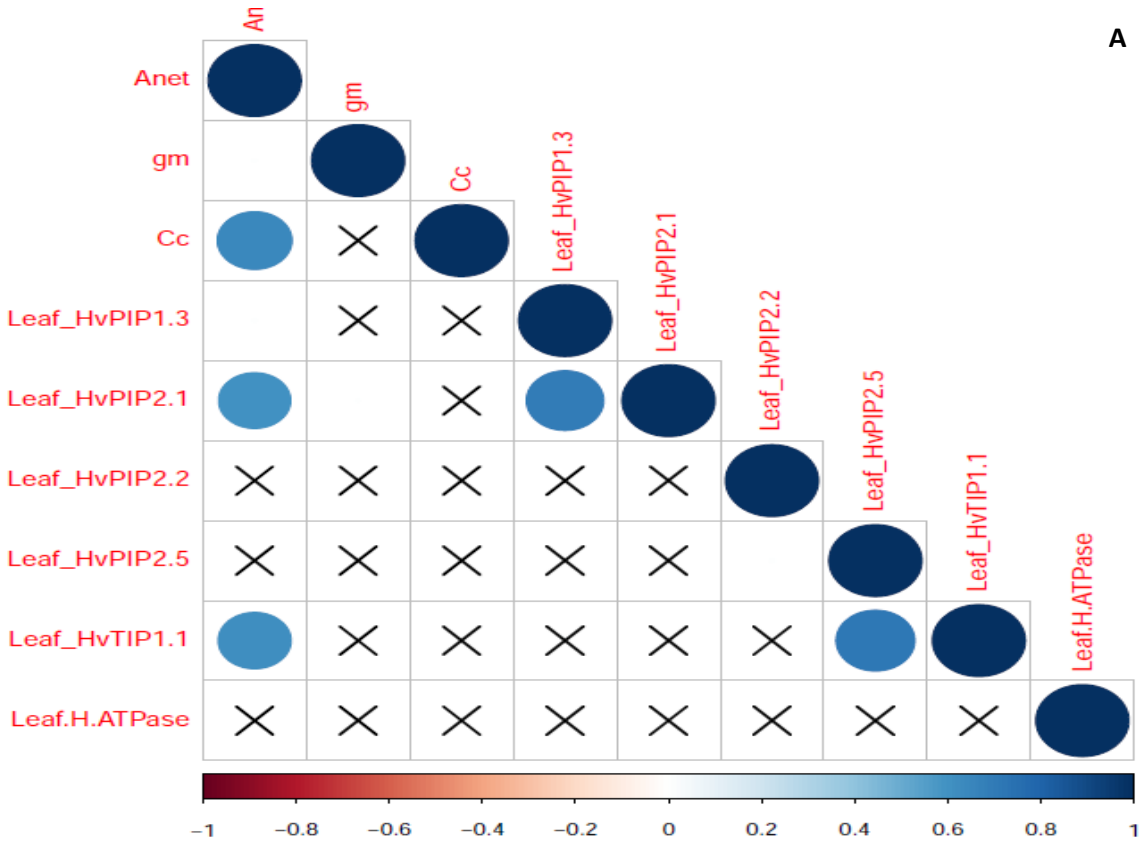


Fig. S1. Correlation matrixes for water related physiological parameters (*K, gs, E*) and root and leaf aquaporin expression data. A= all treatments; B= Control; C= Droughts; D= Vegetative; E= Anthesis; F= CATA; G= CETE; H= Plant memory; I= Recovery; J= Priming. Growth conditions are as described