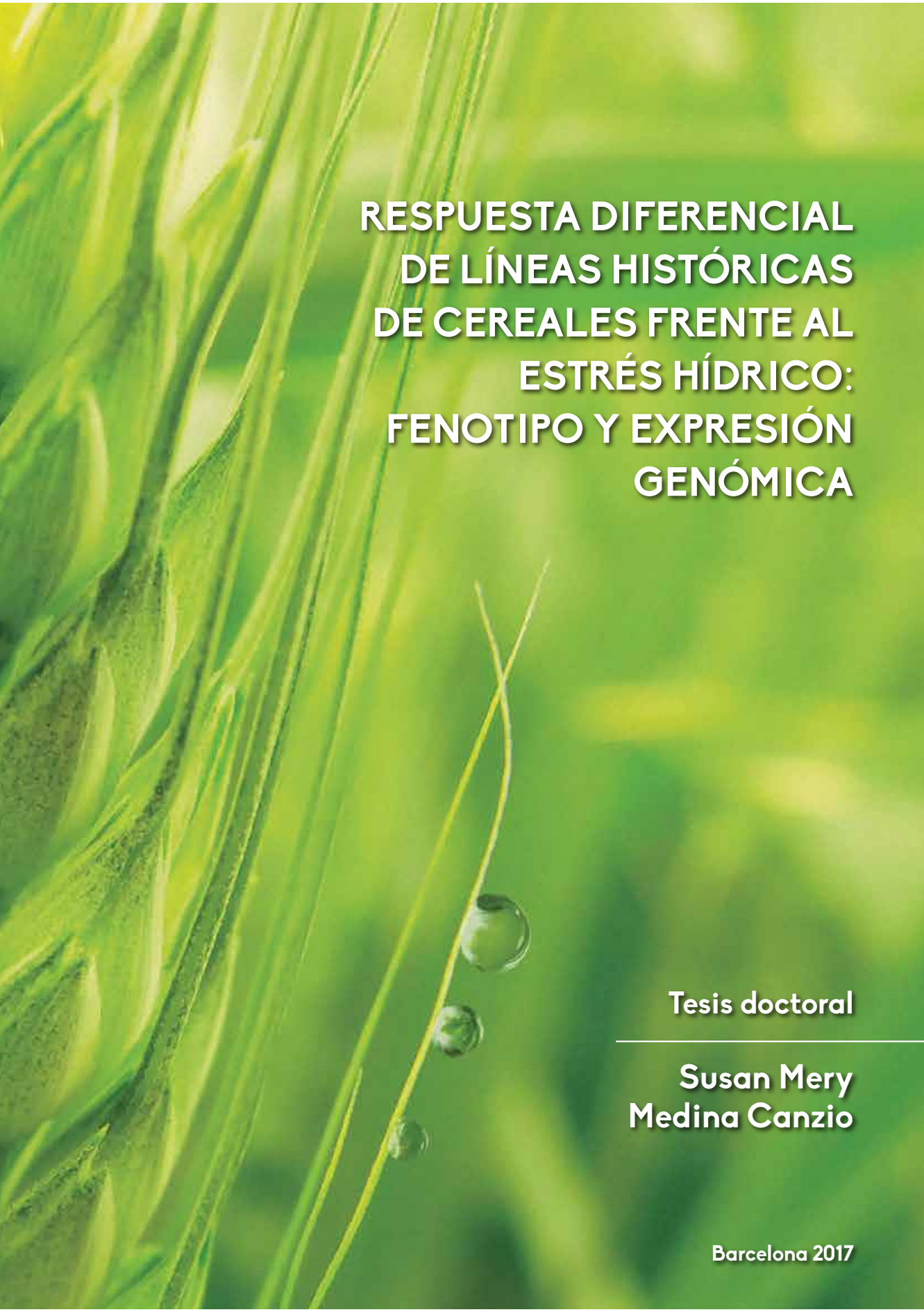
UNIVERSITAT DE BARCELONA

Differential responses of historical cereal lines to water stress: phenotype and gene expression

Respuesta diferencial de líneas históricas de cereales frente al estrés hídrico: fenotipo y expresión genómica

Susan Mery Medina Canzio



RESPUESTA DIFERENCIAL DE LÍNEAS HISTÓRICAS DE CEREALES FRENTE AL ESTRÉS HÍDRICO: FENOTIPO Y EXPRESIÓN GENÓMICA

Tesis doctoral

Susan Mery
Medina Canzio

Barcelona 2017

DIFFERENTIAL RESPONSES OF HISTORICAL CEREAL LINES TO WATER STRESS: PHENOTYPE AND GENE EXPRESSION.

RESPUESTA DIFERENCIAL DE LÍNEAS HISTÓRICAS DE CEREALES FRENTE AL ESTRÉS HÍDRICO: FENOTIPO Y EXPRESIÓN GENÓMICA.

Memoria presentada por Susan Mery Medina Canzio para optar al título de Doctor por la Universitat de Barcelona. Este trabajo se enmarca dentro del programa de doctorado de Biología Vegetal de la Facultad de Biología de la Universidad de Barcelona. El trabajo se ha realizado en el Departamento de Biología Evolutiva, Ecología y Ciencias Ambientales, Unidad de Fisiología Vegetal de la Facultad de Biología de la Universidad de Barcelona (UB) bajo la dirección del Dr. José Luis Araus y en el Departamento de Fisiología de Cultivos del Instituto Internacional de investigación de cultivos del trópico semiárido (ICRISAT) bajo la dirección del Dr. Vincent Vadez.

Doctoranda

Susan Mery Medina Canzio

Director y tutor

Dr. Jose Luis Araus Ortega

Director

Dr. Vicent Vadez

Barcelona, Mayo 2017

A mi familia,

cerca o lejos siempre les tendré en mi corazón.

A mis padres Maria Antonieta y Zoilo,

son lo más bonito que tengo y les quiero infinitamente.

To my family,

near or far, you will always be in my heart.

To my parents Maria Antonieta and Zoilo

they are the most beautiful treasure that I have, and I love them so much.

Cualesquiera que hayan sido nuestros logros,

alguien nos ayudó siempre a alcanzarlos.

Althea Gibson

ACKNOWLEDGMENT

Sencillo es todo lo

verdaderamente grande.

Honoré de Balzac

First, I would like to thank all people that contributed in some way to the development of this thesis:

To Jose Luis Araus as director and tutor of the thesis, for trusting me in order to carry out this investigation in wheat at the University of Barcelona. More than a director he has been a guide for me, to perform the experiments, analyses the data and drafting of articles. We spent pleasant moments together with the research team in the field trips and arduous sessions, where he worked together with us. He had supported me in all difficult times at personal and professional levels, he taught me to be strong, perseverant and ambitious. Perhaps I have been his most rebellious student, but like a responsible father he turned me back to the road, and run to the final goal. Jose Luis thank you very much for your patience and confidence, I truly appreciate you!

To Vincent Vadez as director of the thesis, by the confidence that he always put in me since the first moment, despite of my little experience in plant physiology by the time I began this thesis. He used to be always busy with ICRISAT issues, but he never doubted in giving me some time when I had any problem with the experiments, or when I was performing the endless measures of leaf area or transpiration, he gave me lot of encouragement. More than just a thesis director, he was a friend who supported me a lot at both professional and personal levels. Moreover, he taught me how to focus well the experiments and the results, likewise to write the erticles in a better way, he always made the corrections with a pleasant attitude. Thank you Vincent for your confidence and appreciation, I also appreciate you a lot!

Thank a lot to both Jose Luis and Vincent; thanks for all the efforts that were made to finish this thesis, for your great support in the moments that my health has crippled a bit, and especially by their absolute commitment in the last phase of the thesis

writing. Also I would thank you for your infinite patience while helping me to write the thesis and articles. I enjoyed too much the thesis development with both directors; I had matured and learned lot. Now, I think that I am better than three and a half years ago when I started the doctorate. Thanks for everything!

To my family, my parents who were always supporting me, with your great love you made me feel that it was not far from home, I always had them in my heart. My mom with her sweetness always made me feel encouraged and loved at any time; my dad who always gave me strength and never let me to give up. Both mom and dad will be always my biggest love. My brother and sisters: Cinty, Miki and Janet that in the same way like my parents allways encouraged me and at any moment were worried about me, I missed them a lot, but the love that join us is larger than the sea we had in the middle. To my grandparents Rosa and Manlio that now are my brightest stars in the sky next to my mom. To my cousin Renzo who accompanied me on this last stage and always made me feel that I am not alone, also by all the funny moments we spent together while I was writing this thesis.

To the group of Integrated crop ecophysiology or Araus's group at UB, where I made one half of this thesis. I spent pleasant moments in the office and during the sampling journeys with Omar, Fadia, Shawn, Mariate, Nieves, Jordi and Dolors; it was an enjoyable and enriching experience. Especially I would like to thank Ruth, Ruben, Adrian, Jose Armando, Cristina and Bangwei with whom I had shared funny moments, and when I fell down they always helped me to get up again. Appreciate you a lot guys!

To the fellows in the Department of plant physiology at UB, to the munis group: Barbara, Xavi, Eva, Laura, Veronica and Jana who were neighbors of the office and the nicest friends during this time. Likewise to Susana, Marta Renato, Xavi Serret,

Edu, Carmen, Esther, Jordi, Isis, Alex and Mireia with whom I shared funny moments at lunch time, in the garden or drinking a coffee. They were very helpful people that always made me switch off for a moment of the wheat and millet issues. On the other hand, to the professors of the Department who always supported me, gave me encouragement and had a nice smile for me: Ramón Vallejo, Luisa Moysset, Nuria Roca, Marta Lopes and Teresa Sauras; and a special thanks to Josep Matas for helping me always with the experiments in the greenhouse and growth chambers. In the same way to the Genomics team of the Parc Científic: Ramon, Elia and Amaya, who helped me and gave much encouragement when I had the largest PCR plates trial. I really appreciate all of you!

On the other hand, I also want to thank the members of the Group of Crop Physiology at ICRISAT (India), the GEMS group: Jana, Rekha, Madina, Siva, Karthika, Purush, Pushpa and technicians for their help with the larger experiments during the stages that I made there, and for the nice moments we spent despite of the stress that carries the experimental work. Especially to Sunita, Tharanya, Kritikha and Aparna, who were my inseparable friends during all the seasons I spent at ICRISAT. I thank your support and affection! Furthermore, I would thank all the other friends I did made there, people who helped me to spend the holidays in this very different country. To the international students Jalime, Antoine, Laura, William, Moureen, Jorick, Christian, Virginia, Hugo and Sara, with them I shared trips and tours in Hyderabad. And to the residents of the Icrisat campus, friends who always had a warm smile and made me feel that I was not so far away from home: Estefania, Salvatore, Vicky, July, Rebecca and Diogo. All my appreciation to you, there was a nice experience to enjoy India with you, thank you very much for all the good times we lived there!

Then I would like to thank all the friends with whom I shared many adventures and pleasant moments here in Barcelona while doing the doctorate: Juan Diego and

Monica as kind of my elder brother and sister, they always took care of me and we spent together very good moments. To Paty, Carlos, Jose, Paula, Elisa, Karolina, Johana, Cristina and Esti, they are friends with whom I lived and I met during this period, they always supported me and listened me when I had existential problems with this thesis, as well as sharing the funniest moments since I lived here. In the same way, thank Jose Vall and Nuria Martinez that treated me as if I were a daughter and always took care of me. To my friends from the whole life: Gabriela, Marianella, Evelyn, Ccori, Nora and Anita that supported me in each single moment despite the distance, they were the kind of friends that no matter which distance separated us, our heart stayed side by side. To all the friends I mentioned here, thank you for the good moments, for the good feelings and for being my support outside the university, you helped me to go far during this three years and a half of PhD!

A special thanks to Miguel, who has accompanied me this last year. He stayed with me during the stressful times; he was my support when my health had broken down and the reason for my biggest smiles. Thank you for your love and support!

To the government of Perú who gave me the scholarship "Presidente de la República -2013 III" along these four years, also the grants that founded this research: to the Spanish MINECO project grant No. AGL2016-76527-R and the USAID grant (Feed the Future Innovation lab for Climate Resilient Pearl Millet).

At the end of this road, I want to express to all of you my sincere affection and give thanks for all the great moments that we shared, each one has helped me much in this adventure of the doctorate, in difficult and nice moments, and taught me to look ahead with a big smile.

Thanks a lot!

Agradecimientos

Primero, agradezco a todos los que han contribuido de alguna manera a la realización de esta tesis:

A José Luis Araus por ser el director y tutor de la tesis, por haber confiado en mí para realizar las investigaciones de trigo aquí en la Universidad de Barcelona. Más que un director ha sido una guía al realizar los experimentos, análisis y redacción de los artículos. Hemos pasado momentos agradables junto al equipo de investigación en las salidas de campo y las arduas jornadas en que él tambien trabajaba junto a nosotros. Así mismo ha sido un apoyo en los momentos difíciles a nivel personal y profesional; me ha enseñado a ser fuerte, perseverante y ambiciosa. Quizá he sido su estudiante más rebelde, pero cual padre responsable me ha hecho volver al camino y llegar a buen puerto. Muchas gracias José Luis por tu confianza y paciencia, verdaderamente te aprecio mucho.

A Vincent Vadez por ser tambien director de la tesis, por la confianza que siempre tuvo en mí, desde el primer momento, a pesar de mi poca experiencia en fisiología vegetal cuando comencé la tesis. A pesar de que él siempre estaba ocupado en ICRISAT, nunca dudó en dedicarme tiempo cuando yo tenía algun contratiempo con los experimentos, y siempre me animaba cuando tenía que hacer las medidas interminables de área foliar o de transpiración. Más que solo un director de tesis fue un amigo que me apoyó mucho a nivel profesional y personal. También me enseñó a enfocar bien los experimentos y los resultados, así mismo a escribir mejor, siempre con una actitud muy agradable. Muchas gracias Vincent por la confianza y el aprecio que es mutuo.

A ambos Jose Luis y Vincent les agradezco todos los esfuerzos que hicieron para que terminemos esta tesis, por su apoyo en los momentos en que mi salud ha cojeado un poco y especialmente por su compormiso absoluto en la ultima fase. Tambien he de agradecerles por su infinita paciencia para ayudarme a escribir ala tesis y los artículos. He disfrutado mucho al realizar esta tesis con ambos como directores. He aprendido y madurado muchísimo; creo que ahora soy mejor que hace tres años y medio cuando comencé el doctorado. ¡Gracias por todo!

A mi familia, a mis padres que siempre me estuvieron apoyando y con su gran cariño me hicieron sentir que no estaba lejos de casa; siempre les he tenido en mi corazón. Mi mamá con su dulzura siempre me hizo sentir reconfortada y amada en los momentos duros, y mi papá que a pesar de todo me daba fortaleza y nunca me dejó rendirme. A mis hermanos Cinty, Miki y Janet que de la misma manera me daban ánimos y siempre se preocupaban por mí, yo les he hechado de menos, pero el cariño que nos une ha sido más grande que el mar que teniamos por medio. A mis abuelitos Rosa y Manlio que hoy son mis estrellas más brillantes en el cielo junto a mi mamá. A mi primo Renzo que me acompañó en esta última etapa y siempre me hizo sentir que no estoy sola, y tambien por todos los momentos graciosos que pasamos mientras yo escribía la tesis.

Al grupo de Ecofisiologia Integrada de Cultivos o grupo Araus de la UB donde realicé la mitad de la tesis, pasé momentos agradables en la oficina y en los viajes de muestreos con Omar, Fadia, Shawn, Mariate, Nieves, Jordi y Dolors; ha sido una experiencia agradable y enriquecedora. En especial quiero agradecer a Rut, Ruben, Adrian, Jose Armando, Cristina y Bangwei con quienes he pasado momentos divertidos, y cuando he decaido me han ayudado a levantarme nuevamente. ¡Les aprecio muchísimo!.

A los compañeros de la Sección de Fisiología Vegetal, a los munis: Barbara, Xavi, Eva, Laura, Veronica y Jana quienes fueron los vecinos de despacho y los amigos más agradables durante todo este tiempo. Así mismo a Susana, Marta Renato, Xavi Serrat, Edu, Carmen, Esther, Jordi, Isis, Alex y Mireia con quienes compartí momentos divertidos a la hora de comer, en el huerto o bajando a tomar un café. Son personas muy simpáticas que siempre me hicieron desconectar por un momento del trigo y del mijo. Por otro lado a los profesores del departamento quienes siempre me apoyaron, me dieron ánimos y tuvieron una sonrisa agradable para mí: Ramón Vallejo, Luisa Moysset, Nuria Roca, Marta Lopes y Teresa Sauras; y un agradecimiento especial a Josep Matas por ayudarme siempre con los experimentos en el invernadero y las cámaras. También al grupo de genómica del Parc Cientific: Ramon, Elia y Amaya quienes me ayudaban y daban muchos ánimos cuando no veía fin a las placas de PCR. ¡A todos les tengo mucho aprecio y cariño, les agradezco su calidez y todo lo que hicieron por mí!

Por otro lado quiero también agradecer a los miembros del grupo de Fisiología de cultivos de ICRISAT (India), al grupo GEMS: a Jana, Rekha, Madina, Siva, Karthika, Purush, Pushpa y a los técnicos por su ayuda con los experimentos tan grandes que realicé ahí, y por los momentos divertidos que pasamos a pesar del estrés y de no tener momentos de descanzo cuando comenzábamos un experimento. En especial a Sunita, Tharanya, Kritikha y Aparna que fueron mis amigas inseparables durante todas las temporadas que pasé ahí, les agradezco su apoyo y cariño. También a todos los otros amigos que hice ahí y que me ayudaron a disfrutar de las estancias a pesar de estar en un país muy diferente; a los estudiantes internacionales Jalime, Antoine, Laura, William, Moureen, Jorick, Christian, Virginia, Hugo y Sara con quienes compartimos viajes y salidas en Hyderabad. Y a los residentes del campus de ICRISAT que siempre tuvieron una cálida sonrisa y me hicieron sentir que no estaba tan lejos de casa: Estefania, Salvatore, Vicky, July, Rebeca y Diogo. ¡A todos les tengo mucho

cariño y fué una experiencia bonita disfrutar de India con ustedes, les agradezco mucho los buenos momentos que vivimos ahí!

También agradecer a todos los amigos con los que compartí muchas aventuras y momentos agradables aquí en Barcelona mientras hacía el doctorado: a Juan Diego y Mónica que son como mis hermanos mayores aquí, siempre me cuidaron y pasamos momentos muy bonitos. A Patricia, Carlos, Jose, Paula, Elisa, Karolina, Johana, Cristina y Esti. Son amigos con los que viví y conocí durante todo este tiempo; siempre me apoyaron y me ecucharon cuando tenía problemas existenciales con la tesis, asi como siempre estuvimos juntos para pasar los momentos más divertidos que viví aquí. De la misma manera agradecer a Jose Vall y a Nuria Martinez que me trataron como si fuera una hija y siempre se preocuparon por mí. También a mis amigas de toda la vida Gabriela, Marianella, Evelyn, Ccori, Nora y Anita que a pesar de la distancia me apoyaron en cada momento; son amigas que aunque la distancia nos separó, el corazón siempre estuvo uno al lado del otro. ¡A todos muchas gracias por los momentos vividos, el cariño compatido y por ser el apoyo fuera de la uni que me ayudó a avanzar durante estos tres años y medio de doctorado!

A una persona muy especial, a Miguel quien me acompañó este último año, me soportó durante los momentos de estrés, fué mi apoyo cuando mi salud se quebró y también fué y es el motivo de las sonrisas más grandes. ¡Gracias por tu cariño y comprensión!

Al gobierno de Perú que me concedió la beca "Presidente de la República -2013 III" durante estos cuatro años. Tambien a los proyectos que financiaron la investigación: el proyecto nacional Español MINECO AGL2016-76527-R y el proyecto USAID (Feed the Future Innovation lab for Climate Resilient Pearl Millet).

Al final de este camino les quiero expresar a todos mi cariño sincero; también les doy mil gracias por todos los gratos momentos que compartimos, cada uno ayudó mucho en esta aventura del doctorado, en los momentos difíciles y alegres, y me enseñó a mirar hacia delante con una sonrisa.

¡Muchas gracias!

INDEX

Ha triunfado quien unió

Lo útil a lo agradable.

Horacio

ABBREVIATIONS INDEX

a*, b*, u*, v*, vegetation indices

ATPase, chloroplastic ATP synthase β-subunit

AUC, area under the curve

C%, carbon content

CAT, catalase

CTD, canopy temperature depression

DHN16, dehydrin Td16

DM, dry matter

DW, dry weight

DREB, dehydration responsive element binding-transcription factor

Ex, exudate

Ex-RL, Exudate normalized by root length

LA, leaf area

LDW, leaf dry weight

GA, greenness- vegetation index

GOGAT, ferredoxin-dependent glutamate synthase

g_s, stomata conductance

GS1, cytosolic glutamine synthetase

GS2, chloroplastic glutamine synthetase

HR, higher rainfall

HY, high yielding

Int, Intensity - vegetation index

Light, Lightness- vegetation index

LR, lower rainfall

LSD, least significant difference

LY, low yielding

MX, metaxylem

N%, nitrogen content

NDVI; normalized difference vegetation index,

NR, non-restrictive

PEPC, phosphoenolpyruvate carboxylase

PIP, Protoplasm intrinsic aquaporin

PK, pyruvate kinase

qRT-PCR, quantitative retro-transcriptase polymerase chain reaction

R, restrictive

R-; mid including

R+, very restrictive

RBCL, Rubisco large subunit

RBCS, Rubisco small subunit

RDW, root dry weight

RL, root length

Sat, Saturation - vegetation index

Slope 1, TR slope at low VPD

Slope 2, TR slope at high VPD

SOD, superoxide dismutase

SPAD, chlorophyll content

SLA, specific leaf area

TIP , Tonoplast intrinsic aquaporin

TR, transpiration response

VPD, vapor pressure deficit

WCOR, dehydrin/ cold regulated gene WCOR719

WS, water stress

$[CO_2]$, carbon dioxide concentration

X_0, VPD breakpoint

$\delta^{13}C$, carbon isotope composition

Δslope, variation of slope1-slope2

INTRODUCTION

Las ideas son capitales que solo ganan intereses

en las manos del talento.