in Fig. 3.1. The information related for each correlation matrix is explained in Table 3.2. The crosses significate that the correlation did not meet P< 0.05 and the blank boxes significate that the correlation did not meet r> ±0.55. Legend is depicted in the figure.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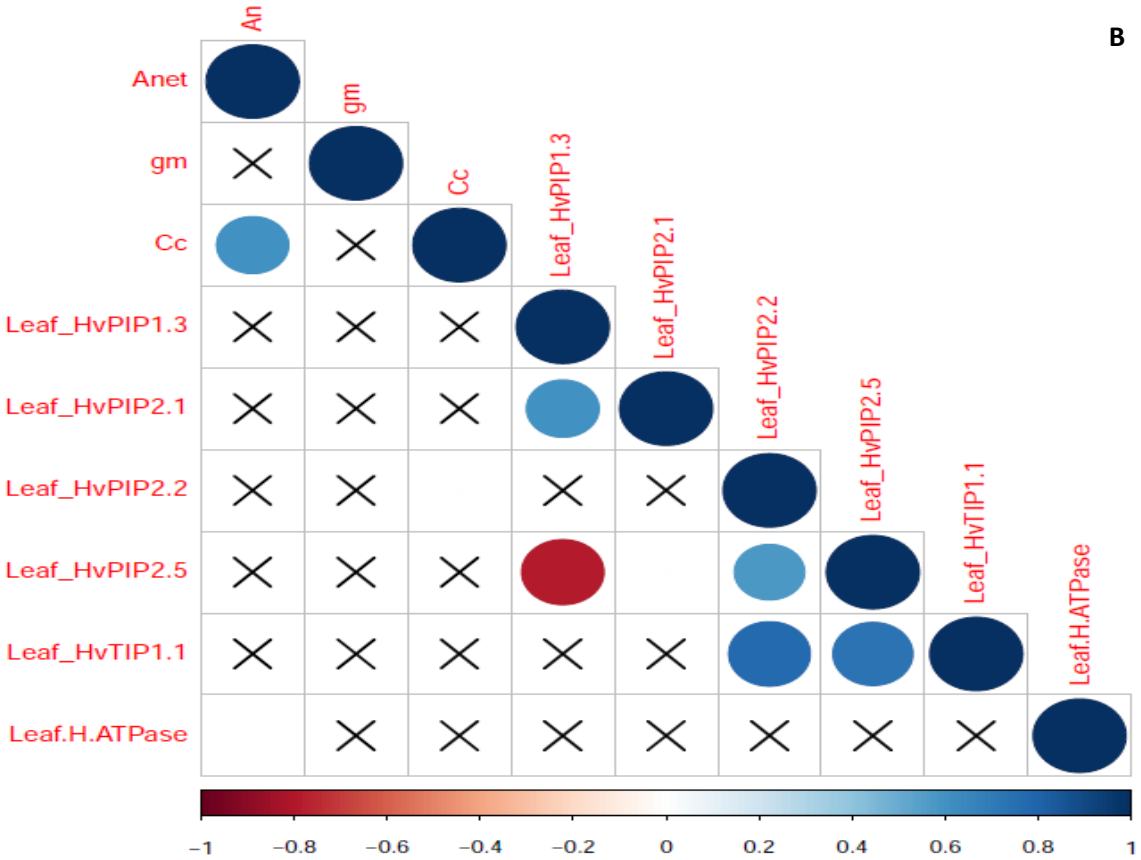