Rivarol

1 Climate change and crops

Agriculture is highly vulnerable to climate change due to the predicted changes in environmental conditions and stress factors, which they will affect crop yield, food production and food quality (Toscano *et al.*, 2014). The changes on environmental conditions, i.e. water scarcity, are the main constraints for crop productivity of the major crops over the world, such as wheat in the Mediterranean basin and pearl millet in the Asian and African countries. A rise of environmental temperatures and changes in precipitation patterns will eventually decrease crop yields, probably causing stresses like heat or drought and favouring weed and disease attacks (Ceccarelli *et al.*, 2010). The global estimation of drought disasters risk for the next century is about 44%, in the major crops the 50% of these negative impacts will affect directly the yield production due to irrigation deficit (Li *et al.*, 2009). The projections of wheat production assume that the growth rate will be lower than the historical growth rates reported in the second half of the twentieth century (Bort *et al.*, 2014). It is unlikely that any improvements will support the increase in world population or mitigate against future extreme weather events (Araus *et al.*, 2002; Ray *et al.*, 2013; Trnka *et al.*, 2014)

Actual progress is being conducted to mitigate the negative impacts of the environmental conditions under near-future climate change scenario. Any improvement in crop production needs a combination of classical breeding approaches that target a specific and well-defined process at whole plant levels and modern biotechnology tools that identify genotypes which express the desired character (Ghanem *et al.*, 2015) and enhanced yield (McKersie, 2015). This goal can be achieved by understanding the complex physiological mechanisms of adaptation where molecular information plays an important role (Araus *et al.*, 2008), assuming that several genes may also determine the plant phenotype. Thus, this research targets the integration of classical phenotyping with molecular screening in the scope of understanding the plant behaviour for the future water limited scenarios

facing the responses to acclimation to elevated CO_2 concentration [CO_2] and water stress (Ceccarelli *et al.*, 2010; Tardieu *et al.*, 2011; Mwadzingeni *et al.*, 2016).

1.1 Durum Wheat

Durum wheat (*Triticum turgidum* L. ssp. *durum* Desf.) is a monocotyledonous plant belonging to the Poaceae family (grass plants). It is one of the most important crops worldwide and the principal crop in the Mediterranean basin. It is a major economic and cultural factor in this region, being used for the production of staple food, such as pasta, couscous or bourghul (Habash *et al.*, 2009; Vicente *et al.*, 2016*b*). During the last four years, wheat production and consumption have raised, accounting for 752 million of tons global production in the last year (IGC, 2017). Its yield increment in the Mediterranean basin was 1-35 $kg.ha^{-1}$ per year between 1930 and 2000. This genetic gain had environmental influence that was positive associated with daily temperatures within the period of sowing-heading. The local durum wheat landraces were replaced by improved semi-dwarf cultivars around 1950s, which led to an improvement of durum wheat yield of about 30% associated with a higher harvest index (Soriano *et al.*, 2016). Then, in 1970s after the post-green revolution a new introduction of germplasm from CIMMYT (International Maize and Wheat Improvement Centre) raised the grain yield in 37 % (Sanchez-Garcia *et al.*, 2013), then yields have remained constant during the last decades. So the collection of genotypes studied in this thesis is compound by 20 semi-dwarf durum wheat lines developed after post-green revolution. At the moment, global efforts are being done to increase wheat yield gains for the next 10-20 years based on selecting traits for better tolerance to water and heat stresses in order to support the future demand (Parry *et al.*, 2005); in this sense, the efforts to develop biotechnology tools along with better means of doing phenotyping in Australia could possibly increase wheat yield around 20 kg/ha over the next 20 years (Robertson *et al.*, 2016).

1.2 Pearl Millet

Pearl millet (*Pennisetum glaucum L.*) is a monocotyledonous plant belonging to the Poaceae family; it is the second most important crop in India. This cereal is able to grow in most arid zones, and its higher crop culture is being developed in the north arid and semi-arid regions of this country, these agro-ecological zones vary principally in the rainfall level. The lower rainfall zone is located in Northern India, it is known as A1 zone (most arid zone or primary zone) and covers the territories of Western part of Rajasthan, and parts of the states of Haryana and Gujarat, with an annual rainfall of 320-400 mm; its soil composition is sand and entysol (59%). On the other hand, the higher rainfall zones (A and B, being less arid than zone A1) are located in the northern-central part of India. The A zone (secondary zone) comprises the northern and north western part of India including the eastern Rajasthan and parts of Haryana, Gujarat and Uttar Pradesh. It has an annual rainfall level near to 400 mm with fine sand and entysol (31%) soil composition accounting low organic matter content. The B zone (tertiary zone) comprises the Peninsular Indian states of Maharashtra, Tamil Nadu and Karnataka; its annual rainfall level is among 400-520 mm and has heavy soil composition as entysol (28%) and alfisol (26%) (Manga and Kumar, 2011; Rai *et al.*, 2015; Vadez *et al.*, 2015). In effect those differences between soil profile and rainfall intensity and distribution in both zones may cause an effect on pearl millet adaptation and its breeding history.

2 Adaptation to climate change

2.1 CO_2 acclimation: its concentration increment in the atmosphere

The atmospheric [CO_2] raised more than 40% since the beginning of the industrial revolution and is expected to double by the end of this century (IPCC, 2014). The accumulation of greenhouse gases, mainly CO_2, is leading to an increment of mean Earth temperature, which may negatively affect crop production due to heat stress.

The model projection for the next 50 years suggest that there will be a direct fertilization effect due to the rising of atmospheric [CO_2], which may compensate the effects of high temperatures and water limitation (Martins *et al.*, 2016). Nevertheless, not always the positive effects of elevated CO_2 ameliorate the negative effects of higher temperatures and lower water availability(Stitt and Krapp, 1999). The CO_2 concentration in the atmosphere is currently a limiting factor for C_3 photosynthesis. The short-term effect of the exposure to elevated [CO_2] stimulates the photosynthesis by increasing the substrate [CO_2] for ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) carboxylation and by the inhibition of competitive Rubisco oxygenation, which could contribute to generate higher biomass (Stitt and Krapp, 1999; Long *et al.*, 2006; Bencze *et al.*, 2014). This increment of [CO_2] induces a stomatal closure, which leads to a better leaf and plant water status. Recent studies demonstrated that the increment of [CO_2] in open-air field conditions may cause disadvantages in the increment of yield, being 50% less than reported in enclosure studies (Long *et al.*, 2006), in part explained by a lower photosynthetic capacity due to a decline in Rubisco protein content and activity (Aranjuelo *et al.*, 2011; Vicente *et al.*, 2015, 2016*a*). This may be regulated by plant mechanisms like carbon sink limitation, biomass dilution effects, or a decline in nutrient uptake or assimilation (Stitt and Krapp, 1999). This increment in [CO_2] also leads to altered gene expression patterns in the photosynthetic related mechanisms such as C distribution, respiration, and N uptake and re-mobilization in durum wheat (Vicente *et al.*, 2015). Moreover, the increment of emissions of greenhouse gases is causing warming and reduction of rainfall levels, in the near future this will enhance the drought intensity in many parts of the world. (Morison *et al.*, 2008; Habash *et al.*, 2009; IPCC, 2014; McKersie, 2015).

2.2 Water Stress: soil water availability

Water stress is the unbalance between soil water availability and transpiration water needs that are driven by the evaporative demand, and whose consequence is the

decrease on carbon accumulation, tissue expansion and cell number, it also gather a several number of processes (parallel or serial) at plant level which may define the growth based on the entry of water into the growing cells depending of the variation of water potential and hydraulic conductivities(Tardieu *et al.*, 2014). This water stress cause a reduction in the leaf water content, water potentials and photosynthesis (Habash *et al.*, 2014; Liu *et al.*, 2016). Drought stress can occur at any growth stage of crops (Russo *et al.*, 2015), numerous studies have been focused in this stress, especially at grain filling phase, whereas few research was focused in the early vegetative stage. At early stages, the water supply is important for plant growth and the acquisition of resources that later will be mobilized to the grain. Water limitation may also affect the tiller development and stem elongation (Guo *et al.*, 2016). Moreover, to face the water limitations the cereals develop some strategies such as capture more soil water, economize water use, use stem resources for grain filling (Araus *et al.*, 2008). All this plant responses mentioned above are the result of changes in the metabolism, those changes are driven by the synthesis of proteins and metabolites which probably are regulated at the transcriptional stage of gene expression, like the response to water stress driven via transcription factors (Sheshadri *et al.*, 2016).

3 Gene regulation

The gene regulation of the main metabolic pathways like nitrogen (N) and carbon (C) metabolism, together with stress-responsive and stress-perception genes are the precursor mechanisms that regulates processes involved in water management and leaf senescence, such as detoxification, osmoprotection and water movement. These processes in cereals are directly linked with the plant water status and its capacity to assimilate more carbohydrates in the grain, in order to succeed under stress episodes (Reynolds *et al.*, 2005; Blum, 2013; Vicente *et al.*, 2015; Medina *et al.*, 2016).

The plant responses under abiotic stresses, i.e. water stress can be better explained with molecular characterization in field assessments, under different conditions the plants display diverse strategies where the functional components are regulated by numerous genes that are involved in a network of many pathways (Hu and Xiong, 2014; Langridge and Reynolds, 2015).

The expression-profiling assessments showed that a set of genes for a particular stress level can be elucidated by using technologies such as RT-PCR (reverse transcriptase polymerase chain reaction). This technique allows the amplification of mRNA from the target gene to evaluate its expression. Those changes in mRNA could let us clarify the metabolic pathways affected by any stress as a first approach, although it does not always correspond to changes in protein or enzymatic activity (Vicente *et al.*, 2015). Nevertheless, it is a good approach to identify key genes regulated at transcript level by different environmental conditions. This regulation approach enhances the understanding of one part of the plant adaptation to abiotic stresses, which requires a linkage with physiological traits to have a large impact in crop improvement (Reynolds *et al.*, 2005).

The genes selected for this thesis were key genes encoding for enzymes involved in many metabolic pathways of C and N metabolism, like Rubisco (RCBL and RCBS, large and small subunit respectively), phosphoenolpyruvate carboxylase (PK), pyruvate kinase (PK), cytosolic and plastidial glutamine synthetase (GS1 and GS2, respectively), gluferredoxin-dependent glutamate synthase (GOGAT), as well as the proteins involved in stress responses like dehydrins (DHN11, DHN16 and WCOR), catalase (CAT), chloroplastic ATP synthase β-subunit (ATPase), superoxide dismutase (SOD), the transcription factors DREB1 and DREB2, and the aquaporins: tonoplast intrinsic proteins TIP1.1 and TIP 1;2, protoplasm intrinsic proteins PIP 2;3 and PIP 2;6, those aquaporins are responsible for water movements across membranes and are hypothesized to play an important role in phenotypes related to drought adaptation. These will be seen in the last part of this section.

3.1 Genes involved in Nitrogen and Carbon Metabolism

The nitrogen (N) and carbon (C) metabolism are the main pathways for plant development during all stages of growth. C is the main molecule which is assimilated by photosynthesis to produce plant biomass. After C, N is the element required in largest amounts by plants (around 1–5% of total plant dry matter), which is an integral constituent of proteins, nucleic acids, chlorophyll, co-enzymes, phytohormones and secondary metabolites (Marschner, 2011). It drives the cell production and growth processes by the function of two main enzymes: GS1 and GS2. The N leaf content is closely related to photosynthesis rates while N grain content enhances the seed quality of cereals; if N is reduced in the meristems it causes penalties in the synthesis of proteins and nucleic acids; (Sinclair and Rufty, 2012; Thomsen *et al.*, 2014). Three main enzymes play important roles in this pathway: (i) GS1 located in the cytosol is mainly involved in the N remobilization through the plant and N recycling from catabolic processes which is important for the maintenance of N flow from the root to the grain. (ii) GS2 located in the chloroplast is thought to be involved in the primary assimilation of ammonium (NH_4^+) from nitrate reduction, and the re-assimilation of photorespiratory NH_4^+. And (iii) the glutamate synthase (GOGAT) which supplies the GS substrate through the conversion of glutamine to glutamate. All these enzymes contribute to the N uptake and assimilation during the growth cycle, together with nitrate and nitrite reductases. These enzymes are related with the raise of water acquisition to enhance crop production (Sinclair and Rufty, 2012; Nagy *et al.*, 2013; Zhang *et al.*, 2017), and overexpression of GS1 was reported as a good as indicator of grain yield improvement (Thomsen *et al.*, 2014).

The C metabolism involves several pathways and key enzymes regulated at transcriptional, translational and post-translational levels. In these sense, we have focused on three key enzymes. (i) Rubisco is the most abundant protein in plants located in the chloroplast, inside the stroma, which play a key function in photosynthetic CO_2 assimilation, and its activity is highly responsive to atmospheric

[CO_2] (Carmo-Silva *et al.*, 2015). Its expression is 50% of all chloroplast proteins and plays an important role in CO_2 fixation and photorespiration of C_3 and C_4 plants (Calsa and Figueira, 2007; Tambussi *et al.*, 2007). A decline in Rubisco content and activity is the main negative effect contributing to a decrease of photosynthesis under elevated CO_2 and water stress (Komatsu *et al.*, 2014; Vicente *et al.*, 2015, 2016*a*). (ii) Pyruvate kinase (PEPC) is a key enzyme in the transformation of pyruvate to Acetil-coA and the production of oxaloacetate, linked with the N assimilation process and anapleurotic functions. It supplies C skeletons to drive nitrate reduction and amino acid biosynthesis. Under stress conditions this enzyme is degraded (Sinha *et al.*, 2015) which could reduce plant growth and development due to its interaction with the K^+ availability (Sugiyama *et al.*, 1968). And (iii) piruvate kynase (PK) participates in the provision of C skeletons for amino acid biosynthesis mediated by the GS-GOGAT pathway (Andre *et al.*, 2007), in parallel to PEPC, although PK is predominant in most of the tissues (Taiz and Zeiger, 2010).

3.2 Genes involved in Stress Response

Many transcription factors and stress-inducible genes have been identified under drought conditions. The stress responsive pathways are driven by many genes like: (i) the dehydration-responsive element-binding proteins (DREB1 and DREB2) which are transcription factors (TFs) which belong to the AP2/EREBP multigene family, they play an important role regulating several developmental mechanisms of stress response (Gahlaut *et al.*, 2016). These genes are molecular breeding targets. Previous studies in Arabidopsis used TaDREB1A and TaDREB2B as transcriptional regulators of constitutive drought-tolerance mechanism to enhance water use in crops like wheat (Salekdeh *et al.*, 2009; Yousfi *et al.*, 2016). (ii) The dehydrin gene families such as Cor and LEA (late embryogenesis abundant proteins) were studied in cereals and other plants. Dehydrins, belonging to LEA family (such as DNH11 and DNH16) are involved in temperature and dehydration responses under stress conditions (Tsvetanov *et al.*, 2000; Kosová *et al.*, 2014*a*). The actin-binding protein

Wcor719 is another dehydrin from cofilin proteins that is involved in the low temperature response involving the cytoskeleton reorganization, to control the extracellular ice formation under non- freezing cold acclimation. these three dehydrins are involved in dehydration and temperature response under water limited conditions (Danyluk *et al.*, 1996; Tsvetanov *et al.*, 2000). (iii) Catalase (CAT) and superoxide dismutase (SOD) are primary antioxidant enzymes involved in the elimination of reactive oxygen species (ROS) such as the cytotoxic H_2O_2 produced by photorespiration and the superoxide generated during photosynthetic electron transport (Xu *et al.*, 2010; Huseynova *et al.*, 2015). And (iv) the mitochondrial ATPase which play a role in the stress tolerances, it is the key enzyme in ATP synthesis driven by the transmembrane electrochemical proton gradient, reports in Arabidopsis attribute its high expression to several abiotic stresses (Zhang *et al.*, 2008). Also relevant under water stress is the water flow from the root to the shoot accompanied with other small solutes such as CO_2, ammonia and urea, which is mediated by the water channel proteins known as aquaporins (PIP and TIP) that belong to major intrinsic protein superfamily (Forrest and Bhave, 2007; Hove *et al.*, 2015). All the genes mentioned above may play a regulation role in the C and N metabolic pathways and the plant protection under water stress or elevated CO_2. Our study combines the physiological traits with gene expression which will integrate the plant responses to changes in environmental conditions during vegetative growth and late growth stages.

4 Efficiency of Water Use

The efficiency of water use (EUW) have been studied as an avenue to increase adaptation and improve yield under stress conditions (Slafer *et al.*, 2005; Reynolds *et al.*, 2005; Araus *et al.*, 2008; Blum, 2011; Vadez *et al.*, 2011; Kumar *et al.*, 2012; Mwadzingeni *et al.*, 2016). This new concept refers to the genotypic capacity to

manage the amount of water which is available in the soil matrix, in order to sustain the plant transpiration under water limited environments (Lopes *et al.*, 2011).

The water use efficiency (WUE) is a ratio estimating the quantity of biomass produced per unit of water used. This trait is closely related with the transpiration efficiency (TE). This trait is influenced by different plant factors (species variety and their sensitivity to stress) and environmental factors (variation in climate conditions).

The WUE improvement is favourable for the Mediterranean crops and highly important for the arid zones based on the critical availability of water resources in these regions, the water taken by the plant is released as water vapour (transpiration) into the atmosphere in a rate of 90% and through evapotranspiration in a 10%. At field level, the WUE is the relation between grain yield and water received, while at plant level it is defined as the transpiration efficiency (TE) which is the relation between biomass and water transpired (Vadez *et al.*, 2014). And at leaf level it is related with the ratio of instantaneous CO_2 assimilation, driven by the C isotope discrimination of Rubisco (CID) against the heavy form of ^{13}C in the stomata (Katerji et al., 2008; Reynolds et al., 2005) which also is an indicator of water status.

4.1 Transpiration response to the water deficit pressure

The transpiration response has been shown to be sensitive to changes of the vapour pressure deficit (VPD) which is a combined function of air temperature and relative humidity.

Several studies have described negative impacts of warm climates on yield, although much less attention has been focused on the effects of increases in temperature, as those under climate change, on the vapour pressure deficit, for instance under Mediterranean environments. Moderate temperature variation affects plant gas exchange and transpiration response, as reported in crops like cereals and legumes (Belko et al., 2013; Choudhary et al., 2013; Kholová et al., 2012; Schoppach and Sadok, 2013). The transpiration response to increasing VPD is a plant attribute that

drives the restriction of water losses under high vapour-pressure deficits which is influenced by genotypic and environmental control; plant hydraulics and aquaporins may be involved in stomatal closure and changes in transpiration. It is known that limiting the transpiration rate at high VPD in water-limited environments could result in significant yield increment (Gholipoor *et al.*, 2010), with a large genetic variability and variable acclimation strategies with respect to warmer and water limited environments and linked to drought adaptation mechanisms in wheat and pearl millet (Kholová and Vadez, 2013; Schoppach and Sadok, 2013). While in well-watered environments the plants increased transpiration as the VPD conditions were increased that led to a decline on leaf area expansion (Fletcher *et al.*, 2008). Reymond et al., (2003) have also shown an effect on VPD on the leaf expansion of maize. Previous reports on the transpiration response to increasing VPD (Gholipoor *et al.*, 2010; Kholová *et al.*, 2010*b*; Vadez *et al.*, 2011, 2014; Zaman-Allah *et al.*, 2011; Choudhary *et al.*, 2013; Schoppach and Sadok, 2013; Kholova *et al.*, 2016), have not paid attention to the possible effects of increasing VPD on the leaf expansion of these crops. So this study is going to integrate plant and leaf levels of TE and water use traits in order to understand this complex mechanism to improve crop water productivity.

4.2 Plant water status

The carbon isotope discrimination involve stable isotopes of carbon, which can be analysed in all plant tissues, especially in leaves and grain give that will give as an idea of the water status, photosynthetic efficiency of the plant in the crop cycle (Araus *et al.*, 2013); and indirect related with transpiration efficiency as the relation between biomass and water transpired described elsewhere (Monneveux *et al.*, 2006; Vadez *et al.*, 2014). The CO_2 uptake comes directly from the air, the plants take the lighter ^{12}C and discriminate the heavier ^{13}C, so $\delta^{13}C$ ratios are more negative than in air, this ratio is relative to the CO_2 assimilation (Dawson *et al.*, 2002) which is important for the biomass development (Sanchez-Bragado *et al.*, 2014). These ratios

can be affected by the water availability because the plants will close the stomata to avoid dehydration (Elazab *et al.*, 2012), and is also driven by the genetic variability (Monneveux *et al.*, 2006). This $\delta^{13}C$ ratios also provide information about the long term transpiration in the plants, and could be related with the WUE of the crops (Farquhar and Richards, 1984; Martin and Thorstenson, 1988; Cabrera-Bosquet *et al.*, 2007). The stomata closure and opening are very important for the regulation of water status which is reflected in the leaf conductance (g_s) which depends largely on the plant water status; this regulation also leads to changes in the leaf temperature where the canopy temperature deficit (CTD) indicate how the plant is cooling the leaves under changes in environment conditions where lower canopy temperature means higher capacity to take up soil moisture to maintain a better water status. Both g_s and CTD favour net photosynthesis and crop duration (Araus *et al.*, 2008).

5 Water transport

The plant growth dynamic depends on the water and nutrient flow. The plants face rapid changes matching the evaporative demand and soil water content. The water transport consist in taking it by roots through radial and axial paths, then delivered to the aerial part of the plant, and will be released by the leave through the stomata. The axial path accounts for the xylem vessels, which a priori present no or limited resistance to water flow, whereas the radial path allows water flux from the soil through the root cylinder to the xylem vessels. This radial flux involves three interactive pathways: (i) apoplastic path which is across the cell walls, (ii) symplastic path through plasmodesmata and cytoplasm, and (iii) transcellular path across membranes, where aquaporins play a significant role (Steudle and Peterson, 1998). Time ago, the water diffusion across the membranes was thought to be sufficient to support water exchanges in living plant cells and tissues; nowadays it is known that water transport through membranes is mediated by aquaporin proteins and is submitted to metabolic control (Maurel, 1997; Hose *et al.*, 2001; Javot and Maurel,

2002), together with root hydraulics which may play a role supplying the water needed for transpiration (Vadez, 2014).

5.1 Roots

Roots architecture is traditionally thought to be important for drought adaptation, via traits like root depth and root length density (RLD), which were related with water extraction in deep soils. However, higher root growth per se does not necessarily correlate with more water extraction and does not necessarily explain differences in crop yield (Ho *et al.*, 2005; Vadez, 2014). Moreover, root traits like small fine diameters, very long roots with small xylem diameters may also improve the root water acquisition (Comas *et al.*, 2013). Other important characteristics is the root tip zone, which lays between the root cap and lateral root formation and is the most permeable region. In this sense, high radial and axial conductivities suggest an enhancement of water (Segal *et al.*, 2008) and N uptake (Lynch, 2013). Therefore the water uptake not only depends on root architecture, but also hydraulic conductance and functional root tips, which may significantly contribute to water extraction independently of root depth and RLD (Watt et al., 2008). These root traits are also regulated by stress markers like abscisic acid (ABA) and genes like LRD2 (Lateral root density), which is a regulator of root intrinsic development (Deak and Malamy, 2005). The root growth may be also coordinated with shoot growth. In general, the roots are very responsive to environmental and growth conditions, regulating their permeability in response to day/night cycles, nutrient deficiency or stress; this plasticity may explain the lack of universal 'rules' for plants nutrient management (Javot and Maurel, 2002; Hodge, 2009).

5.2 Water flux

When transpiration rates are high, the apoplastic path will be partially used and the root hydraulic resistance will be low allowing a rapid uptake of water, being driven by gradients of water potential between the transpiring leaf and the root. Then the

symplastic and transcellular paths respond to the interaction between the flow of nutrients and water which responds to driving force conducting a coarse regulation of water uptake by roots. Oppositely, when transpiration rates are low (i.e. during the night or during stress conditions), the apoplastic path is less used and the hydraulic resistance is high. Hence, the role of water channels (aquaporins) in the cell-to-cell path is in the fine adjustment of water flow (Steudle and Peterson, 1998; Steudle, 2000; Vadez, 2014). Moreover, recent studies had showed that the dynamics of the xylem flow also relay on CO_2 transport from the root to the shoot (Bloemen *et al.*, 2016). All this responses to soil moisture may favour the water use and water use efficiency of the plant (Rostamza *et al.*, 2013).

5.3 Hydraulic conductivity

Hydraulic conductivity (Lp) determines the water flux through a given interface, like a cell membrane or a root area, being the water flux the volume of water that crosses that interface per unit area in a time frame, accounting the variation of the potential and driving forces between the compartments on either side of this interface.

Root hydraulics determines water uptake intensities but also water potential gradients within the plant. Its dynamics contribute in many nutritional and growth functions. The variability of soil water content, nutrient availability and root hydraulic properties feed each other and all play critical roles in root transport functions from roots to shoots, coordinated growth and water-saving responses (Maurel *et al.*, 2010). The differences in the root hydraulic properties of roots affect the water use (Hose *et al.*, 2001), and the hydraulic resistances in the root cylinder, such as exodermis and/or endodermis, change during the root development (Steudle, 2000; Vadez, 2014). The Lp in the roots decreases when plants are grown under abiotic stresses such as salinity, oxygen deprivation or nutrient starvation (Aroca *et al.*, 2012), also under root aging or environmental constrains (variations in temperature, humidity, or irradiance) which create a resistance to water flow (Steudle and

Peterson, 1998; Clarkson, 2000), and most of this resistance is located in the root cylinder (radial resistance) (Steudle, 2000).

In leaves, Lp can be affected by hormones like ABA, which regulate the stomata, transpiration and aquaporins (Li *et al.*, 2014). Leaf Lp usually increases under water deficit (Steudle, 2000), and it is also known that many aquaporin isoforms of PIPs can contribute from 20 to 70% to the Lp in roots and leaves described elsewhere (Quigley *et al.*, 2001; Chaumont *et al.*, 2005; Nardini and Salleo, 2005; Li *et al.*, 2014; Sutka *et al.*, 2016).

5.4 Aquaporins

Plant aquaporins are membrane channels proteins that facilitate selective transport of water and many other small molecules across biological membranes, and may contribute to several plant growth and developmental processes (Li *et al.*, 2014). Aquaporins are major intrinsic proteins (MIPs) which have larger diversity of isoforms and cellular localizations, structurally conformed by six transmembrane helices that selectively allow water or other small uncharged molecules to pass along the osmotic gradient; they usually form tetramers and each monomer defines a single pore (Kruse *et al.*, 2006). Three main families present in non-vascular and vascular plants: (i) the Plasma membrane Intrinsic Proteins (PIPs) localized in the plasma membrane with two subclasses, PIP1 and PIP2, which exhibit highly conserved and narrow pore typical of high water-selective activity (Li *et al.*, 2014), (ii) the Tonoplast Intrinsic Proteins (TIPs) localized in the vacuolar membrane (tonoplast), and (iii) the Nodulin26 like Intrinsic Proteins (NIPs) localized in the plasma membrane or in the endoplasmic reticulum. These NIPs and TIPs have much higher diversity of pore configurations. Several studies have focused on identifying the subcellular localizations for the aquaporins, but they seem to be located in more than one specific site (Maurel, 1997; Javot and Maurel, 2002; Li *et al.*, 2014).

These proteins may be regulated by an electrostatic positive potential allowing rapid water flux, whereas a negative potential reduces the single-channel water permeability (Hub *et al.*, 2010). Aquaporins represent the major pathways for transcellular and intracellular water transport (Hub *et al.*, 2010), playing a key role in hydraulic regulation and nutrient transport in roots and leaves, in all facets of plant growth and development (Forrest and Bhave, 2007), especially emergence of lateral roots (Li *et al.*, 2014). In leaves, they are involved in water and CO_2 permeability, xylem conductance, embolism refilling and transport of dissolved gases such as CO_2 or metalloids such as boric or silicic acid (Kaldenhoff *et al.*, 2008; Li *et al.*, 2014) and ammonia and urea across the membranes for its compartmentalization in the vacuole (Loque *et al.*, 2005; Kruse *et al.*, 2006).

Its function related to plant growth relays on nutrient acquisition, carbon fixation, cell signalling and stress response (Maurel, 2007), as well as opening and closing the water channel pore. Its expression is modulated by multiple environmental and hormonal stimuli (Li *et al.*, 2014), which is critical important for plant survival under water limitations (Forrest and Bhave, 2007). Aquaporins are related specifically with cell enlargement in organs like roots, hypocotyls, leaves and flower stems (Ludevid *et al.*, 1992). They are also involved in the solutes and water exchange across the tonoplast leading to large central vacuoles, in lateral root emergence they favour the water influx into the root primordium forcing it way through the surrounding cell layers in the main root (Péret *et al.*, 2012).

Aquaporin inhibition

The aquaporin capacity to enhance water transport can be inhibited by metal compounds such as mercury, silver and gold. It is known that the hydraulic architecture, water relationships, and gas exchange of leaves under different conditions of stress are affected by inhibitors of water fluxes which block the water transport pathways (Nardini and Salleo, 2005). Mercurial compounds like $HgCl_2$

inhibit aquaporins by a steric mechanism which binds the aquaporin structure leading to channel inhibition. This inhibitor is attached in two mercury-sensitive sites of the protein, it bounds at two Cys183 sites and occludes the pore through conformational changes (Niemietz and Tyerman, 2002; Savage and Stroud, 2007). By other side Silver as $AgNO_3$ and gold as $HAuCl_4$ are also potent inhibitors of the water permeability in the plasma membrane of roots, i.e. the peribacteroid membrane from soybean (Niemietz and Tyerman, 2002). Those permeability blocking systems may cause penalties in the rate of water which is passing through the plant cylinders and reduce the transpiration. Therefore, these aquaporin inhibitors have been used in pharmacological studies consisting in testing the effects of aquaporin inhibition in processes such as hydraulic conductance regulation, transpiration, where aquaporins are thought to have a key role.

Aquaporin gene expression

Aquaporin role in resistance to abiotic stress can be targeted from the molecular view that reveals a complex mechanism of transcriptional and post-transcriptional responses with variable profiles within aquaporin isoforms and the root hydraulics. Its overexpression is widely used strategy to understand plant water relations under stress, which was associated with high water permeability (Maurel, 2007). Previous studies (Li *et al.*, 2014) showed that high aquaporin expression on transgenic plants may confer either higher resistance or higher sensitivity to stress (Maurel, 2007; Li *et al.*, 2014). Likewise the expression of OsPIP1;3 in a drought-resistant rice enhanced its water stress resistance (Lian *et al.*, 2004), as SlTIP2;2 in tomato altered the plant water relations, enhancing transpiration and modifying leaf water potential (Sade *et al.*, 2014). Hence the regulation of aquaporin expression showed beneficial effects on plant growth.

OBJECTIVES

Para triunfar en la lucha por la vida,

el hombre ha de tener una gran inteligencia

o un corazón de piedra.

Máximo Gorki

The main objective of this thesis was to investigate the physiological and molecular mechanisms that confer drought adaptation in a C_3 (durum wheat) and a C_4 (pearl millet) cereals. In the case of durum wheat an historical collection of semi dwarf (i.e. post green revolution) cultivars released in Spain during the past decades was used. For pearl millet F1 hybrids and their parental lines bred in different rainfall environments were deployed.

The specific objectves were:

1. Compare the molecular and physiological mechanisms which may confer better performance of durum wheat to water stress and elevated atmospheric CO_2.

2. Asses the genotypic variability on plan transpiration response to vapour pressure deficit in durum wheat, the physiological and molecular mechanism involved and the potential impact on grain yield and crop adaptation to Mediterranean conditions.

3. Compare the transpiration response and physiological mechanisms involved in shoot and root development of pearl millet associated with their breeding history.

4. Compare the transpiration response and physiological mechanisms associated with aquaporin gene expression and inhibition, with roots hydraulics of pearl millet bred for different rainfall environments

REPORT OF THE THESIS DIRECTORS

Para las cosas grandes

y arduas necesitan

una combinación sosegada,

voluntad decidida,

acción vigorosa,

cabeza de hielo,

corazon de fuego

y mano de hierro..

Jaime Balmes

Group of Integrative Crop Ecophysiology
Plant Physiology Unit.
Evolution, Ecology and Environmental Sciences Department
Faculty of Biology
(UB)
643 Diagonal Av, 08028 - Barcelona - Spain.
https://integrativecropecophysiology.com

Crop Physiology Laboratory
Building No: 302
System Analysis for Climate Smart Agriculture,
Innovation System for Drylands (ISD)
ICRISAT
Patancheru 502324 - Telangana – India
https://www.gems.icrisat.org

Dr. José Luis Araus and Dr. Vincent Vadez as directors of the thesis entitled ¨Differential responses of historical cereal lines to water stress: Phenotype and Gene expression" – "Respuesta diferencial de líneas históricas de cereales frente al estrés hídrico: phenotipo y expresión genómica" which was developed by the doctoral student Susan Mery Medina Canzio.

REPORT about the impact factor and the participation of the doctoral student in the articles included in this Doctoral Thesis.

Chapter 1. Article "*Transpiration efficiency: new insights into an old story*" published in the Journal of Experimental Botany which has an impact factor of 5.526 in 2014. In this review, the efforts to harness transpiration efficiency (TE); as a genetic component of water-use efficiency were discussed. As TE is difficult to measure, a new lysimetric method for assessing TE gravimetrically throughout the entire cropping cycle was reported. This provided new insight into the genetics of TE, such as the involvement of plant hydraulics, aquaporins for achieving genetic gains via breeding focused on this trait, and especially about the possible relationship between differences in TE and the capacity of the plant to restrict transpiration under high evaporative demand (vapor pressure deficit, VPD). This was a review where the contribution of the doctoral student has been to provide the first tangible evidence for a tight linkage between the capacity to restrict stomatal conductance under increasing VPD and higher transpiration efficiency values. Then the doctoral student was also involved in a follow up activity of looking at gene expression in some of the lines contrasting for the transpiration response to increasing VPD (additional results from this work are currently the object of a paper under review in Plant Cell and Environment). In addition, the doctoral student has tested the response of some contrasting lines for the transpiration response to increasing VPD and has provided additional evidence of a tight linkage to aquaporin functioning. Therefore, while the review in which the doctoral student has been part has remained largely at a theoretical level, the quality of the datasets generated by the doctoral student has deeply influenced the way the review has been written, especially in terms of future research prospects. This is part of these concepts and hypotheses that the doctoral student has applied later on in two chapters of the thesis (4-5).

Chapter 2. Article "Interactive Effects of Elevated [CO_2] and Water stress on physiological traits and gene expression during vegetative growth in four durum wheat genotypes" published in the journal Frontiers in Plant Science which had an impact factor of 4.495 in 2016. In this study, the interactive effects of elevated [CO2] and moderate to severe water stress during the first part of the growth cycle on physiological traits and gene expression in four modern durum wheat genotypes was investigated. The results of this study showed that the increase in plant development was closely linked to the raise of N content together with the highly expression of N metabolism-related genes and down-regulation of genes related to the antioxidant system. Hence the combination of both factors elevated [CO_2] and severe water stress depended basically of the genotypic variability, which may suggest specific genotypic adaptation strategies to the different environmental conditions that we assayed. The doctoral student conceived this study and performed the experimental work analysis; she has shown dedication and responsibility.

Chapter 3. Article "Plant-transpiration response to VPD is associated to differential yield performance and gene expression in durum wheat" will be submitted to Journal of Experimental and Environmental Botany which impact factor is 4.369. This study compares the agronomical, physiological and gene expression responses of a set of 20 commercial (semi dwarf) durum wheat cultivars released during the past four decades in Spain. These varieties were clustered based in their whole-plant transpirative responses to increasing VPD, and different categories of genotypes were identified: restrictive (mild and very restrictive) and non-restrictive categories of durum wheat cultivars. These lines were assayed in field conditions during two consecutive crop seasons in different sites and water conditions accounting for a wide range of growing conditions ranging the grain yield between 2816 Tn ha^{-1} to 7194 Tn ha^{-1}, respectively. Differences between the different set of genotypes were more evident at the high yielding conditions, while no differences in grain yield between the different subset of genotypes were identified. Non-restrictive lines

showed greater yield and biomass than restrictive lines under optimal conditions; this better performance was associated with higher transcript levels of the genes involved in N metabolism such as GS1 and GOGAT, and genes involved in C metabolism like Rubisco, also the aquaporins. In this set of plants the strategy to restrict transpiration was only observed in drought conditions where the yield rate was lower, here it was assured higher carbon assimilates in the grain as yield gains, linked to decreases in N content while the water management ($\delta^{13}C$ and g_s) was better. Thus, the modern durum wheat lines varied in their response to water loss, which was regulated at physiological level as at transcript level DREB transcription factors. The doctoral student has conceived the study and undertaken the related experimental work, showing initiative in conductive all the experimental parts with dedication and efforts, then she showed interest and responsibility on analysing the results and writing the draft of the article. In spite of her health troubles she carried with all the parts of these study showing good experimental skills. These results may be interesting for the selection of genotypes that are better adapted for desirable water conditions.

Chapter 4. Article "*Transpiration response and growth in pearl millet parental lines and hybrids bred for contrasting rainfall environments*" submitted to the journal Frontiers in Plant Science which has an impact factor of 4.298. This study compares the transpiration response under conditions of high evaporative demand (hot and dry air) and soil drying, where restricting transpiration is an important avenue to gain in efficiency of water use, in hybrids and parental lines that have been bred for different agro-ecological zones of India and varying in rainfall quantities. This article elucidated if breeding for environments (varying in rainfall zones) that differ for the evaporative demand had selected for traits that control plant water use, measured in pearl millet material (hybrids and parental). The doctoral student has conceived the study and undertaken the related experimental work. In carrying that work, the doctoral student has shown great dedication to generating high quality datasets –

her experimental skills are absolutely outstanding. This undertaking was also risky since there was no prior experience of the kind for the traits she proposed to follow. She also managed great maturity and skill in dealing with experience at different level of plant organization (from root hydraulics to whole plant transpiration response). The results coming out of this work are extremely interesting and open a scope for the selection of the breeding material for the different agro-ecological zones of pearl millet in India.

Chapter 5. Article "Water flux patterns and aquaporin dynamics from transpiration demand to root hydraulics in Pearl millet hybrids ", article in preparation for publication. This study compares the transpiration responses to increasing VPD, the transpiration response to aquaporin inhibitors and its consequence on the root hydraulic conductance of Pearl millet bred for different agro-ecological zones of India, varying for rainfall. It follows on analyzing possible differences in the aquaporin expression of several pearl millet hybrids bred for higher and lower rainfall zones of India. This work has shown again a close linkage between the transpiration response to increasing VPD and the degree to which transpiration is inhibited following aquaporin inhibitor application, showing that low rainfall hybrids are more dependent on aquaporin-mediated pathways for water transport. The doctoral student has conceived the study and undertaken the related experimental work, showing great initiative in adding measurement, great experimental planning in terms of logistics, and independence in carrying out the experimental work. In doing so she has shown again outstanding experimental skills. She has also generated very nice root anatomical data, showing significant difference between the high and low rainfall hybrids in the amount of metaxylem and the size and shape of the endodermis cell – and these data are extremely interesting.

It should be noted that Susan has undertaken her PhD in a complex setup, working on two cereal crops in two experimental conditions, and in disciplines ranging from gene expression to whole plant physiology. At ICRISAT she has quickly adapted to her new cultural environment and has thrived to develop her experimental work with a high degree of professionalism. At the University of Barcelona she has worked basically in durum wheat integrated also in a multidisciplinary and multicultural team (including researchers and PhD students from Spain, USA, Colombia, Egypt, Tunisia, China). She has brought new skills to the lab, for example to automate some of the data collection processes, or around logistical aspects of collecting sap exudates or field sampling and further stable isotope analyses. As detailed above, she has great experimental skills and these have impacted the work in the lab. During her work she has shown also the capacity to work simultaneously at different levels, either with plants growing in the glasshouse and tested in the growth chamber, with plants grown in soil or hydroponics, or in outdoors conditions. Susan has shown excellent integration in the group and is easy to relate with. She has matured a lot in these years and while she still needs to work on her writing she has the maturity and experience to lead independent research. There are clearly areas of work that she excels at and where she could expand in the future.

To certify this for corresponding purposes

Dr. Jose Luis Araus

Dr. Vincent Vadez

Barcelona May 17st

RESULTS

La confianza en si mismo

es el primer secreto del éxito.

Emerson

Chapter 1

Transpiration efficiency: new insights into an old story

Eficiencia de tranpiración: nuevas ideas de una vieja historia

Vincent Vadez[1,*], Jana Kholova[1], Susan Medina[1], Aparna Kakkera[1] and Hanna Anderberg[2]

[1]International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Crop Physiology Laboratory, Patancheru 502324, Greater Hyderabad, Andhra Pradesh, India

[2]Department of Biochemistry and Structural Biology, Center for Molecular Protein Science, Lund University, Sweden

Published in / Publicado en:

Journal of Experimental Botany (2014), Vol. 65, No. 21, pp. 6141–6153

ABSTRACT

Producing more food per unit of water has never been as important as it is at present, and the demand for water by economic sectors other than agriculture will necessarily put a great deal of pressure on a dwindling resource, leading to a call for increases in the productivity of water in agriculture. This topic has been given high priority in the research agenda for the last 30 years, but with the exception of a few specific cases, such as water-use-efficient wheat in Australia, breeding crops for water-use efficiency has yet to be accomplished. Here, we review the efforts to harness transpiration efficiency (TE); that is, the genetic component of water-use efficiency. As TE is difficult to measure, especially in the field, evaluations of TE have relied mostly on surrogate traits, although this has most likely resulted in over-dependence on the surrogates. A new lysimetric method for assessing TE gravimetrically throughout the entire cropping cycle has revealed high genetic variation in different cereals and legumes. Across species, water regimes, and a wide range of genotypes, this method has clearly established an absence of relationships between TE and total water use, which dismisses previous claims that high TE may lead to a lower production potential. More excitingly, a tight link has been found between these large differences in TE in several crops and attributes of plants that make them restrict water losses under high vapour-pressure deficits. This trait provides new insight into the genetics of TE, especially from the perspective of plant hydraulics, probably with close involvement of aquaporins, and opens new possibilities for achieving genetic gains via breeding focused on this trait. Last but not least, small amounts of water used in specific periods of the crop cycle, such as during grain filling, may be critical. We assessed the efficiency of water use at these critical stages.