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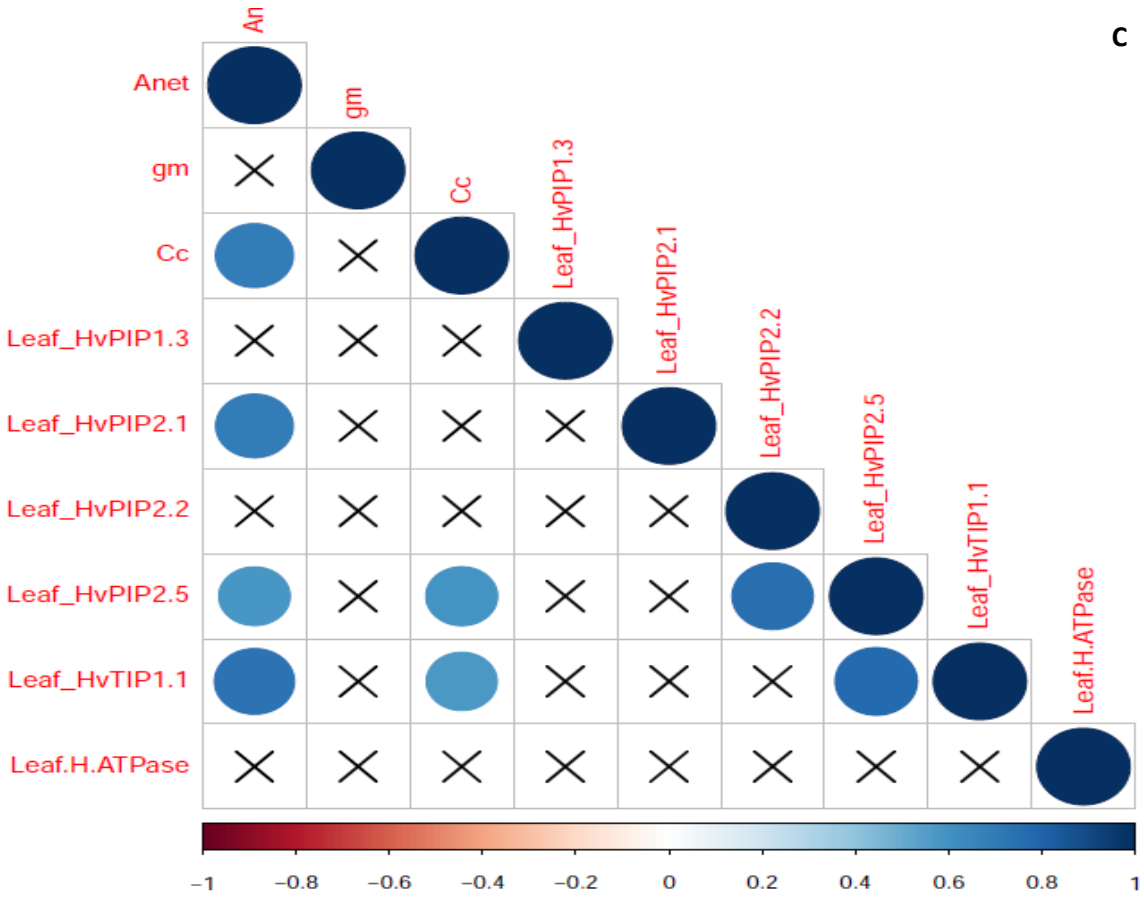
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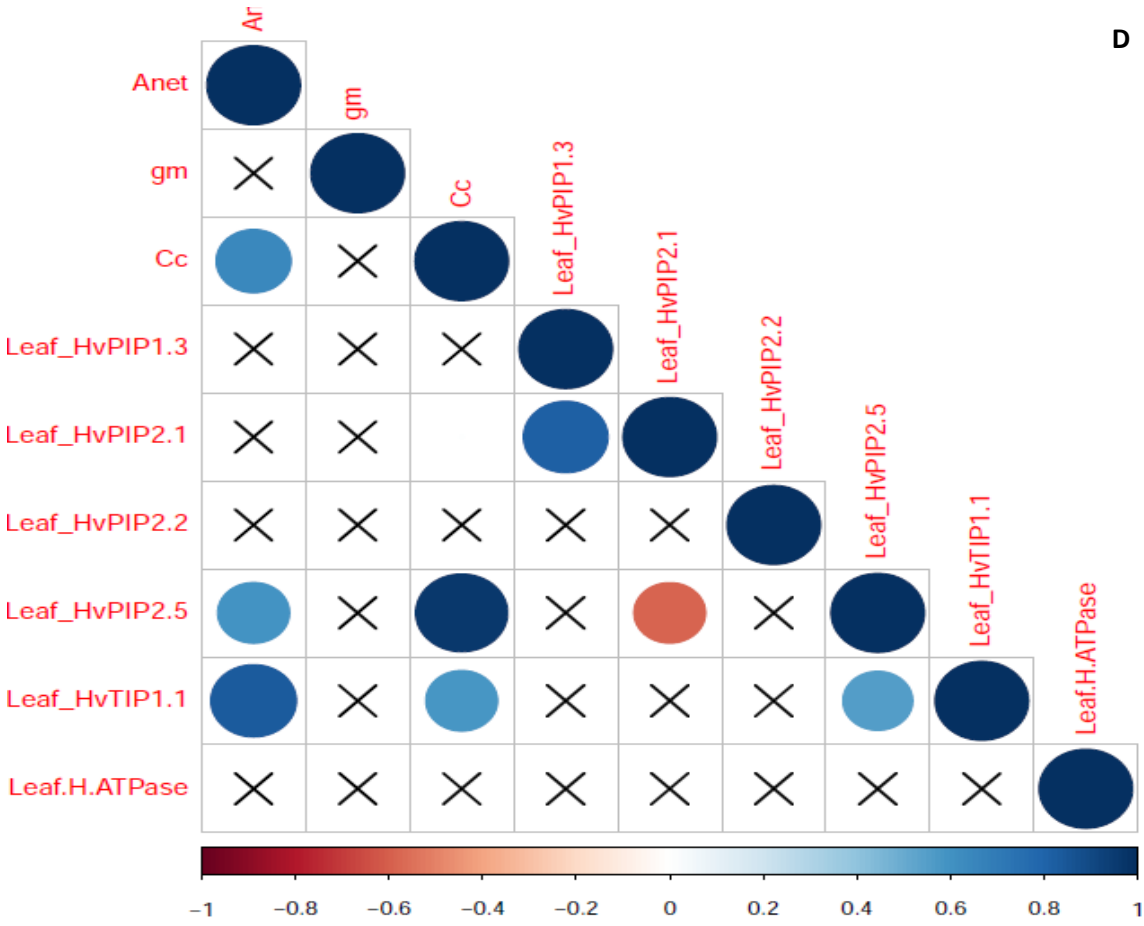
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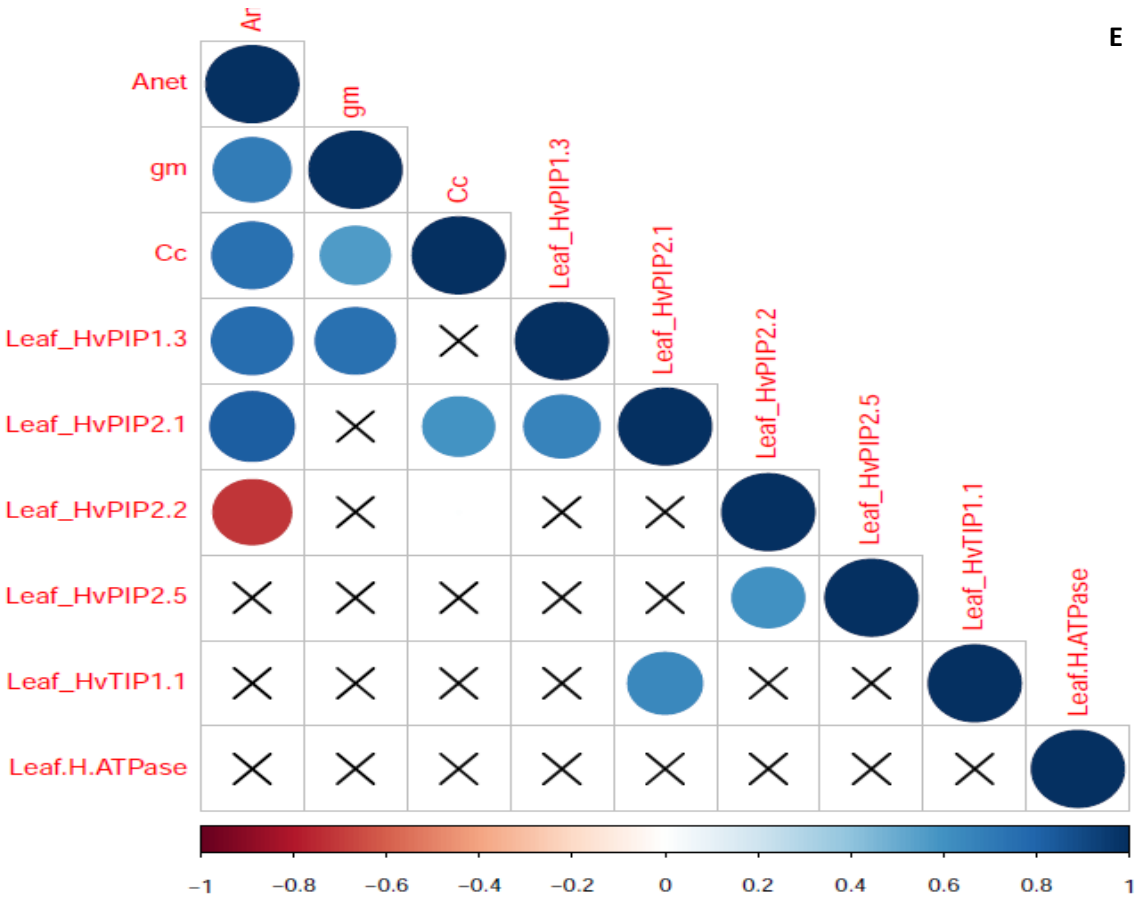
−0.6
−0.4
−0.2
0
0.2
0.4
0.6
0.8
1


E
Ar
Anet
gm
Cc
Leaf_HvPIP1.3
Leaf_HvPIP2.1
Leaf_HvPIP2.2
Leaf_HvPIP2.5
Leaf_HvTIP1.1
Leaf.H.ATPase
−1
−0.8
−0.6
−0.4
−0.2
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0.4
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0.8
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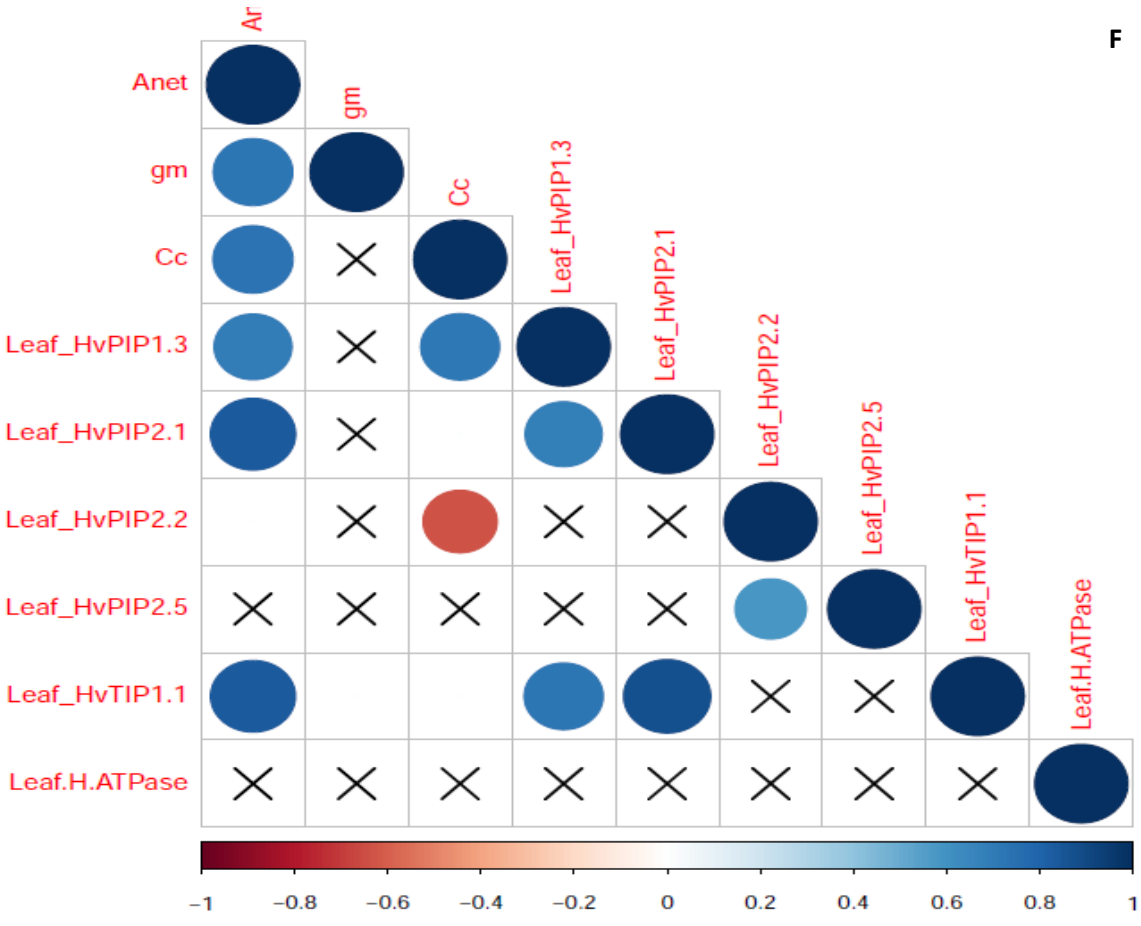
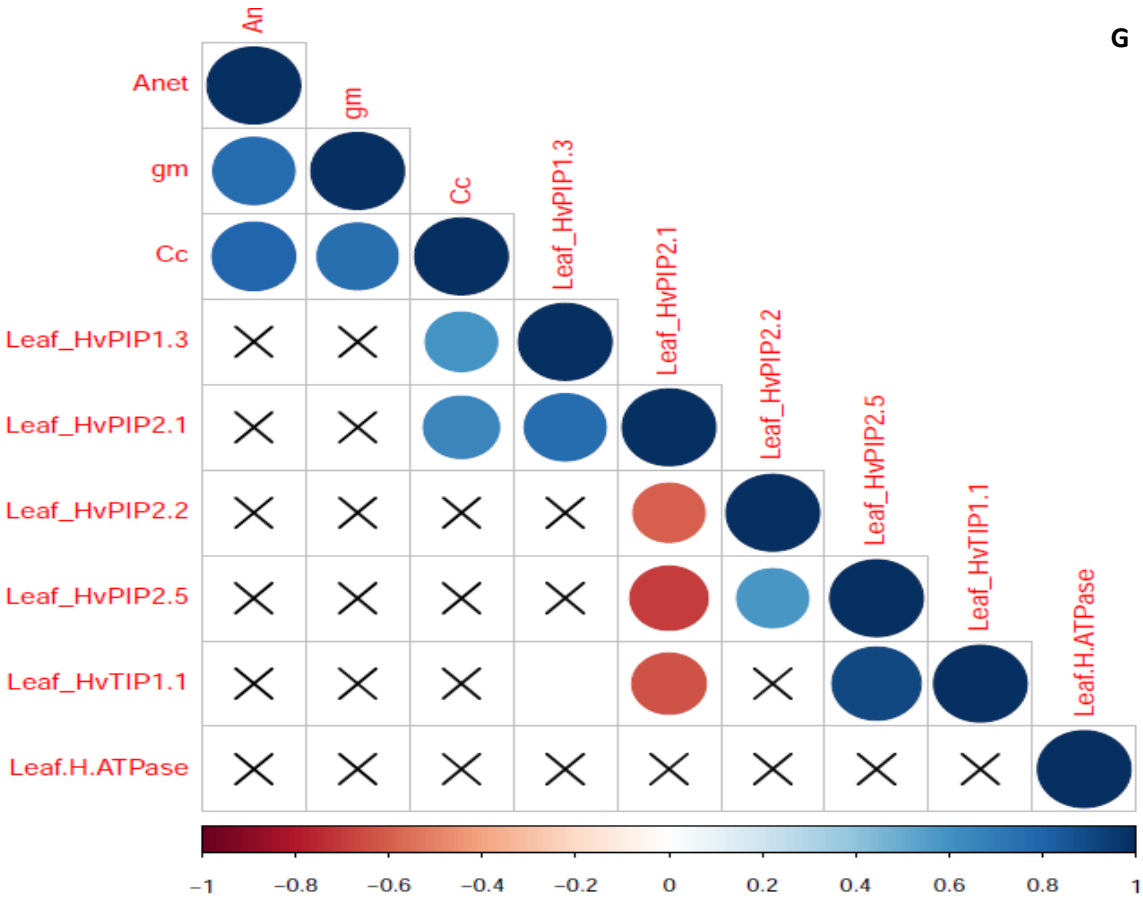