Journal of Experimental Botany, Vol. 65, No. 21, pp. 6141–6153, 2014
doi:10.1093/jxb/eru040 Advance Access publication 5 March, 2014

Journal of Experimental Botany
www.jxb.oxfordjournals.org

REVIEW PAPER

Transpiration efficiency: new insights into an old story

Vincent Vadez[1,*], Jana Kholova[1], Susan Medina[1], Aparna Kakkera[1] and Hanna Anderberg[2]

[1] International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Crop Physiology Laboratory, Patancheru 502324, Greater Hyderabad, Andhra Pradesh, India
[2] Department of Biochemistry and Structural Biology, Center for Molecular Protein Science, Lund University, Sweden

* To whom correspondence should be addressed. E-mail: v.vadez@cgiar.org

Received 28 October 2013; Revised 6 January 2014; Accepted 16 January 2014

Abstract

Producing more food per unit of water has never been as important as it is at present, and the demand for water by economic sectors other than agriculture will necessarily put a great deal of pressure on a dwindling resource, leading to a call for increases in the productivity of water in agriculture. This topic has been given high priority in the research agenda for the last 30 years, but with the exception of a few specific cases, such as water-use-efficient wheat in Australia, breeding crops for water-use efficiency has yet to be accomplished. Here, we review the efforts to harness transpiration efficiency (TE); that is, the genetic component of water-use efficiency. As TE is difficult to measure, especially in the field, evaluations of TE have relied mostly on surrogate traits, although this has most likely resulted in over-dependence on the surrogates. A new lysimetric method for assessing TE gravimetrically throughout the entire cropping cycle has revealed high genetic variation in different cereals and legumes. Across species, water regimes, and a wide range of genotypes, this method has clearly established an absence of relationships between TE and total water use, which dismisses previous claims that high TE may lead to a lower production potential. More excitingly, a tight link has been found between these large differences in TE in several crops and attributes of plants that make them restrict water losses under high vapour-pressure deficits. This trait provides new insight into the genetics of TE, especially from the perspective of plant hydraulics, probably with close involvement of aquaporins, and opens new possibilities for achieving genetic gains via breeding focused on this trait. Last but not least, small amounts of water used in specific periods of the crop cycle, such as during grain filling, may be critical. We assessed the efficiency of water use at these critical stages.

Key words: Aquaporins, carbon-isotope discrimination, CID, drought, grain filling, hydraulics, post-anthesis water use, vapour-pressure deficit, VPD, water stress.

Introduction

Producing more food per unit of water has become a major concern for humanity because fresh water resources are finite in nature and scarce in areas with large populations and increasing population growth rates. The agriculture sector consumes approximately 75% of the total fresh water, although this proportion falls to approximately 50% in industrial nations where the domestic and industrial use of water is much higher. Therefore, as societies develop there will be increasing competition for water resources between the agricultural and development sectors, which will undoubtedly increase pressure on the agriculture sector to produce more food with less water. Water productivity can be defined at different levels (e.g. see Condon *et al.*, 2002). At a plot level, it can be defined as water-use efficiency (WUE) = grain yield/water received (in millimetres; through either irrigation or rain) or as WUE = total biomass/evapotranspiration. At a

Abbreviations: CIE, carbon-isotope discrimination; FTSW, fraction of transpirable soil water; SCMR, Soil Plant Analysis Development chlorophyll meter reading; SLA, specific leaf area; TE, transpiration efficiency; VPD, vapour-pressure deficit; WUE, water-use efficiency.

plant level, the transpiration efficiency (TE), an important component of WUE, is defined as TE = biomass/water transpired. At the leaf level, TE is defined as the intrinsic WUE; that is, the ratio of instantaneous CO_2 assimilation (A) to transpiration (T) = A/T. These definitions illustrate that water productivity can be approached from different perspectives, broadly in terms of agronomic and genetic aspects. In this review we will focus exclusively on the genetic aspects of TE. The agronomic perspective of WUE has been covered in other reviews (e.g. Hsiao *et al.*, 2007; Kirkegaard and Hunt, 2010; Passioura and Angus, 2010).

The first section of this review will address earlier works on TE, especially concerning the discovery of discrimination against the heavy form of carbon, ^{13}C, by Rubisco and by the stomata and the realization that this discrimination could be used as a screening method for TE analyses (Farquhar *et al.*, 1982). One reason for using surrogate measures of TE is the difficulty of measuring TE gravimetrically, by assessing biomass increases and plant water use on a long-term basis. Because of the cost of carbon-isotope discrimination (CID) and the fact that such measurements are not immediate, other surrogates were subsequently identified, such as specific leaf area (SLA) or Soil Plant Analysis Development chlorophyll meter readings (SCMRs), as proxies of CID (Rao *et al.*, 2001). We review the physiological significance and the use of these different surrogates, especially in relation to whether it the stomata or the carboxylation efficiency that is the main driver of CID variations, and we attempt to draw some conclusions and insights concerning their success. This section will also review new and recent methods that allow robust and accurate gravimetric measurements of TE using a lysimetric approach (Vadez *et al.*, 2008, 2011*a*, 2011b).

The TE ratio is actually quite complex, as it depends on both genetic and environmental components [$TE = k_d/(e^*_a - e)_d$], where the term in parentheses reflects the vapour-pressure deficit (VPD) and k_d is a coefficient that reflects the CO_2 concentration in the stomatal chamber (Sinclair *et al.*, 1984; Sinclair, 2012). Hence, TE shows an inverse relationship with the VPD (Bierhuizen and Slatyer, 1965; Tanner and Sinclair, 1983). However, much of the research on the genetics of TE over the last three decades has disregarded the aspects of TE that concern this environmental factor, instead concentrating on aspects related to CO_2 in the leaf. Nevertheless, a modelling study showed that setting a maximum transpiration rate during the mid-day period, when VPD is highest, would improve the yield of sorghum under water-limited conditions and increase TE (Sinclair *et al.*, 2005). This genetic trait would confer the capacity to alter the effective VPD value that drives TE. In the past few years, large genotypic variations in the restriction of transpiration under a high VPD have been reported in several legume species, such as soybean (Fletcher *et al.*, 2007), chickpea (Zaman-Allah *et al.*, 2011*a*), cowpea (Belko *et al.*, 2012), and peanut (Devi *et al.*, 2010), as well as in cereals such as sorghum (Gholipoor *et al.*, 2010), pearl millet (Kholova *et al.*, 2010b), and wheat (Schoppach and Sadok, 2012). The discovery of these large genetic differences therefore re-oriented research on TE, providing new and exciting insights into ways to alter the negative influence of VPD on TE. Therefore, the second section will present the physiological basis for this trait, how it can indeed improve WUE, and how it may explain some of the earlier results that did not fit the CID theory. We will also discuss its mechanistic basis, particularly concerning its relationship with the characteristics of plant hydraulics, and its possible link to the functioning of aquaporins.

In the third section we will refer to the Passioura equation (Passioura, 1977), in which TE is one of the components (Yield = WU × TE × HI; where WU is the water used for transpiration and HI is the harvest index), and discuss the strength and limitations of the equation in approaching TE. Part of the weakness lies in assuming that the TE component is constant throughout a plant's life cycle. The previous paragraph and the second section of the paper discuss the greatly varying influence of VPD on TE. Beyond this, it has become increasingly evident that there are certain stages of the crop life cycle that critically require water, such as the grain-filling period. As such, there are now data showing the very high water productivity of the water used during these specific stages (Manschadi *et al.*, 2006; Kirkegaard *et al.*, 2007; Zaman-Allah *et al.*, 2011*a*; Vadez *et al.*, 2013). Therefore, in this section, we will invert the WUE acronym to examine the efficiency of water use (EWU) and will analyse the importance of securing water for key stages and the genetic means of achieving this. This analysis will be further linked to the second section and traits that, through increasing TE, also contribute to delaying plant water use for later critical stages.

The story since the 1980s, definition, and how to decrease C_i/C_a

Theoretical basis of TE

The definition of the intrinsic TE from equation 4 of (Condon *et al.*, 2002) is that:

$$TE = 0.6C_a^*(1-C_i/C_a)/(W_i - W_a) \quad (1)$$

where C_i and C_a are the stomatal chamber and ambient CO_2 concentrations, respectively, and W_i and W_a are the stomatal chamber and ambient vapour pressures, respectively.

This simple equation lays out the terms that affect TE. First, the C_i/C_a ratio must be kept low to achieve a high intrinsic TE. Because C_a can be considered constant for a given crop, a low ratio comes from a low C_i, which can be achieved in two ways: through (i) a high photosynthetic efficiency or (ii) a low stomatal conductance. As an aside, climate change conditions and the gradual increase in C_a would logically lead to an increase in the TE of crops in the future and partially compensate for the adverse effects of a changing climate (Muchow and Sinclair, 1991). Second, TE shows an inverse relationship with the difference in vapour pressure between the stomatal chamber and the environment (i.e. VPD), whereby low water-vapour gradients would increase TE. This definition is akin to the earlier definition of TE provided by Sinclair *et al.* (1984): $G_d/T = k_d/(e^*_a - e)_d$, where G_d/T is the ratio of daily growth to transpiration, and k_d is a coefficient that reflects the CO_2

concentration in the stomatal chamber. This definition arises from earlier founding work by de Wit (1958).

Equation 1 shows that TE is variable and can be influenced by genetic (the C_i/C_a ratio) and environmental (the $W_i - W_a$ gradient, or VPD) factors. Most, if not all, research on TE over the last few decades has focused on the genetic component of this equation and on finding means of increasing photosynthetic efficiency or decreasing stomatal conductance. In the early 1980s it was discovered that the heavy form of carbon, ^{13}C, is discriminated against by the enzyme Rubisco and by the stomata (Farquhar *et al.*, 1982). An empirical relationship was later established for wheat (Farquhar and Richards, 1984) ($\Delta^{13}C = 4.4 + 22.6\ ^{13}CO_{2int}/^{13}CO_{2ext}$), and measuring the CID was then conceived as a way to assess TE, by comparing ^{13}C values in plant tissues and in the atmosphere. According to this theory, $\Delta^{13}C$ is expected to be negatively related to TE, which has been confirmed by a number of studies (Hubick *et al.*, 1986; Hubick and Farquhar, 1989; Condon *et al.*, 1990; Ehleringer *et al.*, 1991), although other studies have not found such a relationship (e.g. Morgan *et al.* 1993 or Monneveux *et al.* 2006 in wheat; Ismail and Hall 1992 in cowpea; Comstock and Ehleringer 1993 in bean). In the study in cowpea, the conclusion was drawn that the differences in $\Delta^{13}C$ between genotypes were more consistent than the intrinsic TE measurements, although the physiological basis for the differences in $\Delta^{13}C$ was not established and CID would then represent a better screening method for breeding purposes.

Carbon isotope discrimination

This spectrometric assessment method has been used successfully to develop wheat cultivars with a lower $\Delta^{13}C$ and therefore higher TE, leading to higher yields under water-stressed conditions (Condon *et al.*, 2002; Rebetzke *et al.*, 2006; Richards *et al.*, 2010). The method is less efficient, though, in C_4 plants, where CO_2 leakage occurs between the mesophyll and the bundle sheath, resulting in reduced discrimination (Henderson *et al.*, 1998). However, the benefits tend to be limited to conditions of severe water stress, whereas under slightly milder water stress conditions a low $\Delta^{13}C$ would lead to yield penalties (Condon *et al.*, 2002). Indeed, a low $\Delta^{13}C$ in wheat has been related to low plant vigour and small plant size, which would reduce light interception and lead to yield penalties under more favourable conditions. The use of CID was expanded as an indirect way to assess TE at the plant level (biomass/water transpired). For instance, a negative relationship was found between TE and CID in four peanut genotypes (Wright *et al.*, 1994) and ten peanut genotypes (Rao *et al.*, 1993). Similar results have been obtained in different crops, such as cowpea (Ismail and Hall, 1992), wheat (Ehdaie *et al.*, 1991), barley (Anyia *et al.*, 2007), and sunflower (Lambrides *et al.*, 2004). In the study in cowpea (Ismail and Hall, 1992), where the relationship between TE and CID held under both water-stressed and well-watered conditions, the genotype-by-treatment interactions observed for $\Delta^{13}C$ indicated that $\Delta^{13}C$ was not only under genetic control but was subject to the complex effects of the water regime. A similar conclusion was drawn from a study in wheat (Monneveux *et al.*, 2006), where the relationship between the grain $\Delta^{13}C$ and yields showed a strong association only under post-anthesis water stress, whereas no or a weak relationship was found under conditions of residual moisture, pre-anthesis water stress, or full irrigation. Fertility levels have also been shown to affect the value of $\Delta^{13}C$, with an increase in $\Delta^{13}C$ being observed upon P fertilization in pearl millet (Bruck *et al.*, 2000) and a decrease in $\Delta^{13}C$ being found upon N fertilization of N-deficient wheat (Clay *et al.*, 2001). A number of studies have reported the absence of a relationship between TE and CID (Devi *et al.*, 2011; Turner *et al.*, 2007), or a weak relationship (Zacharisen *et al.*, 1999; Krishnamurthy *et al.*, 2007). In summary, while the CID method offers the potential to indirectly assess TE, there are situations where its use has limits, the causes of which are still unclear, and it cannot be employed as a stand-alone tool. To date, CID has only been successfully applied in the case of wheat breeding in relatively dry environments in Australia. We discuss the possible reasons for the inconsistencies in these relationships below.

SCMR and SLA

Measurements of CID are not immediate, and they are quite expensive, which has triggered a search for alternative surrogates that are cheaper and faster to measure. A study by Wright *et al.* (1994) showed a strong negative relationship between $\Delta^{13}C$ and the SLA, indicating that genotypes with thicker leaves would show a higher TE. Subsequently, a significant negative correlation was reported between SLA and SCMR, which can be performed on many leaves relatively quickly, providing a value that serves as a proxy of the chlorophyll content of the leaf (Rao *et al.*, 2001). Similar associations between TE and SLA were identified in a recombinant F_2 population of *Stylosanthes*, and co-mapped quantitative trait loci for SLA and TE were identified, indicating a causal relationship (Thumma *et al.*, 1998). SLA and SCMR have been shown to be related to TE in a number of studies (Comstock and Ehleringer, 1993; Sheshshayee *et al.*, 2006; Thompson *et al.*, 2007). However, other studies have found poor relationships between these surrogates and gravimetric TE measurements (Krishnamurthy *et al.*, 2007; Devi *et al.*, 2011). Additionally, only minor quantitative trait loci were identified for these surrogates in a population of a recombinant inbred line of peanut (Varshney *et al.*, 2009). Thus, the common use of these surrogates, applied in breeding programmes, has not improved the rate of genetic gains in peanut (Nigam *et al.*, 2005).

Limitations on the use of surrogates

Many studies have used these surrogates as factors that are less prone to environmental variation. For instance, $\Delta^{13}C$ values appear to be more consistent across conditions than TE measurements, from which it was concluded that CID should be a more effective tool than TE measurements for breeding purposes (e.g. Hall *et al.*, 1992; Ismail and Hall, 1992). However, this conclusion overlooks the large genotype-by-treatment interaction reported for the leaf gas exchange

efficiency. In another case, in peanut, SCMR and SLA were used to screen for 'drought tolerance' (Upadhyaya, 2005), although these experiments were carried out under fully irrigated conditions, and no TE measurements were performed in even a subset of germplasm. These are cases where the simplicity of a surrogate trait overshadows its ecophysiological meaning. A study in sorghum found large differences in TE in the investigated germplasm (Hammer *et al.*, 1997) but also detected a weak association between TE and CID and suggested that screening for TE should not be based on a single screen but, rather, on establishing the physiological basis of the differences in TE. CID measurements are indeed known to be very closely dependent on the environment, which reflects different responses of specific physiological traits associated with CID to environmental conditions (e.g. Misra *et al.*, 2010; Tambussi *et al.*, 2007; Condon and Richards, 1992).

Another limiting factor in the use of surrogates is the time required. While measurements of intrinsic TE are instantaneous, CID involves integration over time, which depends on the sampled plant parts, which are either flag leaves or grain in many studies. Therefore, unless the conditions under which the intrinsic TE is measured are environmentally stable, there is a chance of genotype-by-environment interactions occurring. For instance in cowpea, gravimetric and intrinsic TE were not found to be correlated (Anyia and Herzog, 2004). In fact, while $\Delta^{13}C$ does not always correlate with TE measurements under a given water regime, $\Delta^{13}C$ always decreases under water stress, indicating the tight dependence of CID on leaf conductance. The stomatal aperture is regulated by a number of environmental factors in addition to soil moisture, including light, temperature, nutrition, and relative humidity (Comstock *et al.*, 2005), and any of these factors would therefore transiently alter CID. Hence, it is interesting that a good correlation between $\Delta^{13}C$ and TE has been identified under environmentally stable conditions (e.g. Ismail and Hall, 1992).

Stomata or photosynthesis: what drives differences in CID?

Both SLA and SCMR are surrogates that serve as indirect proxies for the carboxylation efficiency of the mesophyll, where higher chlorophyll content from more packed mesophyll cells (lower SLA) leads to more active removal of C_i from the stomatal chamber, thus reducing the C_i/C_a ratio and increasing TE. These two surrogates are therefore not useful in situations where a low leaf conductance is driving differences in TE. The CID assay does not distinguish between whether differences in CID are driven by mesophyll efficiency or leaf conductance. The lack of a relationship between TE and CID could be derived from these two mechanisms operating in opposition. This might have been the case in a study in bean, where CID was indeed found to be positively related to both photosynthetic activity and leaf conductance but was not correlated with TE (Comstock and Ehleringer, 1993). The differences in the degree of discrimination against ^{13}C between Rubisco and the stomata are another source of confusion in interpreting these results. The discrimination against ^{13}C by Rubisco is to a factor of 2.7% (Farquhar *et al.*, 1989), whereas the discrimination by stomata is to a factor of 0.4%, which is approximately seven times less. Therefore, stomatal conductance-driven differences in $\Delta^{13}C$ would lead to much weaker CID signatures than those driven by differences in carboxylation efficiency. In cowpea, the differences in the gas exchange efficiency were found to be more strongly driven by differences in the assimilation capacity (A) than by stomatal conductance (Gs) (Hall *et al.*, 1992). A pathway analysis also showed that the main factor explaining the differences in TE evolved over the life of the plant (Hui *et al.*, 2008). In peanut, many authors have argued that differences in TE were driven by the mesophyll efficiency (Hubick *et al.*, 1986; Rao *et al.*, 1993; Wright *et al.*, 1994; Udayakumar *et al.*, 1998). However, more recent results reveal a close relationship between stomatal conductance and TE (Bhatnagar-Mathur *et al.*, 2007). An additional dimension is provided by the sensitivity of stomatal conductance to VPD, which varies across genotype (details in the next section), and the fact that genetic differences have been found in peanut (Devi *et al.*, 2010). Interestingly, the study in bean cited above (Comstock and Ehleringer, 1993) also found high variation among genotypes in the response to decreasing air humidity, and the authors attributed the lack of a relationship between TE and CID to differences in leaf temperature.

In summary, while there is no question about the validity of the theory regarding the differences in discrimination against ^{13}C and its drivers, whether differences in CID are consequences of differences in the photosynthesis or the leaf conductance is still unclear. From a breeding point of view, it would be more effective to decipher the physiology and genetics of both components of discrimination, rather than attempting to harness the genetics of CID as a whole. Until there is a better understanding of what role environmental conditions play in CID, especially for VPD, it will be difficult to use this method on a routine basis in breeding programmes. More research is therefore needed on the ecophysiological meaning and significance of this discrimination phenomenon.

A new method for evaluating TE

The use of indirect methods arose due to the difficulty of precisely measuring transpiration under field conditions or measuring plant transpiration over the long term in pot experiments. A lysimetric method, involving long, large PVC tubes installed outdoors, has been developed (Vadez *et al.*, 2008). The tube size and spacing are designed to provide the plants with a similar space and soil volume to be explored to those found in field conditions (Fig. 1). This approach allows the monitoring of plant water use and biomass accumulation (both vegetative and grain) from very early plant stages until maturity, and it allows extremely robust TE assessments to be conducted with very low experimental error (Ratnakumar *et al.*, 2009; Vadez *et al.*, 2011*a*, 2011*b*). Using this system, transpiration is measured over almost the entire crop cycle, avoiding possible artefacts found in short-term experiments when there is high T variation. This experimental setup has

Fig. 1. Description of the lysimetric system. Panel (a) shows the layer of beads applied on top of the tubes (in addition to a plastic sheet below the beads in other crops than peanut) to prevent soil evaporation. Tubes are lifted for weighing with an extension load cell (b). The lysimetric system is versatile and usable for many crops; e.g. cowpea (c), sorghum (d), and peanut (e). Panels (d) and (e) show that the plants are cultivated individually but are part of a crop canopy, and cultivated at a density that reflects the plant population in the field.

been shown to be suited to a wide range of crops and has been designed to cater for breeding programmes due to its several-thousand-tube capacity. Additionally, this system allows plant water use to be monitored at different times during the crop cycle. Last but not least, it is the first system that allows measurements of all terms of the Passioura equation (Yield = WU × TE × HI) to be conducted on the same plants and then allows the weight of each term on yield to be assessed. Both of these last factors are discussed at length in the third section of the paper.

How to decrease ($W_i - W_a$), the transpiration response to high VPD

Theoretical considerations underlying the VPD response

Here, we come back to the equation of Sinclair *et al.* (1984), where $G_d/T = k_d/(e^*_a - e)_d$, with G_d representing the daily increase in biomass and TE showing an inverse relationship with the VPD faced by the plants. The term $(e^*_a - e)_d$ represents the difference between the saturated vapour pressure (e^*_a) and the ambient one (e), calculated as the mean over 1 day. To measure TE over a long period, this term needs to be integrated over a season and then to be weighted by the transpiration rate of the plant over the course of the measurement period (Tanner and Sinclair, 1983). Applying the same logic to a single day, the weighting of this component based on the transpiration rate throughout the day leads to the possibility of obtaining altered values if there are genotypic variations in the transpiration profile throughout the day (Sinclair, 2012). The sensitivity of the stomatal aperture to the VPD has long been established (Turner *et al.*, 1984; Grantz, 1990), but it was not until fairly recently that genotypic variation was demonstrated in various species (e.g. Fletcher *et al.*, 2007; Kholova *et al.*, 2010b). This search was triggered by evidence from simulation modelling using weather data from a sorghum production area, showing that restricting the maximum transpiration would increase the TE and yield of sorghum (Sinclair *et al.*, 2005). Therefore, the significance of this trait is that if a given genotype possesses the attributes to restrict transpiration at the time when VPD is highest, the proportion of transpiration taking place under a high VPD is lower, which decreases the integrated value of the VPD [the $(e^*_a - e)_d$ component] used to calculate TE and therefore increases TE. Of course, restriction of transpiration under high VPD conditions would also partially increase the leaf temperature and, hence, the e^*_a term, which would partially offset the benefit of a reduced effective VPD. However, simulations have shown a large beneficial effect of that trait on yields in soybean (Sinclair *et al.*, 2010), and more similar evidence has come from peanut (Vadez *et al.*, unpublished data) and sorghum (Kholova *et al.*, unpublished data).