F
Ar
Anet
gm
Cc
Leaf_HvPIP1.3
Leaf_HvPIP2.1
Leaf_HvPIP2.2
Leaf_HvPIP2.5
Leaf_HvTIP1.1
Leaf.H.ATPase
−1
−0.8
−0.6
−0.4
−0.2
0
0.2
0.4
0.6
0.8
1

G


An
gm
Cc
Leaf_HvPIP1.3
Leaf_HvPIP2.1
Leaf_HvPIP2.2
Leaf_HvPIP2.5
Leaf_HvTIP1.1
Leaf.H.ATPase
Anet
gm
Cc
Leaf_HvPIP1.3
Leaf_HvPIP2.1
Leaf_HvPIP2.2
Leaf_HvPIP2.5
Leaf_HvTIP1.1
Leaf.H.ATPase
−1
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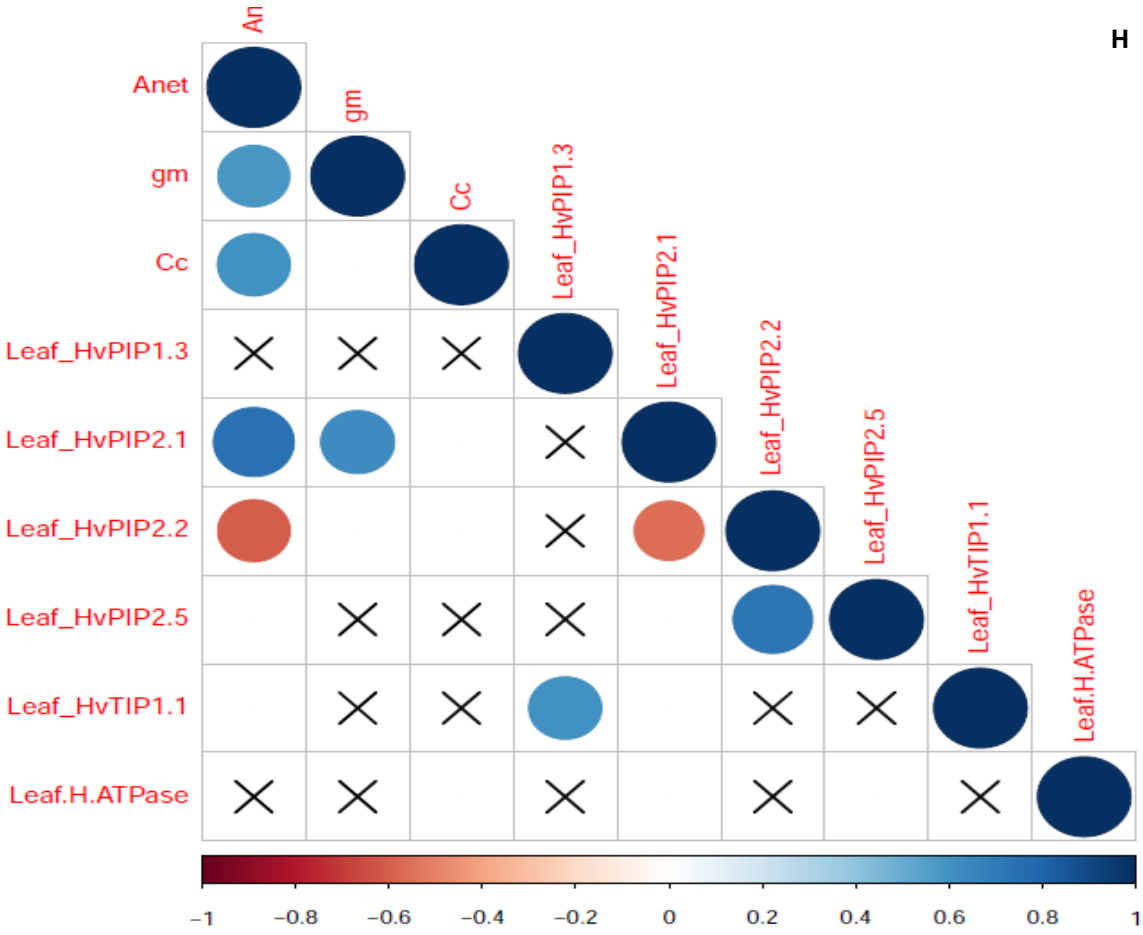
−0.6
−0.4
−0.2
0
0.2
0.4
0.6
0.8
1

H


An
gm
Cc
Leaf_HvPIP1.3
Leaf_HvPIP2.1
Leaf_HvPIP2.2
Leaf_HvPIP2.5
Leaf_HvTIP1.1
Leaf.H.ATPase
Anet
gm
Cc
Leaf_HvPIP1.3
Leaf_HvPIP2.1
Leaf_HvPIP2.2
Leaf_HvPIP2.5
Leaf_HvTIP1.1
Leaf.H.ATPase
−1
−0.8
−0.6
−0.4
−0.2
0
0.2
0.4
0.6
0.8
1