In summary, the restriction of transpiration under conditions of high VPD in certain genotypes across different species decreases the $(e^*_a - e)_d$ term of the TE equation, thereby presenting the opportunity of including a genetic component in a term that had long been seen as purely dependent on the environment and therefore 'out of genetic reach'. There is clearly a possibility of using the genetic component of that term of the equation to enhance TE, specifically for crops that are most likely to face high-VPD conditions.

Genetic differences in the VPD response and its hydraulic basis

Large genetic variation in the capacity to restrict transpiration under a high VPD has been identified in the past 5 years in soybean (Fletcher *et al.*, 2007), chickpea (Zaman-Allah *et al.*, 2011*a*), peanut (Devi *et al.*, 2010), cowpea (Belko *et al.*, 2012), pearl millet (Kholova *et al.*, 2010b), maize (Yang *et al.*, 2012), sorghum (Gholipoor *et al.*, 2010), and wheat (Schoppach and Sadok, 2012). Figure 2 shows an example of the VPD response from a recent study (Kholova *et al.*, 2010b). The fact that rapid changes in transpiration occur once certain VPD thresholds are crossed implies that hydraulic signals are needed to induce fairly rapid stomatal closure and avoid loss of turgor. In soybean, the sensitivity of transpiration to a high VPD of genotype PI416937 was found to be related to a lower leaf hydraulic conductance (Sinclair *et al.*, 2008), and there is preliminary evidence of a similar process in peanut (Devi *et al.*, 2012). In other crops, the source of hydraulic limitation is instead the roots, for instance in wheat (Comstock, 2000; Schoppach *et al.*, 2014; Tharanya *et al.*, unpublished data). In a study in which 9-*cis*-epoxycarotenoid dioxygenase (NCED) enzyme activity was enhanced, the concentration of abscisic acid increased in the shoots while stomatal conductance decreased, and TE and root hydraulic conductivity were increased (Thompson *et al.*, 2007). Additionally, in a study where root hydraulic conductivity was decreased through aquaporin inhibitor treatments, the stomatal conductance was not affected under a low VPD, but the stomata closed under a high VPD (Ehlert *et al.*, 2009).

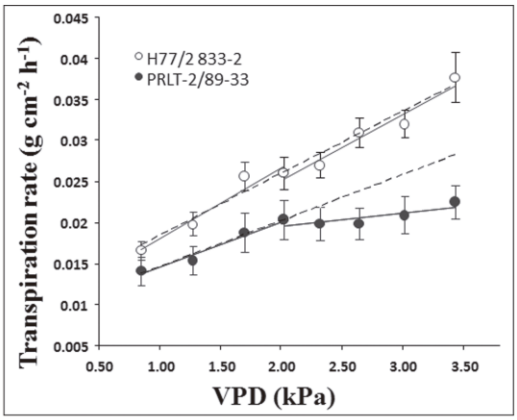


Fig. 2. Transpiration response to a ladder of increasing VPD conditions, in a terminal-drought-sensitive (H77/833-2, open circles) and a terminal-drought-tolerant (PRLT-2/89-33, closed circles) pearl millet genotype. Transpiration was measured gravimetrically at each VPD level by regularly weighing potted plants (wrapped in a plastic bag around the stem to avoid soil evaporation). Leaf area was destructively measured at the end of the VPD response experiment and the transpiration rate computed as the ratio of transpiration values divided by the leaf area. Redrawn from Kholova J, Hash CT, Kumar PL, Yadav RS, Kocova M, Vadez V. 2010*b*. Terminal drought-tolerant pearl millet Pennisetum glaucum (L.) R. Br. have high leaf ABA and limit transpiration at high vapour pressure deficit. *Journal of Experimental Botany* **61,** 1431–1440.

Therefore, there is a hydraulic basis for the differences in the transpiration response to a high VPD. In soybean, genotypes showing contrasting responses to the VPD also display a contrasting sensitivity to silver nitrate in a population of aquaporins present in the leaves (Sadok and Sinclair, 2010). Similarly, peanut genotypes exhibiting differing transpiration responses to a high VPD also differed in their transpiration response to silver nitrate in de-rooted plants (Devi *et al.*, 2012). In both studies, the genotypes of soybean or peanut in which transpiration was constrained by a high VPD did not show inhibition by the aquaporin inhibitor, suggesting that transpiration constraint due to plant hydraulics was related to the absence of some forms of aquaporins. We have also found that differences in the response of transpiration to high VPD in sorghum was related to differences in the gene expression of several PIP isoforms (our unpublished data). Similar evidence was recently shown for an increase in aquaporin gene expression under higher evaporative demand (Laur *et al.*, 2013) or osmotic stress (Hachez *et al.*, 2012). This fits well with the role of aquaporins, which have been described as facilitators of water movement in different organs (Maurel, 1997; Tyerman *et al.*, 1999; Chrispeels *et al.*, 2001; Javot and Maurel, 2002; Li *et al.*, 2013). Aquaporins are also well known in their response to water stress (e.g. Aroca *et al.*, 2012; Henry *et al.*, 2012). Of course, there are many aquaporin isoforms in the different crop species, which are expressed in different plant compartments, with expression varying with age and time of the day. Therefore the challenge still remains to explain which of these, if any, has any direct functional effect on the VPD response phenotype.

In summary, genetic differences in the response of transpiration to a high VPD have been identified in different crop species and this response appears to have a hydraulic basis, in which aquaporin might play a role. However, the conditions under which altered hydraulic properties lead to stomatal closure and increases in TE are still not clear, and more work is required to elucidate them. Additionally, soil hydraulic properties may play a critical role that needs to be better understood, especially concerning how they are translated through root signalling to the shoots, in a hydraulic or biochemical manner, to alter plant development and/or stomatal opening. However, the possibility of harnessing genetic components of these mechanisms in a way that improves water productivity opens an exciting arena of research.

Transpiration response to soil drying

A strong water stress effect on CID has generally been reported (see citations above), and plants being exposed to intermittent water stress would therefore experience alternating periods of sufficient and insufficient water supplies. The plant response to progressive soil drying follows a fairly common pattern across crop species, displaying an initial phase in which the water supply is still sufficient and a second phase in which the water supply to transpiring leaves is insufficient, and stomatal closure begins to avoid loss of turgor and leads to a decline in transpiration (Fig. 3). Plants under water stress (in phase 2) would indeed likely transpire in the early hours

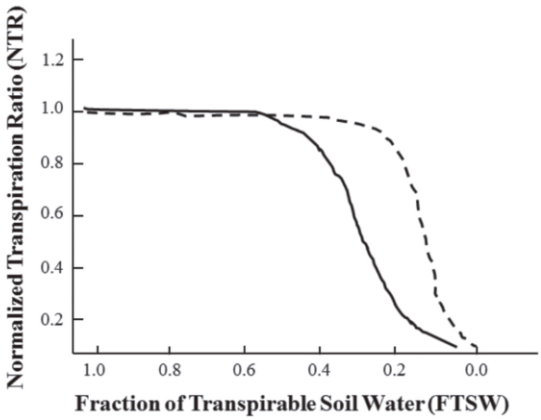


Fig. 3. Typical response of transpiration to progressive soil drying in two hypothetical genotypes: a genotype with an early decline in transpiration at a fraction of transpirable soil water (FTSW) of approximately 0.5 (solid line) and a genotype with a late decline in transpiration at FTSW of approximately 0.2 (dashed line). The *y* axis depicts the normalized transpiration ratio (NTR), which derives from the normalization of transpiration data against a fully irrigated control (see Kholova *et al.* 2010*a* or Zaman-Allah *et al.* 2011*a* for details about the normalization). Where NTR is constant at a value of 1.0, water-stressed plants transpire, and therefore grow, as much as fully irrigated controls. NTR drops below 1.0 when roots cannot fully supply transpiration demand. At an NTR value below 0.1 it is considered that stomata are fully closed and there are no more transpiration-related water losses. At this stage, all the water available for transpiration has been depleted and the FTSW reaches a value of zero.

of the morning and close their stomata when approaching higher-VPD conditions later in the day. What is quite critical here is the genetic variation in the soil moisture thresholds (measured based on the fraction of transpirable soil water; FTSW) where water stress starts affecting the plants (Sadras and Milroy, 1996; Devi *et al.*, 2009; Kholova *et al.*, 2010*a*; Zaman-Allah *et al.*, 2011*a*; Belko *et al.*, 2012; Gholipoor *et al.*, 2012) (a representative example is shown in Fig. 3). This plant attribute would offer another means of altering the $(e^*_a - e)_d$ term of the TE equation, as transpiration would be more heavily weighted toward lower VPD periods of the day. Genetic differences in the FTSW threshold at which the decrease in transpiration is initiated are therefore another major factor that might alter this term by genetic means, and there is a possibility of exploring genetic variation for such traits at a larger scale. Although there are now available data documenting the genetic variation in the transpiration response to a high VPD, there is most likely a need to screen a large germplasm for putative variation in the transpiration response to soil drying. It should be mentioned, however, that this is not an easy phenotype to measure. Assessing it on a large scale is possible and would require a phenotyping platform allowing frequent weighing and automatic re-watering.

A consequence of the genetic variation in FTSW thresholds at which transpiration declines is that genotypes with high thresholds are water-stressed at FTSW levels where low-threshold genotypes are not water stressed and function like fully irrigated plants. From a CID perspective, high-threshold genotypes would display a lower $\Delta^{13}C$. From a VPD perspective, higher-threshold genotypes would be conferred a higher TE because of a lower integrated daily VPD value in the calculation of TE. Experimental evidence of this hypothesis was recently reported (Devi *et al.*, 2009), although the obtained polynomial relationship was relatively weak ($R^2 = 0.39$) and was conditioned by one extreme genotype. Nevertheless, more convincing, but contradictory results were also reported recently. A strong negative correlation ($R^2 = 0.88$) was found between the TE and the FTSW thresholds of a set of transgenic (*rd29::DREB1A*) peanut plants grown under a fairly high average VPD in a glasshouse. In cowpea, a strong negative linear relationship ($R^2 = 0.62$) was also found between TE and the FTSW threshold for transpiration declines under conditions of a high VPD, although no relationship was found under low-VPD conditions (Belko *et al.*, 2012). In the last study, the genotypes exhibiting unrestricted transpiration at a high VPD also presented higher FTSW thresholds, and these results are in agreement with those found in pearl millet, where high-threshold genotypes also show unrestricted transpiration at a high VPD (Kholova *et al.*, 2010*a*). In these two studies we argue that the FTSW threshold calculation, which implies normalization to the transpiration of well-watered plants, would be altered if any factor affected the transpiration of well-watered plants, which was the case for the transpiration response to high VPD. Therefore, a high FTSW threshold may not always lead to increases in TE, especially in genotypes that exhibit transpiration restriction at a high VPD.

Stomatal patchiness

One quite intriguing finding is that differences in the transpiration response to a high VPD in a set of pearl millet genotypes did not lead to differences in TE (Kholova *et al.*, 2010b). In this paper it was argued that the experiment had been carried out in a glasshouse and that the plants may not have been exposed to the VPD threshold at which differences in transpiration occur. However, even when TE was measured over the long term in lysimeters and under high-VPD conditions this parameter did not differ between these genotypes (Vadez *et al.*, 2013). Part of the reason for this result could lie in the opposing effects of transpirational sensitivity to VPD and a low FTSW threshold for transpiration declines (e.g. genotype *PRLT/89-33*). Another possible interpretation is that stomatal closure may not take place uniformly at the leaf level. It is indeed assumed that plants showing transpirational sensitivity to a high VPD partially close all of their stomata upon increases in VPD. It is this uniform response that would lead to an effect on TE, from its effect on stomatal conductance (see TE definition in the first section). A similar effect on transpiration under high VPD could be achieved if only part of the stomata were completely closed, while others remained fully open. In such a case there would be no decrease in stomatal conductance in some parts of the leaf, and full closure in others, and then overall no effect on TE. Evidence of stomatal patchiness has long been reported (Pospisilova and Santrucek, 1994), and the stomatal responses to environmental stimuli

are known to be extremely heterogeneous, which appears to be related to hydraulic interactions (e.g. Mott and Buckley, 2000; Peak *et al.*, 2004). Stomata patchiness might create spots in the leaf having different temperature, and how this would affect the VPD of the micro-environment above these spots is unknown. We might speculate that air circulation at the leaf surface and the small size of these spots would make the VPD homogenous across the leaf surface. Although the interpretation of leaf gas exchange data becomes complex if stomata close in patches, it might partly explain the observation that while transpiration under a high VPD could be restricted (because of the complete closure of some stomata), the intrinsic TE at the level of stomata remaining open would be unchanged.

In summary, while the genetic variability of the transpirational sensitivity to VPD and to soil drying may lead to substantial increases in TE and therefore make it possible to include a genetic component in the $(e^*_a - e)_d$ term of the TE equation, the relationship between this mechanism and TE is not straightforward because (i) the sensitivity to VPD and to soil drying can work in opposition (sensitivity to VPD and insensitivity to soil drying) and (ii) stomatal patchiness would make the transpiration response to VPD or to soil drying quite heterogeneous at the leaf canopy level.

How these findings could explain earlier controversial results

In the paragraphs above there are a number of examples that might explain why the earlier CID theory, when put into practice, has not always 'worked'. CID being driven by CO_2 assimilation or intrinsic transpiration would not result in the same CID signature, although both would alter the C_i/C_a ratio. Hence, unless there is a clear predominant driver of the differences in CID, experimental situations that induce variations in stomatal stimuli are bound to have extremely complex consequences for the CID signature. Hence, we have observed the criticality of the $(e^*_a - e)_d$ term of the TE equation (Sinclair *et al.*, 1984) and that there are several genetic components capable of altering that term. Among these components, we have described the sensitivity of transpiration to VPD and to soil drying, and overall the processes that influence stomata opening, from plant hydraulic differences, soil hydraulic conditions and stomatal heterogeneity. We have seen that some of these processes can work in opposition (for instance, the sensitivity to VPD and to soil drying), which would obscure the resulting effect on TE. Therefore, progress in improving the TE of plants can be made by deciphering the individual components that contribute to increasing the intrinsic TE. This starts by clearly distinguishing the role of the photosynthetic capacity in explaining differences in CID. Many authors have argued that CID is driven by A (e.g. Rao and Wright, 1994; Condon *et al.*, 2002; Arunyanark *et al.*, 2008). Although the role of the stomata in altering CID has been reported (Ehleringer *et al.*, 1991; Udayakumar *et al.*, 1998), it has largely been overlooked, as has the fact that stomatal opening is extremely sensitive to environmental stimuli. Thus, in cases where the stomata serve as the major driver of differences in CID, which have been reported to be more complex to interpret (Condon *et al.*, 2002), a number of potential factors that modulate stomatal movements need to be better understood. Only when these components are identified can their genetics be deciphered and put to use to benefit crop-improvement programmes. Positive correlations between grain yields and CID are usually found in situations of no or mild water stress, whereas under terminal stress conditions negative relationships prevail between grain yields and CID (Tambussi *et al.*, 2007).

Timing of plant water use

In this section, we wish to take a different stand with regard to water by exploring possible interactions between the terms of the Passioura equation (Yield = WU × TE × HI) (Passioura, 1977). For instance, it has been reported that selection for high TE might also select plant types with low vigour and productivity and, hence, a low T term (Condon *et al.*, 2002; Blum, 2009). In the terms of the Passioura equation (Passioura, 1977), the variation in water use by the plant over time, the T term is taken as a linear term showing a similar influence on yields across the entire cropping cycle, but recent work demonstrates the importance of having available water for critical stages in crops, such as the reproduction and grain-filling periods. Therefore, we also want to go beyond the discussion on the means of improving TE and explore the means of achieving efficient timing of water use.

Are the terms of the Passioura equation mutually exclusive?

Recent reviews take the stand that a high WUE is certain to be related to low productivity (Blum, 2005, 2009). This notion is supported by a number of studies in which a high yield was achieved by genotypes with high stomatal conductance to maximize plant water use (e.g. Reynolds *et al.*, 1994; Araus *et al.*, 2002). This indeed appears to be the case under Mediterranean environments, where the grain or leaf CID appears to be positively related to grain yields; that is, higher grain yields are achieved in crops with a high $\Delta^{13}C$ and, hence, a low intrinsic TE (e.g. Monneveux *et al.*, 2006). Low $\Delta^{13}C$ wheat lines in Australia were also found to show low vigour and to present a lower yield in higher rainfall environments (Condon *et al.*, 2002), putatively because their lower stomatal conductance led to less biomass accumulation, smaller leaf area, and less light interception. These are cases in which lower stomatal conductance would indeed reduce the potential to maximize soil water use. However, as noted in the previous section, transient alterations of stomatal conductance during high-VPD conditions can dramatically increase TE. In recent studies, a lysimetric system has been used to assess TE, along with the other terms of the Passioura equation, in a large range of genetic materials (germplasm, breeding populations) (Vadez *et al.*, 2008). In a study in sorghum, no relationship was found between the TE

and T components of the equation (Fig. 4a) (derived from Vadez *et al.*, 2011b). Similar data were obtained in other studies in sorghum (Vadez *et al.*, 2011*a*) and pearl millet (Vadez *et al.*, 2013). Figure 4b and c provide similar information for 268 entries of the reference collection of pearl millet and 280 entries of the peanut reference collection (Vadez *et al.*, unpublished data). These studies were conducted under conditions of medium-to-high-VPD conditions, and sorghum genotypes achieving a high TE also displayed transpirational sensitivity to a high VPD (Vadez *et al.*, unpublished data). Additionally, this work in sorghum showed that among the terms of the Passioura equation, besides the obvious importance of the harvest index, yields were closely related to TE, but not to the water-use term (Vadez *et al.*, 2011*a*, 2011*b*), which contradicts earlier claims (Blum, 2009). In other recent studies testing soybean genotypes with contrasting sensitivities to a high VPD, a higher photosynthetic rate was able to compensate for the transiently lower stomatal conductance of VPD-sensitive genotypes (Gilbert *et al.*, 2011*a*, 2011*b*). This is related to the logarithmic shape of the relationship between the photosynthetic rate and stomatal conductance (Wong *et al.*, 1979) and the marginal increases in the photosynthetic rate above a certain stomatal conductance. These examples provide clear contradictory evidence that a high TE is not necessarily related to low productivity and water use.

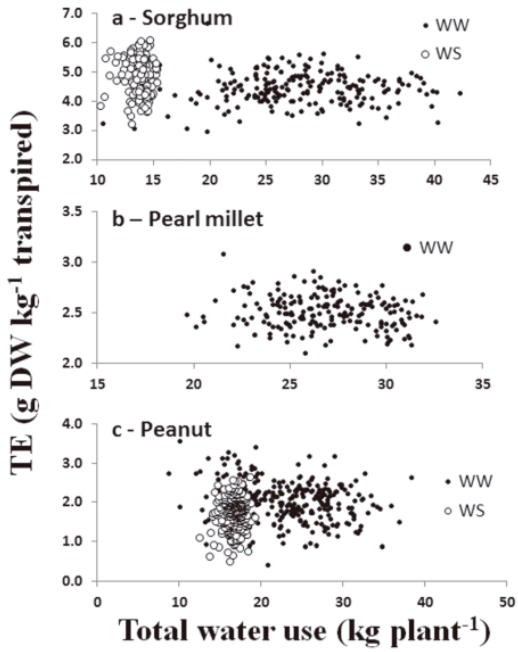


Fig. 4. Relationships between TE in g biomass·kg^{-1} water transpired) and total plant water use (in kg·plant^{-1}) in a set of 152 sorghum germplasm (a), 210 pearl millet germplasm (b), and 280 peanut germplasm (c) under either fully irrigated (well-watered, WW; closed circles) or water-stress (WS; open circles) conditions. Transpiration was monitored throughout most of the cropping cycle, using lysimeters of 2.0 m length and 25 cm diameter (sorghum and pearl millet) or 1.2 m length and 20 cm diameter (peanut).

Again, this situation stresses the need to properly decipher the mechanistic components of TE.

Importance of water availability at key critical stages

It is widely recognized that the reproductive stages are particularly sensitive to water deficits and that water availability during and after anthesis is critical. For instance, higher durum wheat grain yields were found to be closely related to increasing the water input during the post-anthesis period (Araus *et al.*, 2003). Similar results were obtained across 11 grain legume species (Siddique *et al.*, 2001), where increasing water use during the post-anthesis period led to a higher harvest index and grain yield. A finer demonstration of these facts was made in the lysimetric system described at the end of the first section, where water extraction was monitored from the vegetative stage until maturity in pearl millet (Vadez *et al.*, 2013) and chickpea (Zaman-Allah *et al.*, 2011b) exposed to terminal water stress (Fig. 5). In these studies, higher grain yields were achieved by genotypes that had lower plant water use prior to anthesis, which made water available for extraction during the reproductive and grain-filling period. A similar conclusion was drawn from a set of peanut genotypes exposed to intermittent water stress, where higher pod yields were related to higher water extraction during the grain-filling period (Ratnakumar *et al.*, 2009).

The WUE for grain yield production (WUE_{yield}) of 11 grain legumes under a Mediterranean climate ranged from 13 to 16 kg·ha^{-1}·mm^{-1} (Siddique *et al.*, 2001), which is within the range observed for canola under similar conditions (11–15 kg·ha^{-1}·mm^{-1}) (Robertson and Kirkegaard, 2005). These data were computed from the water used during the

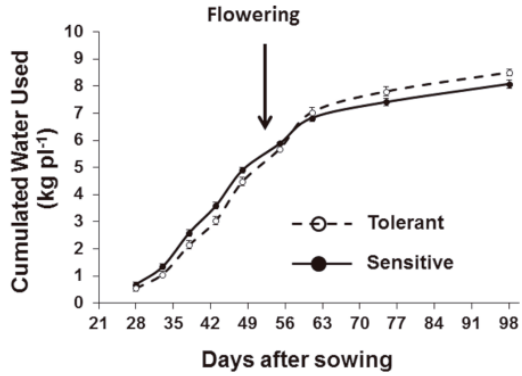


Fig. 5. Cumulated water use (in kg plant^{-1}) as a function of time after sowing and under terminal water stress conditions in chickpea. The plants were grown in lysimeters of 1.2 m length and 20 cm diameter and the last irrigation was applied at 21 days after sowing. The data are the average of the mean values (five replicated plants per line) of 12 tolerant lines (open circles and dashed line) and eight sensitive lines (closed circles and solid line), which were selected from several field experiments. Redrawn from data of Fig. 1 in Zaman-Allah M, Jenkinson DM, Vadez V. 2011*b*. A conservative pattern of water use, rather than deep or profuse rooting, is critical for the terminal drought tolerance of chickpea. *Journal of Experimental Botany* **62,** 4239–4252.

course of the entire crop life cycle, and the WUE for grain yields could be twice as much for water used during the grain-filling period (Wasson *et al.*, 2012). For instance, the slope of the regression between grain yields and the water extracted during the third week after emergence of the flag leaf were observed under two levels of terminal water stress and could be extrapolated to WUE_{yield} values of 37 and 45 $kg \cdot ha^{-1} \cdot mm^{-1}$ in pearl millet (Vadez *et al.*, 2013) or 40 $kg\,kg \cdot ha^{-1} \cdot mm^{-1}$ in chickpea (Zaman-Allah *et al.*, 2011b). Values in a similar range were reported in wheat, for which every millimetre extracted during the grain-filling period showed a WUE_{yield} of 55 (Manschadi *et al.*, 2006) and 59 $kg \cdot ha^{-1} \cdot mm^{-1}$ (Kirkegaard *et al.*, 2007).

In summary, while crops of course require water throughout the cropping cycle and must maximize water use, there are stages in which the water supply is particularly critical. Therefore, the application of the Passioura equation must take into account the interactions between its terms and their different weightings based on yields at different times. Here, the interaction between water use at key times and the harvest index is of particular importance.

Means of securing water at key times

There are several means of securing water availability during the grain-filling period, including those presented in the second section of this paper. In a study in soybean, large yield improvements arose from a higher FTSW threshold for a transpiration decline (Sinclair *et al.*, 2010). The transpirational sensitivity to a high VPD was the factor that made more water available during grain filling in pearl millet genotypes (Kholova *et al.*, 2010b; Vadez *et al.*, 2013). Higher-yield chickpea genotypes show lower leaf conductance during the vegetative stage and present a smaller leaf canopy in the vegetative stage (Zaman-Allah *et al.*, 2011*a*). Therefore, beyond aspects related to leaf conductance and the control of the stomatal aperture, the control of leaf water losses by adjusting the leaf area based on water availability is another critical factor. For instance, genotypes with higher leaf appearance rate would show reduced tillering and therefore a reduced leaf area at anthesis, making more water available after anthesis in sorghum (van Oosterom *et al.*, 2011). Of course, limiting the leaf area would also lead to yield penalties under conditions where there is only mild water stress. Therefore, the key message here is that securing water availability for the reproductive and grain-filling stages relies on the following simple, but fundamental factors: (i) there should be no more water available in the soil profile at maturity and (ii) plant water requirements need to match the water supply. All of these aspects are the topic of a recent review (Vadez *et al.*, 2014). Additionally, we need to consider the case of grain legumes relying on symbiotic nitrogen fixation. Symbiotic activity usually declines during the post-anthesis period because of competition for carbon sources between grain filling and nodule activity. Therefore, high levels of growth and N accumulation prior to anthesis might be needed as well (Sinclair and Vadez 2012).

Conclusions

Improving the productivity of water in agriculture is a necessity. This issue began to be addressed in the early 1980s, and much research on this topic has been undertaken since that time. While the theory concerning the intrinsic WUE in crops has helped investigators to focus on critical issues to achieve a higher TE, successful breeding applications have been limited and are restricted to fairly stable and severe terminal stress situations in wheat in Australia. Here, we argue that a part of this lack of applications is first due to overlooking the high level of interaction between mechanistic traits underlying TE and the environment. There are indeed many factors that can alter CID, and unless their ecophysiological significance is understood there is little possibility of making practical use of this method. The second reason is the subject of this review and involves the terms of equations for determining TE, particularly the VPD term, which has been thought to be purely dependent on the environment. Here, we have reviewed possible ways to 'genetically' alter the effective VPD that is used in the determination of TE, which hold great promise regarding yield increases. The sensitivity of transpiration to soil drying and to VPDs shows a wide range of genotypic variation in a number of crops, and simulation studies predict large possible yield increases. Of course, the use of these traits requires that they be thoroughly understood, especially in relation to hydraulic issues in plants, as should their interactions, with the aim of harnessing the genetics of these traits.

Acknowledgements

The senior author wishes to acknowledge support from the Bill and Melinda Gates Foundation through a grant to the Generation Challenge Program (Tropical Legume I project), the CGIAR Research Program on Dryland Cereals (CRP-DC) and Grain Legumes (CRP-GL), and the Research Program on Climate Change, Agriculture and Food Security (CCAFS), which have supported some of the research presented in this review.

References

Anyia AO, Herzog H. 2004. Water-use efficiency, leaf area and leaf gas exchange of cowpeas under mid-season drought. *European Journal of Agronomy* **20,** 327–339.

Anyia AO, Slaski JJ, Nyachiro JM, Archambault DJ, Juskiw P. 2007. Relationship of carbon isotope discrimination to water use efficiency and productivity of barley under field and greenhouse conditions. *Journal of Agronomy and Crop Science* **193,** 313–323.

Araus JL, Slafer GA, Reynolds MP, Royo C. 2002. Plant breeding and drought in C-3 cereals: what should we breed for? *Annals of Botany* **89,** 925–940.

Araus JL, Villegas D, Aparicio N, del Moral LFG, El Hani S, Rharrabti Y, Ferrio JP, Royo C. 2003. Environmental factors determining carbon isotope discrimination and yield in durum wheat under Mediterranean conditions. *Crop Science* **43,** 170–180.

Aroca R, Porcel R, Luiz-Lozano JM 2012. Regulation of root water uptake under abiotic stress conditions. *Journal of Experimental Botany* **63,** 43–57.

Arunyanark A, Jogloy S, Akkasaeng C, Vorasoot N, Kesmala T, Rao RCN, Wright GC, Patanothai A. 2008. Chlorophyll stability is an indicator of drought tolerance in peanut. *Journal of Agronomy and Crop Science* **194,** 113–125.

Belko N, Zaman-Allah M, Cisse N, Diop NN, Zombre G, Ehlers JD, Vadez V. 2012. Lower soil moisture threshold for transpiration decline

under water deficit correlates with lower canopy conductance and higher transpiration efficiency in drought-tolerant cowpea. *Functional Plant Biology* **39,** 306–322.

Bhatnagar-Mathur P, Devi MJ, Reddy DS, Lavanya M, Vadez V, Serraj R, Yamaguchi-Shinozaki K, Sharma KK. 2007. Stress-inducible expression of At DREB1A in transgenic peanut (Arachis hypogaea L.) increases transpiration efficiency under water-limiting conditions. *Plant Cell Reports* **26,** 2071–2082.

Bierhuizen JF, Slatyer RO. 1965. Effect of atmospheric concentration of water vapor and CO_2 in determining transpiration-photosynthesis relationshiops of cotton leaves. *Agriculture Meteorology* **2,** 259–270.

Blum A. 2005. Drought resistance, water-use efficiency, and yield potential - are they compatible, dissonant, or mutually exclusive? *Australian Journal of Agricultural Research* **56,** 1159–1168.

Blum A. 2009. Effective use of water (EUW) and not water-use efficiency (WUE) is the target of crop yield improvement under drought stress. *Field Crops Research* **112,** 119–123.

Bruck H, Payne WA, Sattelmacher B. 2000. Effects of phosphorus and water supply on yield, transpirational water-use efficiency, and carbon isotope discrimination of pearl millet. *Crop Science* **40,** 120–125.

Chrispeels MJ, Morillon R, Maurel C, Gerbeau P, Kjellbom P, Johansson I. 2001. Aquaporins of plants: Structure, function, regulation, and role in plant water relations. *Aquaporins* **51,** 277–334.

Clay DE, Engel RE, Long DS, Liu Z. 2001. Nitrogen and water stress interact to influence carbon-13 discrimination in wheat. *Soil Science Society of America Journal* **65,** 1823–1828.

Comstock J, Ehleringer J. 1993. Stomatal response to humidity in common bean (Phaseolus vulgaris) – Implications for maximum transpiration rate, water use efficiency and productivity. *Australian Journal of Plant Physiology* **20,** 669–691.

Comstock JP. 2000. Variation in hydraulic architecture and gas-exchange in two desert sub-shrubs, Hymenoclea salsola (T. & G.) and Ambrosia dumosa (Payne). *Oecologia* **125,** 1–10.

Comstock JP, McCouch SR, Martin BC, Tauer CG, Vision TJ, Xu YB, Pausch RC. 2005. The effects of resource availability and environmental conditions on genetic rankings for carbon isotope discrimination during growth in tomato and rice. *Functional Plant Biology* **32,** 1089–1105.

Condon AG, Farquhar GD, Richards RA. 1990. Genotypic variation in carbon isotope discrimination and transpiration efficiency in wheat. Leaf gas exchange and whole plant studies. *Australian Journal of Plant Physiology* **17,** 9–22.

Condon AG, Richards RA. 1992. Broad sense heritability and genotype x environment interaction for carbon isotope discrimination in field-grown wheat. *Australian Journal of Agricultural Research* **43,** 921–934.

Condon AG, Richards RA, Rebetzke GJ, Farquhar GD. 2002. Improving intrinsic water-use efficiency and crop yield. *Crop Science* **42,** 122–131.

Devi MJ, Bhatnagar-Mathur P, Sharma KK, Serraj R, Anwar SY, Vadez V. 2011. Relationships between transpiration efficiency and its surrogate traits in the rd29A:DREB1A transgenic lines of groundnut. *Journal of Agronomy and Crop Science* **197,** 272–283.

Devi MJ, Sadok W, Sinclair TR. 2012. Transpiration response of de-rooted peanut plants to aquaporin inhibitors. *Environmental and Experimental Botany* **78,** 167–172.

Devi MJ, Sinclair TR, Vadez V, Krishnamurthy L. 2009. Peanut genotypic variation in transpiration efficiency and decreased transpiration during progressive soil drying. *Field Crops Research* **114,** 280–285.

Devi MJ, Sinclair TR, Vadez V. 2010. Genotypic variation in peanut for transpiration response to vapor pressure deficit. *Crop Science* **50,** 191–196.

de Wit CT. 1958. *Transpiration and Crop Yields*, vol. 64.6. Versl. Landbouwk. Onderz., Institute of Biological and Chemical Research on Field Crops and Herbage, Wageningen, The Netherlands.

Ehdaie B, Hall AE, Farquhar GD, Nguyen HT, Waines JG. 1991. Water use efficiency and carbon isotope discrimination in wheat. *Crop Science* **31,** 1282–1288.

Ehleringer JR, Klassen S, Clayton S, Sherrill D, Fuller-Holbrook M, Fu QA, Cooper TA. 1991. Carbon isotope discrimination and transpiration efficiency in common bean. *Crop Science* **31,** 1611–1615.

Ehlert C, Maurel C, Tardieu F, Simonneau T. 2009. Aquaporin-mediated reduction in maize root hydraulic conductivity impacts cell turgor and leaf elongation even without changing transpiration. *Plant Physiology* **150,** 1093–1104.

Farquhar GD, Ehleringer JR, Hubick KT. 1989. Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology Plant Molecular Biology* **40,** 503–537.

Farquhar GD, O'Leary MH, Berry JA. 1982. On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Australian Journal of Plant Physiology* **9,** 121–137.

Farquhar GD, Richards RA. 1984. Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. *Australian Journal of Plant Physiology* **11,** 539–552.

Fletcher AL, Sinclair TR, Allen LH. 2007. Transpiration responses to vapor pressure deficit in well watered 'slow-wilting' and commercial soybean. *Environmental and Experimental Botany* **61,** 145–151.

Gholipoor M, Prasad PVV, Mutava RN, Sinclair TR. 2010. Genetic variability of transpiration response to vapor pressure deficit among sorghum genotypes. *Field Crops Research* **119,** 85–90.

Gholipoor M, Sinclair TR, Prasad PVV. 2012. Genotypic variation within sorghum for transpiration response to drying soil. *Plant and Soil* **357,** 35–40.

Gilbert ME, Holbrook NM, Zwieniecki MA, Sadok W, Sinclair TR. 2011a. Field confirmation of genetic variation in soybean transpiration response to vapor pressure deficit and photosynthetic compensation. *Field Crops Research* **124,** 85–92.

Gilbert ME, Zwieniecki MA, Holbrook NM. 2011b. Independent variation in photosynthetic capacity and stomatal conductance leads to differences in intrinsic water use efficiency in 11 soybean genotypes before and during mild drought. *Journal of Experimental Botany* **62,** 2875–2887.

Grantz DA. 1990. Plant response to atmospheric humidity. *Plant Cell and Environment* **13,** 667–679.

Hachez C, Veselov D, Ye Q, Reinhardt H, Knipfer T, Fricke, W, Chaumont F 2012. Short-term control of maize cell and root water permeability through plasma membrane aquaporin isoforms. *Plant Cell and Environment* **35,** 185–198.

Hall AE, Mutters RG, Farquhar GD. 1992. Genotypic and drought-induced differences in carbon isotope discrimination and gas exchange of cowpea. *Crop Science* **32,** 1–6.

Hammer GL, Farquhar GD, Broad IJ. 1997. On the extent of genetic variation for transpiration efficiency in sorghum. *Australian Journal of Agricultural Research* **48,** 649–655.

Henderson S, von Caemmerer S, Farquhar GD, Wade L Hammer G. 1998. Correlation between carbon isotope discrimination and transpiration efficiency in lines of the C4 species Sorghum bicolor in the glasshouse and the field. *Australian Journal of Plant Physiology*, **25,** 111–123

Henry A, Cal AJ, Batoto TC, Torres RO, Serraj R. 2012. Root attributes affecting water uptake of rice (Oryza sativa) under drought. *Journal of Experimental Botany* **63,** 4751–4763.

Hsiao TC, Steduto P, Fereres E. 2007. A systematic and quantitative approach to improve water use efficiency in agriculture. *Irrigation Science* **25,** 209–231.

Hubick KT, Farquhar GD. 1989. Genetic variation in carbon isotope discrimination and the ratio of carbon gained to water lost in barley. *Plant Cell and Environment* **12,** 795–804.

Hubick KT, Farquhar GD, Shorter R. 1986. Correlation between water-use efficiency and carbon isotope discrimination in diverse peanut (Arachis) germplasm. *Australian Journal of Plant Physiology* **13,** 803–816.

Hui Z, Zhang ZB, Shao HB, Ping X, Foulkes MJ. 2008. Genetic correlation and path analysis of transpiration efficiency for wheat flag leaves. *Environmental and Experimental Botany* **64,** 128–134.

Ismail AM, Hall AE. 1992. Correlation between water use efficiency and carbon isotope discrimination in diverse cowpea genotypes and isogenic lines. *Crop Science* **32,** 7–12.

Javot H, Maurel C. 2002. The role of aquaporins in root water uptake. *Annals of Botany* **90,** 301–313.

Kholova J, Hash CT, Kakkera A, Kocova M, Vadez V. 2010a. Constitutive water-conserving mechanisms are correlated with the terminal drought tolerance of pearl millet Pennisetum glaucum (L.) R. Br. *Journal of Experimental Botany* **61,** 369–377.

Kholova J, Hash CT, Kumar PL, Yadav RS, Kocova M, Vadez V. 2010b. Terminal drought-tolerant pearl millet Pennisetum glaucum (L.) R. Br. have high leaf ABA and limit transpiration at high vapour pressure deficit. *Journal of Experimental Botany* **61,** 1431–1440.

Kirkegaard JA, Hunt JR. 2010. Increasing productivity by matching farming system management and genotype in water-limited environments. *Journal of Experimental Botany* **61,** 4129–4143.

Kirkegaard JA, Lilley JM, Howe GN, Graham JM. 2007. Impact of subsoil water use on wheat yield. *Australian Journal of Agricultural Research* **58,** 303–315.

Krishnamurthy L, Vadez V, Devi MJ, Serraj R, Nigam SN, Sheshshayee MS, Chandra S, Aruna R. 2007. Variation in transpiration efficiency and its related traits in a groundnut (Arachis hypogaea L.) mapping population. *Field Crops Research* **103,** 189–197.

Lambrides CJ, Chapman SC, Shorter R. 2004. Genetic variation for carbon isotope discrimination in sunflower: association with transpiration efficiency and evidence for cytoplasmic inheritance. *Crop Science* **44,** 1642–1653.

Laur J, Hacke UG. 2013. Transpirational demand affects aquaporin expression in poplar roots. *Journal of Experimental Botany* **64,** 2283–2293.

Li G, Santoni V, Maurel C. 2013. Plant aquaporins: roles in plant physiology. *Biochimica et Biophysica Acta - General Subjects* doi 10.1016/j.bbagen.2013.11.004.

Manschadi AM, Christopher J, Devoil P, Hammer GL. 2006. The role of root architectural traits in adaptation of wheat to water-limited environments. *Functional Plant Biology* **33,** 823–837.

Maurel C. 1997. Aquaporins and water permeability of plant membranes. *Annual Review of Plant Physiology and Plant Molecular Biology* **48,** 399–429.

Misra SC, Shinde S, Geerts S, Rao VS, Monneveux P. 2010. Can carbon isotope discrimination and ash content predict grain yield and water use efficiency in wheat? *Agricultural Water Management* **97,** 57–65.

Monneveux P, Rekika D, Acevedo E, Merah O. 2006. Effect of drought on leaf gas exchange, carbon isotope discrimination, transpiration efficiency and productivity in field grown durum wheat genotypes. *Plant Science* **170,** 867–872.

Morgan JA, LeCain DR, McCaig TN, Quick JS. 1993. Gas exchange, carbon isotope discrimination, and productivity in winter wheat. *Crop Science* **33,** 178–186.

Mott KA, Buckley TN. 2000. Patchy stomatal conductance: emergent collective behaviour of stomata. *Trends in Plant Science* **5,** 258–262.

Muchow RC, Sinclair TR. 1991. Water deficit effects on maize yields modeled under current and greenhouse climates. *Agronomy Journal* **83,** 1052–1059.

Nigam SN, Chandra S, Sridevi KR, Bhukta M, Reddy AGS, Rachaputi NR, Wright GC, Reddy PV, Deshmukh MP, Mathur RK *et al.* 2005. Efficiency of physiological trait-based and empirical selection approaches for drought tolerance in groundnut. *Annals of Applied Biology* **146,** 433–439.

Passioura JB. 1977. Grain yield, harvest index and water use of wheat. *Journal of the Australian Institute of Agriculture Science* **43,** 117–121.

Passioura JB, Angus JF. 2010. Improving productivity of crops in water-limited environments. In: Sparks DL, ed. *Advances in Agronomy* **106,** 37–75.

Peak D, West JD, Messinger SM, Mott KA. 2004. Evidence for complex, collective dynamics and emergent, distributed computation in plants. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 918–922.

Pospisilova J, Santrucek J. 1994. Stomatal patchiness. *Biologia Plantarum* **36,** 481–510.

Rao RCN, Talwar HS, Wright GC. 2001. Rapid assessment of specific leaf area and leaf nitrogen in peanut (Arachis hypogaea L.) using a chlorophyll meter. *Journal of Agronomy and Crop Science* **186,** 175–182.

Rao RCN, Williams JH, Wadia KDR, Hubick KT, Farquhar GD. 1993. Crop growth, water use efficiency and carbon isotope discrimination in groundnut (Arachis hypogeae L.) genotypes under end of season drought conditions. *Annals of Applied Biology* **122,** 357–367.

Rao RCN, Wright GC. 1994. Stability of the relationship between specific leaf area and carbon isotope discrimination across environments in peanut. *Crop Science* **34,** 98–103.

Ratnakumar P, Vadez V, Nigam SN, Krishnamurthy L. 2009. Assessment of transpiration efficiency in peanut (Arachis hypogaea L.) under drought using a lysimetric system. *Plant Biology* **11,** 124–130.

Rebetzke GJ, Richards RA, Condon AG, Farquhar GD. 2006. Inheritance of carbon isotope discrimination in bread wheat (Triticum aestivum L.). *Euphytica* **150,** 97–106.

Reynolds MP, Acevedo E, Sayre KD, Fischer RA. 1994. Yield potential in modern wheat varieties – its association with a less competitive ideotype. *Field Crops Research* **37,** 149–160.

Richards RA, Rebetzke GJ, Watt M, Condon AG, Spielmeyer W, Dolferus R. 2010. Breeding for improved water productivity in temperate cereals: phenotyping, quantitative trait loci, markers and the selection environment. *Functional Plant Biology* **37,** 85–97.

Robertson MJ, Kirkegaard JA. 2005. Water-use efficiency of dryland canola in an equi-seasonal rainfall environment. *Australian Journal of Agricultural Research* **56,** 1373–1386.

Sadok W, Sinclair TR. 2010. Genetic variability of transpiration response of soybean Glycine max (L.) mem shoots to leaf hydraulic conductance inhibitor AgNO3. *Crop Science* **50,** 1423–1430.

Sadras VO, Milroy SP. 1996. Soil-water thresholds for the responses of leaf expansion and gas exchange. *Field Crops Research* **47,** 253–266.