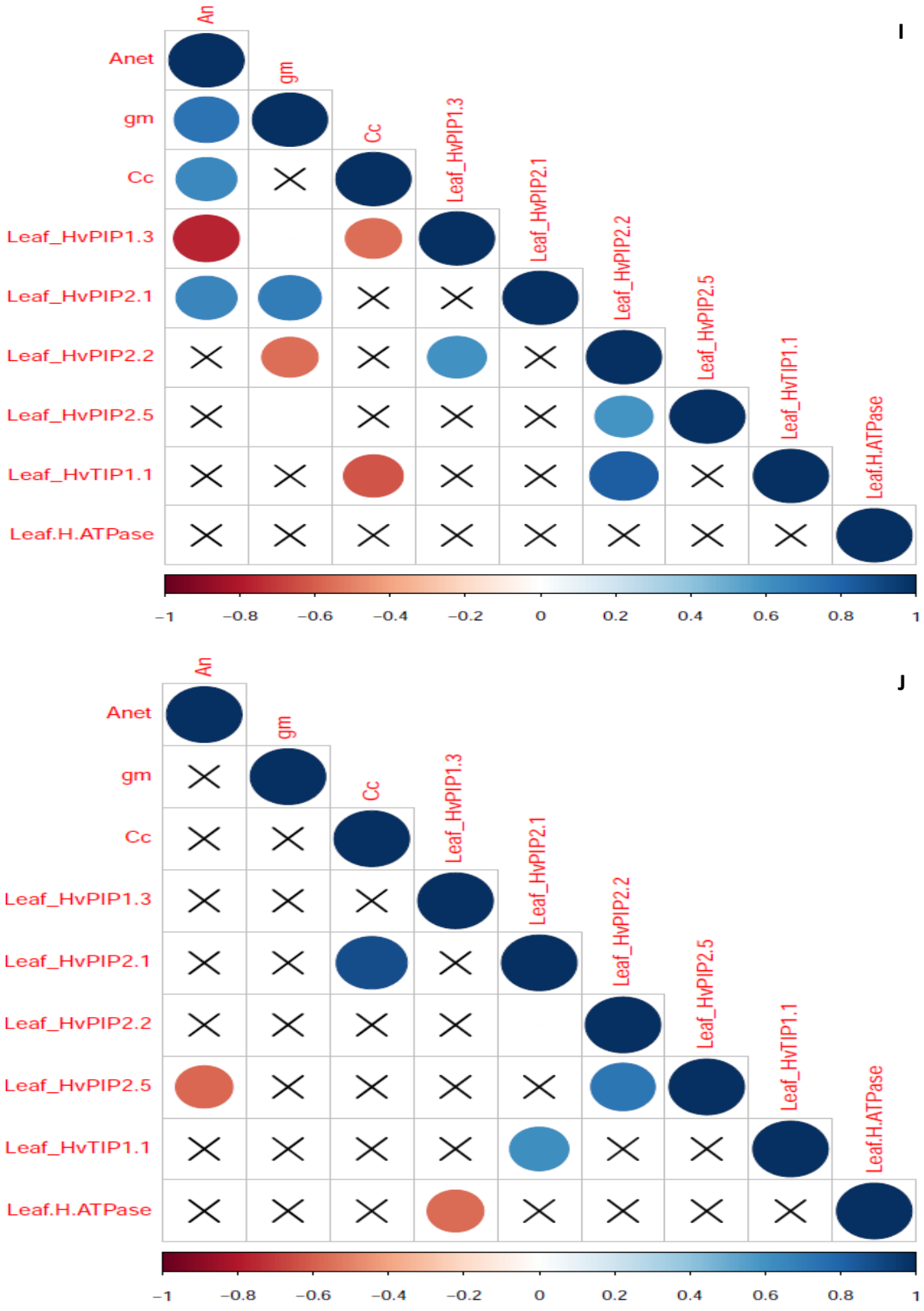


Fig. S2. Correlation matrixes for CO_2 transport related physiological parameters (*Anet, gm, Cc*) and leaf aquaporin expression data. A= all treatments; B= Control; C= Droughts; D= Vegetative; E= Anthesis; F= CATA; G= CETE; H= Plant memory; I= Recovery; J= Priming. Growth conditions are as described in Fig. 3.1. The information related for each correlation matrix si explained in Table 4.2. The crosses significate that the correlation did not meet P< 0.05 and the significate that the correlation did not meet blank boxes r> ±0.55. Legend is depicted in the figure.

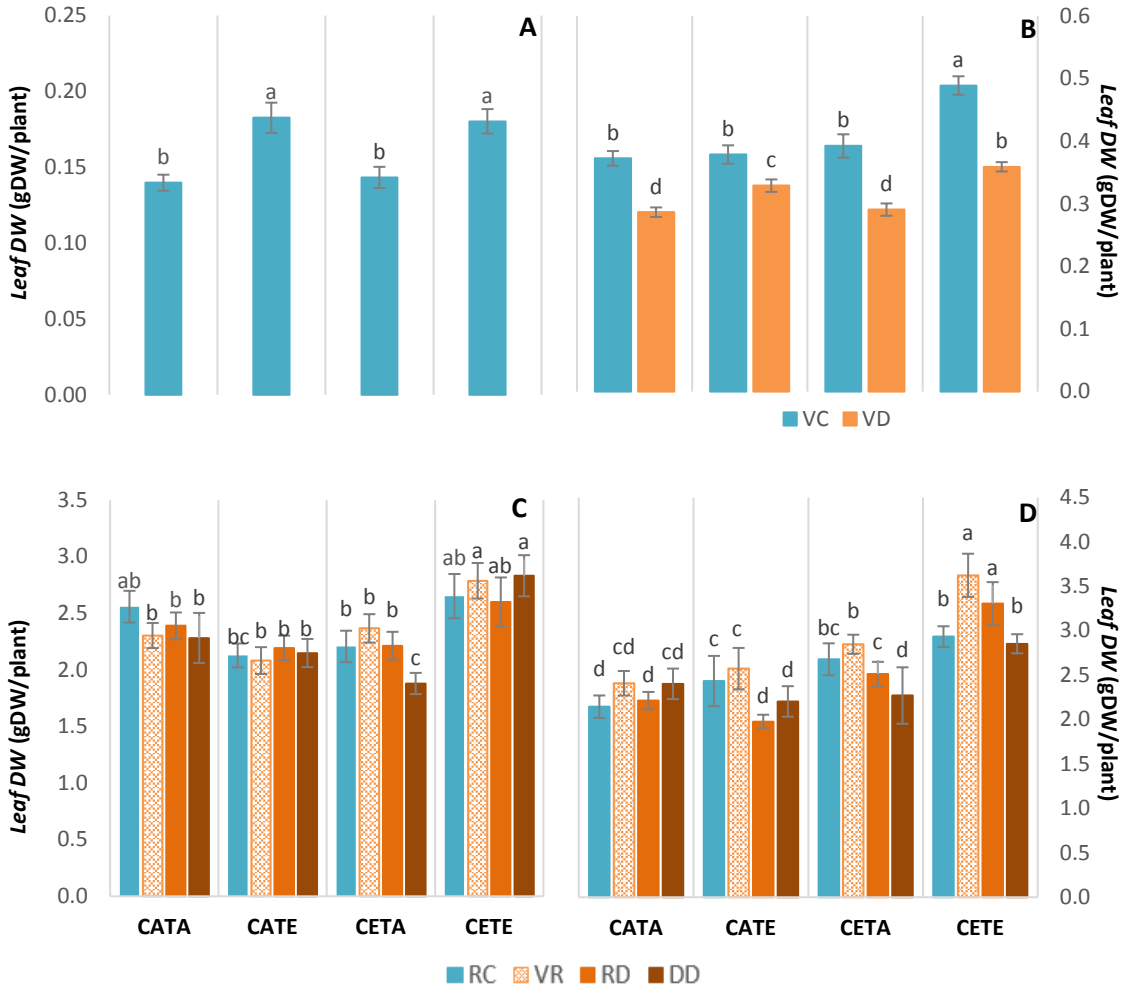


Fig. S3. Leaf dry weight (*Leaf DW*) at the early-vegetative (A), vegetative (B), anthesis (C) and maturity (D) stages. Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.