Schoppach R, Sadok W. 2012. Differential sensitivities of transpiration to evaporative demand and soil water deficit among wheat elite cultivars indicate different strategies for drought tolerance. *Environmental and Experimental Botany* **84,** 1–10.

Schoppach R, Wauthelet D, Jeanguenin L, Sadok W 2014. Conservative water use under high evaporative demand associated with smaller root metaxylem and limited trans-membrane water transport in wheat. *Functional Plant Biology* http://dx.doi.org/10.1071/FP13211.

Sheshshayee MS, Bindumadhava H, Rachaputi NR, Prasad TG, Udayakumar M, Wright GC, Nigam SN. 2006. Leaf chlorophyll concentration relates to transpiration efficiency in peanut. *Annals of Applied Biology* **148,** 7–15.

Siddique KHM, Regan KL, Tennant D, Thomson BD. 2001. Water use and water use efficiency of cool season grain legumes in low rainfall Mediterranean-type environments. *European Journal of Agronomy* **15,** 267–280.

Sinclair TR. 2012. Is transpiration efficiency a viable plant trait in breeding for crop improvement? *Functional Plant Biology* **39,** 359–365.

Sinclair TR, Hammer GL, van Oosterom EJ. 2005. Potential yield and water-use efficiency benefits in sorghum from limited maximum transpiration rate. *Functional Plant Biology* **32,** 945–952.

Sinclair TR, Messina CD, Beatty A, Samples M. 2010. Assessment across the United States of the benefits of altered soybean drought traits. *Agronomy Journal* **102,** 475–482.

Sinclair TR, Tanner CB, Bennett JM. 1984. Water-use efficiency in crop production. *Bio Science* **34,** 36–40.

Sinclair TR, Vadez V. 2012. The future of grain legumes in cropping systems. *Crop and Pasture Science* **63,** 501–512.

Sinclair TR, Zwieniecki MA, Holbrook NM. 2008. Low leaf hydraulic conductance associated with drought tolerance in soybean. *Physiologia Plantarum* **132,** 446–451.

Tambussi EA, Bort J, Araus JL. 2007. Water use efficiency in C(3) cereals under Mediterranean conditions: a review of physiological aspects. *Annals of Applied Biology* **150,** 307–321.

Tanner CB, Sinclair TR. 1983. Efficient water use in crop production: research or re-search? In: HM Taylor ***et al.*** (eds), *Limitations to Efficient Water Use in Crop Production* . Madison. WI: ASA, CSSA and SSSA, pp 1–27.

Thompson AJ, Andrews J, Mulholland BJ *et al.* 2007. Overproduction of abscisic acid in tomato increases transpiration efficiency and root hydraulic conductivity and influences leaf expansion. *Plant Physiology* **143,** 1905–1917.

Thumma BR, Naidu BP, Cameron DF, Bahnisch LM. 1998. Transpiration efficiency and its relationship with carbon isotope discrimination under well-watered and water-stressed conditions in Stylosanthes scabra. *Australian Journal of Agricultural Research* **49,** 1039–1045.

Turner NC, Palta JA, Shrestha R, Ludwig C, Siddique KHM, Turner DW. 2007. Carbon isotope discrimination is not correlated with

transpiration efficiency in three cool-season grain legumes (Pulses). *Journal of Integrative Plant Biology* **49,** 1478–1483.

Turner NC, Schulze ED, Gollan T. 1984. The response of stomata and leaf gas exchange to vapour pressure deficits and soil water content. *Oecologia* **63,** 338–342.

Tyerman SD, Bohnert HJ, Maurel C, Steudle E, Smith JAC. 1999. Plant aquaporins: their molecular biology, biophysics and significance for plant water relations. *Journal of Experimental Botany* **50,** 1055–1071.

Udayakumar M, Sheshshayee MS, Nataraj KN, Madhava HB, Devendra R, Hussain ISA, Prasad TG. 1998. Why has breeding for water use efficiency not been successful? An analysis and alternate approach to exploit this trait for crop improvement. *Current Science* **74,** 994–1000.

Upadhyaya HD. 2005. Variability for drought resistance related traits in the mini core collection of peanut. *Crop Science* **45,** 1432–1440.

Vadez V, Rao S, Kholova J, Krishnamurthy L, Kashiwagi J, Ratnakumar P, Sharma KK, Bhatnagar-Mathur P, Basu PS. 2008. Roots research for legume tolerance to drought: quo vadis? *Journal of Food Legumes* **21** 77–85.

Vadez V, Deshpande SP, Kholova J, Hammer GL, Borrell AK, Talwar HS, Hash CT. 2011a. Stay-green quantitative trait loci's effects on water extraction, transpiration efficiency and seed yield depend on recipient parent background. *Functional Plant Biology* **38,** 553–566.

Vadez V, Krishnamurthy L, Hash CT, Upadhyaya HD, Borrell AK. 2011b. Yield, transpiration efficiency, and water-use variations and their interrelationships in the sorghum reference collection. *Crop & Pasture Science* **62,** 645–655.

Vadez V, Kholova J, Yadav RS, Hash CT. 2013. Small temporal differences in water uptake among varieties of pearl millet (Pennisetum glaucum (L.) R. Br.) are critical for grain yield under terminal drought. *Plant Soil* **371,** 447–462

Vadez V, Kholova J, Zaman-Allah M, Belko N. 2014. Water: the most important "molecular" component of water stress tolerance research. *Functional Plant Biology* **40,** 1310–1322.

van Oosterom EJ, Borrell AK, Deifel KS, Hammer GL. 2011. Does increased leaf appearance rate enhance adaptation to postanthesis drought stress in sorghum? *Crop Science* **51,** 2728–2740.

Varshney RK, Bertioli DJ, Moretzsohn MC *et al.* 2009. The first SSR-based genetic linkage map for cultivated groundnut (Arachis hypogaea L.). *Theoretical and Applied Genetics* **118,** 729–739.

Wasson AP, Richards RA, Chatrath R, Misra SC, Prasad SVS, Rebetzke GJ, Kirkegaard JA, Christopher J, Watt M. 2012. Traits and selection strategies to improve root systems and water uptake in water-limited wheat crops. *Journal of Experimental Botany* **63,** 3485–3498.

Wong SC, Cowan IR, Farquhar GD. 1979. Stomatal conductance correlates with photosynthetic capacity. *Nature* **282,** 424–426.

Wright GC, Rao RCN, Farquhar GD. 1994. Water use efficiency and carbon isotope discrimination in peanut under water deficit conditions. *Crop Science* **34,** 92–97.

Yang ZJ, Sinclair TR, Zhu M, Messina CD, Cooper M, Hammer GL. 2012. Temperature effect on transpiration response of maize plants to vapour pressure deficit. *Environmental and Experimental Botany* **78,** 157–162.

Zacharisen MH, Brick MA, Fisher AG, Ogg JB, Ehleringer JR. 1999. Relationships between productivity and carbon isotope discrimination among dry bean lines and F-2 progeny. *Euphytica* **105,** 239–250.

Zaman-Allah M, Jenkinson DM, Vadez V. 2011a. Chickpea genotypes contrasting for seed yield under terminal drought stress in the field differ for traits related to the control of water use. *Functional Plant Biology* **38,** 270–281.

Zaman-Allah M, Jenkinson DM, Vadez V. 2011b. A conservative pattern of water use, rather than deep or profuse rooting, is critical for the terminal drought tolerance of chickpea. *Journal of Experimental Botany* **62,** 4239–4252.

Chapter 2

Interactive Effects of Elevated [CO_2] and Water Stress on Physiological Traits and Gene Expression during Vegetative Growth in Four Durum Wheat Genotypes

Efectos interactivos de la elevada [CO_2] y el estrés hídrico sobre parámetros fisiológicos y expresión de genes durante el crecimiento vegetativo de cuatro genotipos de trigo duro.

Susan Medina[1, 2], Rubén Vicente[1*], Amaya Amador[3] and José Luis Araus[1]

[1]Integrative Crop Ecophysiology Group, Plant Physiology Section, Faculty of Biology, University of Barcelona, Barcelona, Spain

[2]Crop Physiology Laboratory, International Crops Research Institute for Semi-Arid Tropics, Patancheru, India

[3]Unitat de Genòmica, Centres Científics i Tecnològics, Universitat de Barcelona, Barcelona, Spain

Published in / Publicado en:

Journal Frontiers in Plant Science (2016), Vol. 7, No. 1738

ABSTRACT

The interaction of elevated [CO_2] and water stress will have an effect on the adaptation of durum wheat to future climate scenarios. For the Mediterranean basin these scenarios include the rising occurrence of water stress during the first part of the crop cycle. In this study, we evaluated the interactive effects of elevated [CO_2] and moderate to severe water stress during the first part of the growth cycle on physiological traits and gene expression in four modern durum wheat genotypes. Physiological data showed that elevated [CO_2] promoted plant growth but reduced N content. This was related to a down-regulation of Rubisco and N assimilation genes and up-regulation of genes that take part in C-N remobilization, which might suggest a higher N efficiency. Water restriction limited the stimulation of plant biomass under elevated [CO_2], especially at severe water stress, while stomatal conductance and carbon isotope signature revealed a water saving strategy. Transcript profiles under water stress suggested an inhibition of primary C fixation and N assimilation. Nevertheless, the interactive effects of elevated [CO_2] and water stress depended on the genotype and the severity of the water stress, especially for the expression of drought stress-responsive genes such as dehydrins, catalase, and super oxide dismutase. The network analysis of physiological traits and transcript levels showed coordinated shifts between both categories of parameters and between C and N metabolism at the transcript level, indicating potential genes and traits that could be used as markers for early vigor in durum wheat under future climate change scenarios. Overall the results showed that greater plant growth was linked to an increase in N content and expression of N metabolism-related genes and down-regulation of genes related to the antioxidant system. The combination of elevated [CO_2] and severe water stress was highly dependent on the genotypic variability, suggesting specific genotypic adaptation strategies to environmental conditions.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 22 November 2016
doi: 10.3389/fpls.2016.01738

Interactive Effects of Elevated [CO_2] and Water Stress on Physiological Traits and Gene Expression during Vegetative Growth in Four Durum Wheat Genotypes

Susan Medina[1,2], *Rubén Vicente*[1]*, *Amaya Amador*[3] and *José Luis Araus*[1]

[1] Integrative Crop Ecophysiology Group, Plant Physiology Section, Faculty of Biology, University of Barcelona, Barcelona, Spain, [2] Crop Physiology Laboratory, International Crops Research Institute for Semi-Arid Tropics, Patancheru, India, [3] Unitat de Genòmica, Centres Científics i Tecnològics, Universitat de Barcelona, Barcelona, Spain

OPEN ACCESS

Edited by:
Paul Christiaan Struik,
Wageningen University and Research Centre, Netherlands

Reviewed by:
Iker Aranjuelo,
Instituto de Agrobiotecnología (CSIC-UPNA), Spain
Fulai Liu,
University of Copenhagen, Denmark
Salvatore Ceccarelli,
Rete Semi Rurali, Italy

***Correspondence:**
Rubén Vicente
vicenteperez.ruben@gmail.com

Specialty section:
This article was submitted to Crop Science and Horticulture, a section of the journal Frontiers in Plant Science

Received: 06 June 2016
Accepted: 04 November 2016
Published: 22 November 2016

Citation:
Medina S, Vicente R, Amador A and Araus JL (2016) Interactive Effects of Elevated [CO_2] and Water Stress on Physiological Traits and Gene Expression during Vegetative Growth in Four Durum Wheat Genotypes. Front. Plant Sci. 7:1738. doi: 10.3389/fpls.2016.01738

The interaction of elevated [CO_2] and water stress will have an effect on the adaptation of durum wheat to future climate scenarios. For the Mediterranean basin these scenarios include the rising occurrence of water stress during the first part of the crop cycle. In this study, we evaluated the interactive effects of elevated [CO_2] and moderate to severe water stress during the first part of the growth cycle on physiological traits and gene expression in four modern durum wheat genotypes. Physiological data showed that elevated [CO_2] promoted plant growth but reduced N content. This was related to a down-regulation of Rubisco and N assimilation genes and up-regulation of genes that take part in C-N remobilization, which might suggest a higher N efficiency. Water restriction limited the stimulation of plant biomass under elevated [CO_2], especially at severe water stress, while stomatal conductance and carbon isotope signature revealed a water saving strategy. Transcript profiles under water stress suggested an inhibition of primary C fixation and N assimilation. Nevertheless, the interactive effects of elevated [CO_2] and water stress depended on the genotype and the severity of the water stress, especially for the expression of drought stress-responsive genes such as dehydrins, catalase, and superoxide dismutase. The network analysis of physiological traits and transcript levels showed coordinated shifts between both categories of parameters and between C and N metabolism at the transcript level, indicating potential genes and traits that could be used as markers for early vigor in durum wheat under future climate change scenarios. Overall the results showed that greater plant growth was linked to an increase in N content and expression of N metabolism-related genes and down-regulation of genes related to the antioxidant system. The combination of elevated [CO_2] and severe water stress was highly dependent on the genotypic variability, suggesting specific genotypic adaptation strategies to environmental conditions.

Keywords: climate change, durum wheat, elevated [CO_2], genotypic variability, stable isotopes, transcript levels, vegetative growth, water stress

INTRODUCTION

Food security is facing new challenges nowadays due to the increase in the world population and the impacts of climate change on agriculture and food supply. Wheat is a very important crop for the human diet, ranking in fourth position in terms of the world's most important crops by production quantity after sugarcane, maize, and rice (FAO, 2013). Although bread wheat dominates global wheat production, durum wheat is an economically and culturally important staple crop in the Mediterranean region, used for the production of pasta, bread, burghul, couscous, and freekeh (Habash et al., 2009). In the second half of the twentieth century, local durum wheat landraces were replaced by improved semi-dwarf cultivars, which showed higher yield and harvest index (Soriano et al., 2016). In the early 1970s, introduction of germplasm from CIMMYT (International Maize and Wheat Improvement Centre) increased grain yield (Sanchez-Garcia et al., 2013). Improvement in wheat yield per unit area constitutes one of the largest challenges to be addressed by breeding programs, covering numerous research areas (McKersie, 2015). Projections of wheat production assume that the growth rate will be lower than the historical growth rates reported in the second half of the twentieth century (Bort et al., 2014; Nakhforoosh et al., 2015), with insignificantly higher yields in modern wheat genotypes released in recent years (Sanchez-Garcia et al., 2013). It is unlikely that any improvements will support the increase in world population or mitigate against future extreme weather events (Araus et al., 2002; Alexandratos and Bruinsma, 2012; Trnka et al., 2014).

Observations of the climate system confirm that Earth's mean surface temperature is increasing rapidly as a consequence of the anthropogenic emissions of CO_2 and other greenhouse gases (IPCC, 2013). The atmospheric concentration of CO_2 ($[CO_2]$) has increased by more than 40% since the beginning of the industrial revolution and is expected to double by the end of this century (IPCC, 2013). As atmospheric $[CO_2]$ is currently a limiting factor for C_3 photosynthesis, the primary effect of a short-term exposure to elevated $[CO_2]$ includes an initial stimulation of photosynthesis due to both enrichment of substrate for ribulose bisphosphate carboxylase oxygenase (Rubisco) carboxylation and inhibition of competitive Rubisco oxygenation which may eventually contribute to a higher biomass (Stitt and Krapp, 1999; Long et al., 2006). High $[CO_2]$ also induces a stomatal closure leading to a better leaf water status. However, growth over the long-term under elevated $[CO_2]$ leads to a down-regulation of photosynthetic capacity, which has been related to a decline in Rubisco protein content and activity, together with a higher carbohydrate accumulation and a decline in N concentration and protein content in wheat (Aranjuelo et al., 2011, 2013; Vicente et al., 2015a,b). This phenomenon suggests that regulatory mechanisms may occur in the plant, e.g., end-product inhibition, carbon sink limitation, biomass dilution effects, or a decline in nutrient uptake and/or assimilation (Stitt and Krapp, 1999; Vicente et al., 2015a). Moreover, elevated $[CO_2]$ leads to an altered expression pattern of genes involved in the photosynthetic apparatus, the distribution of C, respiration, and N metabolism in durum wheat (Vicente et al., 2015b).

Increasing greenhouse gas emissions may cause further warming together with rainfall reduction in the next decades, which will increase the frequency and intensity of drought in the Mediterranean basin (Habash et al., 2009; IPCC, 2013; McKersie, 2015). For the Iberian Peninsula it is predicted that drought stress can occur at any growth stage of wheat (Russo et al., 2015), with the grain-filling phase being the most studied. However, the number of studies focusing on drought stress during early growth is limited. Although rainfall has been traditionally most abundant and evapotranspiration the lowest during winter, the occurrence of drought in winter months during the early stages of the crop cycle has been reported in recent times (Russo et al., 2015). This can further constrain wheat growth and thus final grain yield, mostly through a decrease in the ear density and number of kernels per unit crop area (Araus et al., 2008; Rebolledo et al., 2013). In addition, a constitutive (i.e., in absence of water stress) rapid development of wheat plants (early vigor) could be a positive trait and relevant for further avoiding drought stress-related consequences at both early and late growth stages. Early vigor could benefit plant growth and yield by increasing resource acquisition, shading the soil, preventing evaporation from it, and suppressing weeds (Maydup et al., 2012; Bort et al., 2014; Pang et al., 2014). As a consequence, differences in early growth (tillering and further stem elongation) will affect the number of fertile stems (and thus the ear density) and the size of the ears (and thus the potential number of grains per ear), which are the main contributors determining grain yield (Guo et al., 2016).

Plant responses to water stress define a complex and sophisticated regulatory network comprising physiological, biochemical, and molecular mechanisms. In wheat, some of these responses include inhibition of plant growth and photosynthetic capacity, together with a wide range of physiological responses, including changes in stomatal closure and decreases in transpiration, Rubisco efficiency, and chlorophyll content as well as an increase in oxidative stress among other responses (Budak et al., 2013; Nezhadahmadi et al., 2013). Such responses are modulated by stress severity. Cessation of watering showed a progressive reduction in leaf relative water content, water potential and photosynthesis in durum wheat (Habash et al., 2014). Liu et al. (2016) reported a progressive inhibition of photosynthetic activity as water stress is more severe in field-grown bread wheat, probably due to non-stomatal limitations, which led to lower grain yields even at moderate water stress. Furthermore, water stress in wheat leads to complex changes in the expression of some genes, including those involved in photosynthesis, respiration, N metabolism, lipid metabolism, transcription factors, signal transducers, and synthesis of protective proteins (Habash et al., 2009, 2014; Budak et al., 2013; Yousfi et al., 2016). These changes in gene expression occurred mainly in the early phases of the stress (Habash et al., 2014).

Plant responses to elevated $[CO_2]$ or water stress are influenced by the duration and level of the environmental factor, the growth stage, and the genetic variability. Studies carried out with different durum wheat genotypes demonstrated that the responsiveness to elevated $[CO_2]$ (Aranjuelo et al., 2013), water stress (De Leonardis et al., 2007; Aprile et al., 2013; Habash et al., 2014), and the combination of both (Erice et al., 2014) is

genotype specific. Moreover, the growth stage greatly influences the response of durum wheat to elevated [CO_2] (Aranjuelo et al., 2011; Vicente et al., 2015a) and drought (Liu et al., 2016). In addition, the interactive effects of environmental conditions and genotypic variability cannot be anticipated from the individual effects of these treatments (Ceccarelli et al., 1991). Some studies have shown positive effects of elevated [CO_2] on water stress tolerance of different bread wheat varieties (Harnos et al., 2002; Wall et al., 2006; Robredo et al., 2011; Bencze et al., 2014). A positive synergistic effect of elevated [CO_2] and water stress has been reported to decrease g_s, and thus leads to an improvement in water use efficiency at the stomatal and whole plant level (Bencze et al., 2014; Pazzagli et al., 2016). The decrease in photosynthesis under water stress is often mitigated by elevated [CO_2] (Bencze et al., 2014), resulting in increased levels of carbohydrates for the development of new tissues or filling grain (Wall et al., 2006). However, such positive effects of elevated [CO_2] in improving stress tolerance are not always achieved (Hudak et al., 1999; Pleijel et al., 2000). Bencze et al. (2014) reported that drought at elevated [CO_2] led to a stimulation of the antioxidant enzyme system in bread wheat, which suggests a high level of oxidative stress. Erice et al. (2014) showed that the stimulation of plant growth by elevated [CO_2] was only found in durum wheat genotypes with high harvest indices and optimal water supply. Therefore, additional efforts are still necessary to deepen our understanding of the interactive effect of [CO_2] and water regime in durum wheat.