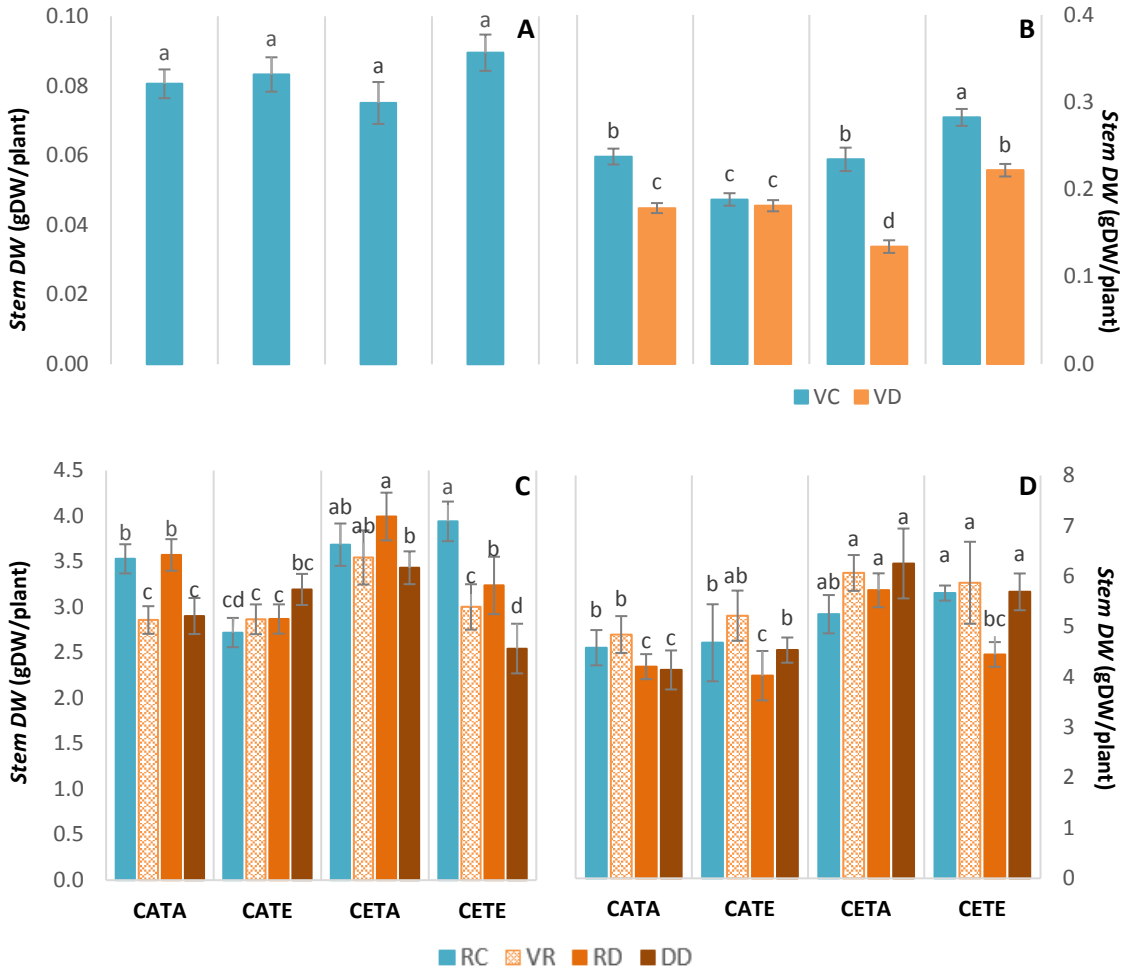


Fig. S4. Stem dry weight (*Stem DW*) at the early-veegtative (A), vegetative (B), anthesis (C) and maturity (D) stages. Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.

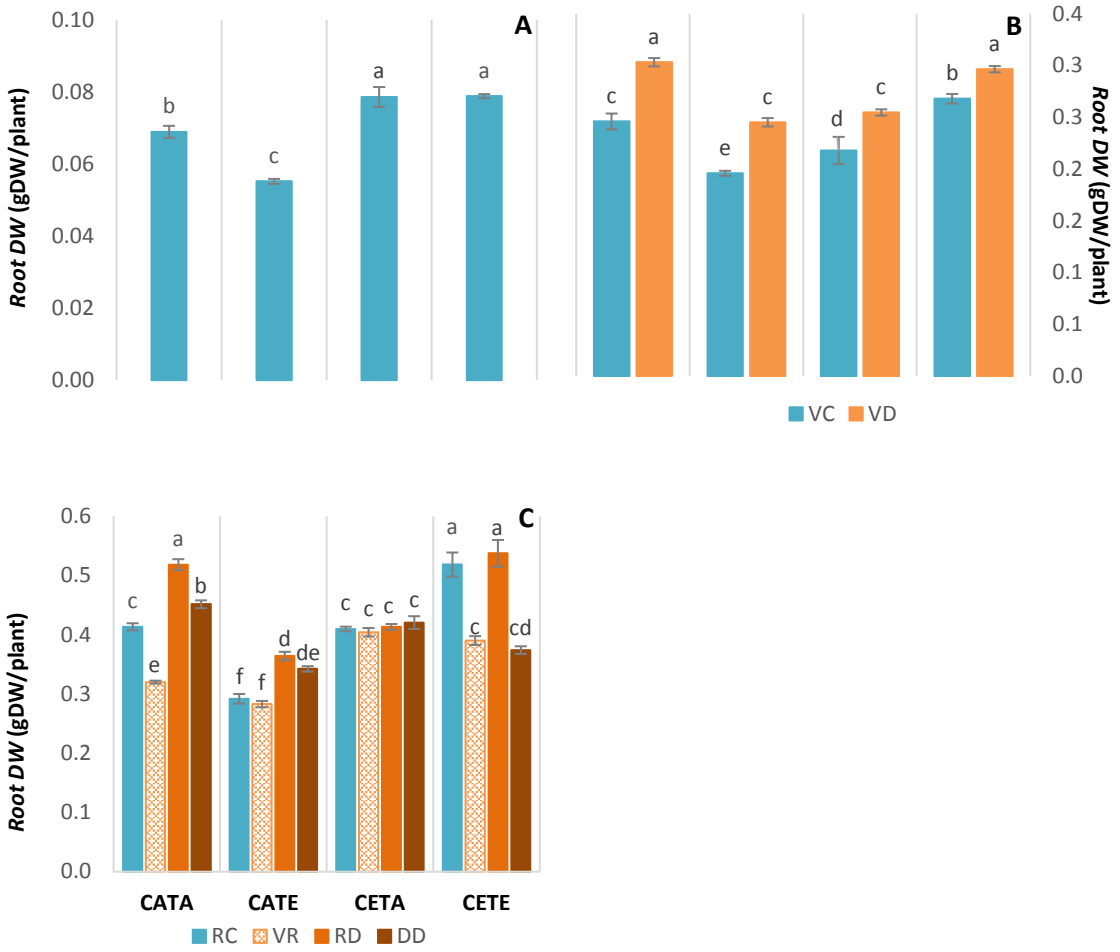


Fig. S5. Root dry weight (*Root DW*) at the early-vegetative (A), vegetative (B) and anthesis (C) stages. Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.

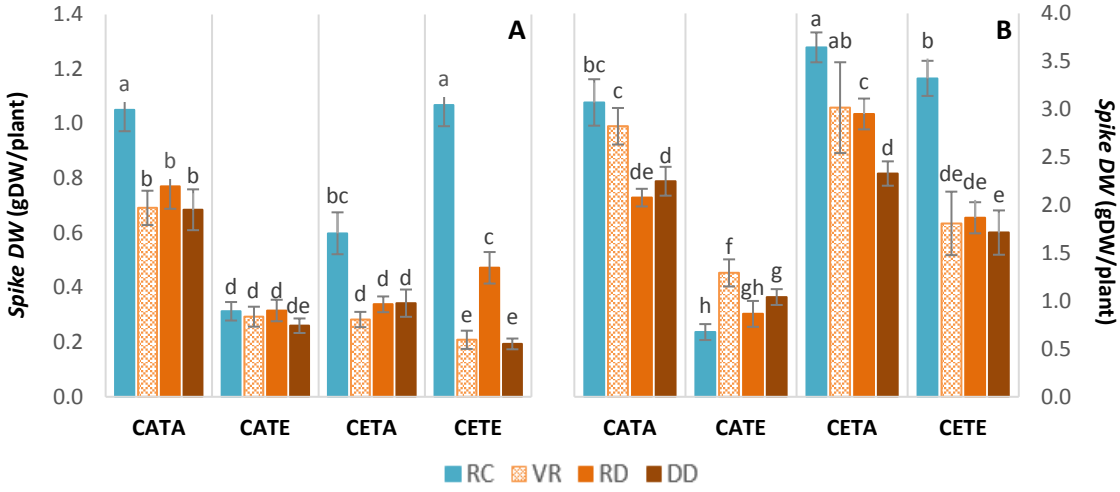


Fig. S6. Spike dry weight (*Spike DW*) at anthesis (A) and maturity (B) stages. Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.

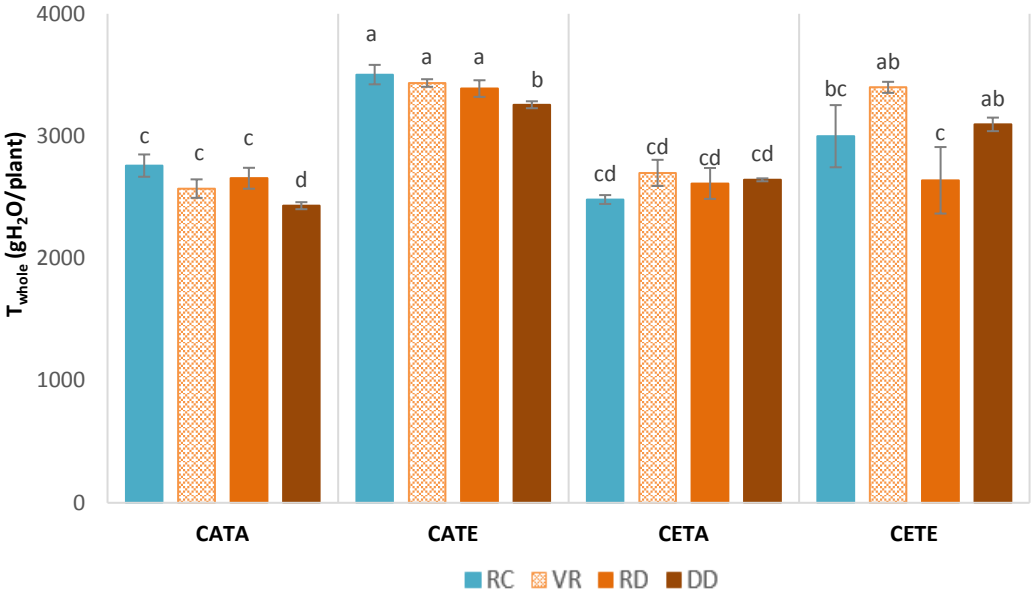


Fig. S7. Plant transpiration along the whole life cycle. Growth environmental conditions, water regime treatments and statistical analysis are as described in Fig. 3.1.