The aim of this work was to determine the physiological and molecular mechanisms involved in the adaptive response of four semi-dwarf (i.e., post-Green Revolution) durum wheat cultivars to different [CO_2] and water regimes. Durum wheat genotypes were grown under controlled conditions at ambient and elevated [CO_2] and two different water regimes (fully irrigated and moderate/severe water stress). We assessed plant growth, physiological traits, stable C and N isotopic signatures, and transcript levels for stress-responsive genes that could be good indicators of durum wheat's adaptation to future climate conditions at vegetative growth stages. The genes selected corresponded to key enzymes in the metabolism of C (the Rubisco large and small subunits, RBCL and RBCS, respectively, and phosphoenolpyruvate carboxylase, PEPC) and N (the cytosolic and plastidial glutamine synthetases, GS1 and GS2, respectively), as well as proteins involved in stress responses (dehydrins 11, DHN11, and 16, DHN16, catalase, CAT, and superoxide dismutase, SOD). Rubisco is the key enzyme for photosynthetic CO_2 assimilation, and its activity is highly responsive to atmospheric [CO_2] (Vicente et al., 2011; Carmo-Silva et al., 2015). PEPC is a cytosolic enzyme that catalyzes the β-carboxylation of phosphoenolpyruvate to produce oxaloacetate, which is involved in anaplerotic functions. GS1 and GS2 play a central role in N metabolism: the former is thought to be involved in the primary assimilation of ammonium from nitrate reduction and photorespiration, while the latter is mainly involved in the transport of N through the plant and N recycling from catabolic processes. The function of the dehydrin family is not completely understood, but these proteins are involved in conferring stress tolerance (Kosová et al., 2014). Catalases and superoxide dismutases are primary antioxidant enzymes involved in the elimination of reactive oxygen species (ROS) such as the cytotoxic H_2O_2 produced by photorespiration (Luna et al., 2005) and the superoxide generated during photosynthetic electron transport (Xu et al., 2010; Huseynova et al., 2014). Thus, our study combines the effects of genotypic variability and future environmental conditions, integrating plant performance with gene expression, and aims to identify traits associated with better performance during vegetative growth.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The experiment was conducted with four semi-dwarf durum wheat [*Triticum turgidum* L. ssp. *durum* (Desf.)] genotypes: Mexa (year of commercial release: 1977), Regallo (1988), Burgos (1997) and Ramirez (2006). These cultivars represent high-yield genotypes released in the last forty years that are (or were) widely cultivated in the Mediterranean regions of Spain. The study of these genotypes could provide information about the adaptation of modern cultivars to climate change and whether there are differences between them associated with the year they were released. The experiment was conducted from May to July 2015 in two controlled environment chambers (Conviron E15; Controlled Environments, Winnipeg, MB, Canada) in the Experimental Facilities of the Faculty of Biology at the University of Barcelona. A total of 96 durum wheat plants (24 for each genotype) were sown in 2 L pots containing a mixture of standard substrate:perlite (1:1, v/v) and were grown with a long light period of 16 h, a photosynthetic photon flux density (PPFD) of 350 $\mu mol\ m^{-2}\ s^{-1}$, a day/night temperature of 23/17°C and a relative humidity of 60%. During the entire experiment, half of the pots were cultivated under atmospheric [CO_2] (400 $\mu mol\ mol^{-1}$) in one chamber, while the other half grew under elevated [CO_2] (790 $\mu mol\ mol^{-1}$) in the other chamber with injection of CO_2 from an external bottle (Carburos Metálicos S.A., Barcelona, Spain). The temperature, relative humidity and [CO_2] within each chamber were continuously monitored by Conviron series controllers (CMP3243 Controlled Environments Ltd., Winnipeg, MB, Canada). The technical staff of the Experimental Facilities of the Faculty of Biology tested the growth conditions of each chamber periodically with external sensors: an HMP75 humidity and temperature probe and a GMP222 CO_2 probe for use with an MI70 series hand-held indicator (Vaisala, Vantaa, Finland). Similarly, the PPFD was periodically verified with an LI-188B quantum/radiometer/photometer (LI-COR Inc., Lincoln, NB, USA).

The plants were uniformly irrigated every 2 days with 50% Hoagland's nutrient solution over a 25 day period. After that (Zadoks 21), the water stress was imposed; one half of the plants of each genotype and [CO_2] were maintained under well-watered conditions (100% pot capacity, PC) until the end of the experiment, while the other half were subjected to water stress conditions. The maximum soil volumetric water content of each pot was evaluated at the beginning of the experiment as the difference between pot weight after watering with the excess water drained and the pot dry weight. Thus, pots were

watered by direct measurements of the pot weight and the water supply was adjusted to the pot water conditions established for each water regime. In the water-stressed plants the watering was progressively restricted by 10% PC every 2 days. First, after 8 days the water-stressed plants received a 60% PC (moderate water stress) and this irrigation regime was strictly maintained for 10 days (see **Figure 1** for a schematic representation of the experimental design). At the end of this period (Zadoks 26), equal numbers (48) of well-watered and water-stressed plants were sampled. The youngest fully expanded leaf was collected, immediately frozen in liquid nitrogen and stored at −80°C for gene expression, C and N content and stable isotope analyses. After that, the whole plant was harvested and dried in an oven at 60°C for 72 h for biomass analysis. Second, in the remaining half of the plants (48), the progressive water limitation continued for 8 more days until water-stressed plants received a 30% PC (severe water stress). As in the moderate water stress, the irrigation conditions in well-watered and water-stressed plants were maintained for 10 days. Later, these 51-day-old plants (Zadoks 28–32) were collected following the procedure described above. The moderate and severe water stresses were defined in this experiment based on similar reductions in irrigation and stomatal conductance used in other studies (Galmes et al., 2007; Liu et al., 2016). The pots were rotated three times a week to avoid edge effects in the growth chambers over the course of the experiment. We used a rotatory randomized complete block design with three replicates (one plant per pot) per factor combination ([CO_2], water level and genotype) at each sampling.

Physiological Traits

Prior to harvest a hand-held portable spectroradiometer (GreenSeeker, NTech Industries, Ukiah, CA, USA) was used to estimate the normalized difference vegetation index (NDVI) of each plant (only at the second sampling date). Relative chlorophyll content was measured with a Minolta SPAD-502 chlorophyll meter (Spectrum Technologies, Plainfield, IL, USA). Stomatal conductance (g_s) was measured using a Decagon SC-1 Leaf Porometer (Decagon Device, Inc., Pullman, WA, USA). Both chlorophyll content and stomatal conductance of the adaxial surface were recorded in the central segment of the same youngest fully expanded leaf between 3 and 5 h after the start of the photoperiod. In addition, plants were collected to determine the leaf, shoot, root, and plant dry weights as indicated above, while the roots were washed in tap water until all substrate was removed. The number of tillers and the root to shoot dry weight ratio (root/shoot) were then determined.

C and N Content and Stable Isotope Signatures

A fraction of the youngest fully expanded leaf was finely powdered and then 1 mg of this leaf material was used for the measurements of total C and N content (as a percentage of

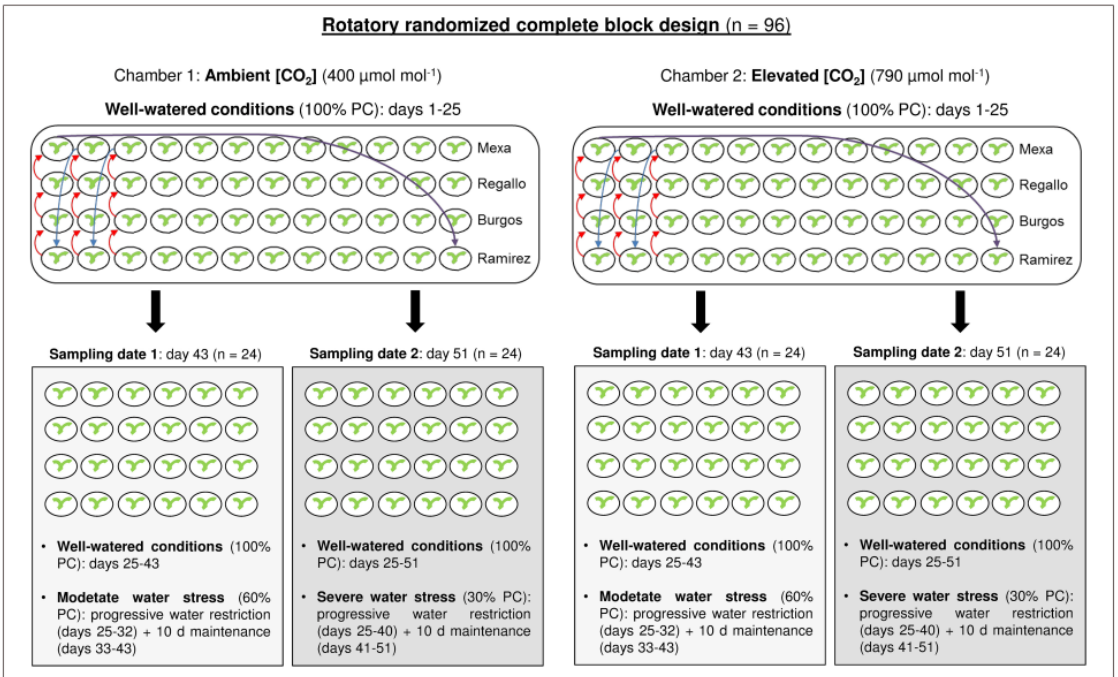


FIGURE 1 | Schematic representation of the experimental design.

leaf dry weight) and the stable C ($^{13}C/^{12}C$) and N ($^{15}N/^{14}N$) isotope ratios. Measurements were carried out using an elemental analyzer (Flash 1112 EA; ThermoFinnigan, Bremen, Germany) coupled with an isotope ratio mass spectrometer (Delta C IRMS; ThermoFinnigan), operating in continuous flow mode, at the Scientific Facilities of the University of Barcelona. As has been described previously (Bort et al., 2014; Yousfi et al., 2016), the $^{13}C/^{12}C$ ratio was expressed in δ notation: $\delta^{13}C$ (‰) = $[(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard} - 1] \times 1000$. The standard refers to international secondary standards of known $^{13}C/^{12}C$ ratios (IAEA CH7 polyethylene foil, IAEA CH6 sucrose, and USGS 40 L-glutamic acid) calibrated against Vienna Pee Dee Belemnite calcium carbonate. The same δ notation was used for the $^{15}N/^{14}N$ ratio ($\delta^{15}N$) using N_2 in air as standard.

Quantitative Reverse Transcriptase PCR Amplification

Frozen leaf samples were ground with liquid nitrogen and subsequently RNA was isolated from 100 mg of this material with Ribozol RNA Extraction Reagents (Amresco, Solon, OH, USA) according to the manufacturer's instructions. RNA quantity and quality was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was checked by 1.5% (w/v) agarose gel electrophoresis. Total RNA (1 μg) was treated with PerfeCTa DNase I RNase-free (Quanta Biosciences, Gaithersburg, MD, USA) to eliminate residual genomic DNA. cDNA was synthesized using a qScript cDNA Synthesis Kit (Quanta Biosciences) following the manufacturer's instructions. The qRT-PCR assays were performed in optical 384-well-plates with the LightCycler 480 System (Roche Applied Science, Penzberg, Germany) in the Centres Científics i Tecnològics de la Universitat de Barcelona (CCiTUB), in a reaction volume of 10 μL: 5 μL of PerfeCTa SYBR Green FastMix (Quanta Biosciences), 200 nM of each gene-specific primer and 1 μL of diluted cDNA (1:10). The thermal profile was as follows: initial denaturation for 30 s at 95°C, PCR cycling (45 cycles) for 5 s at 95°C, 15 s at 60°C, and 10 s at 72°C, and a final step of 95°C for 5 s and 60°C for 60 s to obtain the dissociation curve. Two technical replicates were analyzed per biological replicate. Specific primers for genes encoding the Rubisco large subunit (NC_021762), phosphoenolpyruvate carboxylase (Y15897), plastidial glutamine synthetase (DQ124212), dehydrin 11 (AJ890140), and superoxide dismutase (KP696754) were designed in Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the following criteria: Tm = 60 ± 1°C, primer length of 18–25 bases, GC content of 30–70% and product size of 60–150 bases. The specificity of PCR amplification was confirmed by the presence of unique amplicons of the expected length on 3.5% (w/v) agarose gels. The genes encoding the ADP-ribosylation factor and the RNase L inhibitor-like protein, previously identified as potential reference genes (Vicente et al., 2015b), were used to normalize qRT-PCR data after the evaluation of their expression stability in this study. All primers used for gene expression analysis and their symbols are listed in Supplementary Table S1. The values of the cycle threshold (C_t) were calculated using the LightCycler 1.5 software (Roche Applied Science). The quantification of the relative gene expression was analyzed using the comparative C_t method $2^{-\Delta\Delta Ct}$ (Schmittgen and Livak, 2008), and the data were presented as the $\log_2$ fold change.

Data Analysis

The effects of [CO_2] (ambient and elevated), water regime (well-watered and water stressed), genotype (Mexa, Regallo, Burgos, and Ramirez), and their interaction on plant growth, chlorophyll content, g_s, and C and N contents and isotope composition were determined through a three-factor (2 CO_2 × 2 water regimes × 4 genotypes) analysis of variance (ANOVA) for each sampling date (moderate and severe waters stress; see Supplementary Table S2) with GenStat 6.2 (VSN International Ltd, Hemel Hempstead, UK). Further, and given the implicit complexity of the design, each genotype was analyzed through a two-factor ANOVA (2 CO_2 × 2 water regimes) for both sampling dates. All factors were treated as fixed independent variables. When the F-ratio was significant ($P < 0.05$), the least significant difference (LSD) test was used to assess differences between treatment means. Clustered heat maps of relative gene expression were built in the R statistics environment (R Development Core Team, 2008) to study the effects of elevated [CO_2] and water stress on transcript levels. A correlation matrix was generated in R for evaluating the relationships between all parameters analyzed. Visualization of significant correlations was performed using Cytoscape software (Shannon et al., 2003).

RESULTS

Effect of [CO_2], Water Regime, and Genotype on Plant Growth

Total biomass of the plant and its different fractions (leaves, shoot and root), the root/shoot ratio, and the number of tillers were analyzed through two-factor ANOVA ([CO_2] × water regime) for each genotype (**Tables 1**, **2**). Moderate and severe water stress were established with reductions of 40 and 70% in the water supplied to the pots and average decreases of 34 and 57% in g_s, respectively, compared to well-watered plants (data not shown). Growth under elevated [CO_2] led to significant increases in biomass compared to ambient [CO_2] (**Tables 1**, **2**). At the first sampling date, elevated [CO_2] increased root biomass in Mexa, Regallo and Ramirez (and also in Burgos, $P = 0.074$), but only increased plant biomass in Regallo. The root/shoot ratio also increased in Regallo and Burgos under elevated [CO_2]. At the second sampling date, elevated [CO_2] increased plant biomass due to higher shoot and root biomass compared to ambient [CO_2], with larger increases in plant biomass in Mexa and Regallo under well-watered conditions in comparison to water stressed conditions. As a consequence of the increases in both shoot and root dry weights by elevated [CO_2], the root/shoot ratio was not altered, except in Regallo. Moreover, in this genotype an increase in the tillers per plant was also observed under elevated [CO_2] but only in well-watered conditions.

Moderate water stress did not lead to statistical differences in biomass, the root/shoot ratio, or the number of tillers between

TABLE 1 | Total leaf (LDW), shoot (SDW), root (RDW) and plant (PDW) dry weight, root/shoot ratio, and number of tillers per plant in four durum wheat genotypes grown under ambient or elevated [CO_2] and well-watered or moderate water stress conditions (100 vs. 60% pot capacity).

	Genotype	Ambient [CO_2]		Elevated [CO_2]		P_C	P_W	$P_{C\times W}$
		Well-watered	Water stressed	Well-watered	Water stressed			
LDW (g)	Mexa	1.84	1.29	1.45	1.23	0.527	0.304	0.656
	Regallo	1.26^{a}	1.37^{ab}	1.59^{b}	1.18^{a}	0.402	0.105	**0.013**
	Burgos	1.32	2.36	1.83	1.28	0.562	0.618	0.128
	Ramirez	1.98	1.41	1.70	1.15	0.417	0.109	0.964
SDW (g)	Mexa	3.12	1.85	2.17	2.27	0.732	0.463	0.387
	Regallo	1.75	1.97	2.14	1.90	0.441	0.962	0.258
	Burgos	4.18	3.78	2.47	1.91	0.147	0.679	0.946
	Ramirez	3.27	2.36	2.39	1.64	0.339	0.320	0.924
RDW (g)	Mexa	0.65	0.47	1.24	0.82	**0.023**	0.109	0.512
	Regallo	0.65	0.65	0.96	1.26	**<0.001**	0.080	0.074
	Burgos	0.85	0.49	0.90	1.00	0.074	0.374	0.125
	Ramirez	0.72	0.62	1.10	1.00	**0.022**	0.459	0.981
PDW (g)	Mexa	3.77	2.32	3.41	3.10	0.803	0.298	0.490
	Regallo	2.41	2.62	3.10	3.16	**0.002**	0.351	0.576
	Burgos	5.03	4.27	3.36	2.91	0.211	0.602	0.895
	Ramirez	3.99	2.98	3.50	2.64	0.577	0.232	0.921
Root/shoot	Mexa	0.30	0.26	0.56	0.36	0.055	0.184	0.353
	Regallo	0.38	0.37	0.45	0.69	**0.045**	0.214	0.180
	Burgos	0.28	0.19	0.37	0.52	**0.025**	0.711	0.146
	Ramirez	0.32	0.34	0.47	0.61	0.095	0.484	0.588
Tiller/plant	Mexa	9.3	7.0	8.0	6.7	0.543	0.200	0.713
	Regallo	7.0	8.3	8.3	8.3	0.567	0.567	0.567
	Burgos	8.7	11.0	9.0	9.0	0.620	0.491	0.491
	Ramirez	11.7	8.7	7.0	6.3	**0.045**	0.248	0.451

Significant effects for elevated [CO_2] (C), water stress (W) and their interaction (C × W) were determined by two-factor ANOVA (P). Values with the same letter are not significantly different for the interaction [CO_2] × water level. Significant P values are marked in bold ($P < 0.05$).

well-watered and water-stressed plants (**Table 1**). However, severe water stress led to significant changes in these parameters, while NDVI was also affected (**Table 2**). Plant biomass generally decreased under severe water stress compared to well-watered conditions and was associated with decreases in leaf, shoot, and root dry weights. Water restriction decreased the number of tillers per plant in Burgos under severe water stress, while this reduction was not significant in the other genotypes. Additionally, the NDVI values were lower in water-stressed plants compared to well-watered plants, irrespective of the [CO_2] and the genotype (**Table 2**). In general, at severe water stress the interaction [CO_2] × water regime × genotype showed that the root/shoot ratio strongly increased in Regallo, especially under ambient [CO_2] and well-watered conditions (Supplementary Table S2). The Burgos and Mexa cultivars had higher shoot dry weight than Ramirez and Regallo, while root dry weight was higher in Regallo (Supplementary Table S2). Furthermore, significant [CO_2] × genotype interaction showed that Burgos and Regallo under elevated [CO_2] increased tiller production, whereas Ramirez and Regallo plants under ambient [CO_2] had lower tillering (Supplementary Table S2).

Effect of [CO_2], Water Regime, and Genotype on Chlorophyll Content, g_s, C and N Content and C and N Isotope Composition

The interactive effects of [CO_2] and water regime on chlorophyll content, g_s, and C and N contents and isotope composition were analyzed in the youngest fully expanded leaf through two-factor ANOVA for each genotype during vegetative growth under moderate (**Table 3**) and severe water stress (**Table 4**). At moderate water stress, elevated [CO_2] compared to ambient [CO_2] decreased N content in Mexa and Regallo, and $\delta^{13}C$ regardless of the genotype, while it increased chlorophyll content in Mexa, g_s, and C content in Regallo, and $\delta^{15}N$ in all genotypes except in Regallo. Water stress reduced g_s and increased

TABLE 2 | Total leaf (LDW), shoot (SDW), root (RDW) and plant (PDW) dry weight, root/shoot ratio, number of tillers per plant, and normalized difference vegetation index (NDVI) in four durum wheat genotypes grown under ambient or elevated [CO_2] and well-watered or severe water stress conditions (100 vs. 30% pot capacity).

	Genotype	Ambient [CO_2]		Elevated [CO_2]		P_C	P_W	$P_{C\times W}$
		Well-watered	Water stressed	Well-watered	Water stressed			
LDW (g)	Mexa	2.40^a	2.69^a	4.73^b	1.97^a	0.127	**0.031**	**0.012**
	Regallo	1.09^a	1.75^b	4.56^c	2.23^b	**<0.001**	**0.001**	**<0.001**
	Burgos	2.77	1.70	4.67	1.80	0.082	**0.004**	0.112
	Ramirez	2.27	1.86	3.28	1.98	0.178	0.057	0.282
SDW (g)	Mexa	4.26^a	4.96^a	8.35^b	4.97^a	**0.039**	0.147	**0.040**
	Regallo	1.49^a	3.71^b	6.24^c	3.20^b	**0.001**	0.361	**<0.001**
	Burgos	4.78	4.21	8.43	5.43	0.058	0.143	0.301
	Ramirez	3.59	3.53	6.98	3.73	**0.035**	**0.048**	0.054
RDW (g)	Mexa	1.31^a	1.14^a	2.65^b	1.47^a	**0.002**	**0.008**	**0.030**
	Regallo	2.42	1.92	3.54	2.45	**0.002**	**0.002**	0.139
	Burgos	1.54	1.33	3.01	1.75	**0.003**	**0.012**	0.053
	Ramirez	1.59	1.37	2.33	1.93	**0.013**	0.166	0.689
PDW (g)	Mexa	5.56^a	6.10^a	10.99^b	6.44^a	**0.018**	0.074	**0.031**
	Regallo	3.91^a	5.63^b	9.78^c	5.65^b	**<0.001**	**0.025**	**<0.001**
	Burgos	6.32	5.54	11.43	7.19	**0.011**	**0.039**	0.128
	Ramirez	5.18	4.89	9.32	5.66	**0.012**	**0.032**	0.058
Root/shoot	Mexa	0.33	0.23	0.32	0.30	0.400	0.123	0.267
	Regallo	1.65^b	0.58^a	0.57^a	0.76^a	**0.011**	**0.013**	**0.002**
	Burgos	0.40	0.48	0.36	0.34	0.563	0.850	0.718
	Ramirez	0.58	0.39	0.34	0.53	0.662	0.994	0.136
Tiller/plant	Mexa	9.3	9.3	11.0	7.0	0.852	0.279	0.279
	Regallo	6.3^a	8.0^a	15.7^b	10.0^a	**0.002**	0.138	**0.017**
	Burgos	12.3	7.0	13.0	8.7	0.377	**0.005**	0.699
	Ramirez	8.0	7.7	6.7	6.7	0.773	0.174	0.267
NDVI	Mexa	0.29	0.18	0.36	0.13	0.678	**<0.001**	0.104
	Regallo	0.29	0.18	0.34	0.15	0.610	**<0.001**	0.067
	Burgos	0.25	0.17	0.30	0.16	0.524	**0.006**	0.347
	Ramirez	0.29	0.18	0.25	0.16	0.287	**0.003**	0.651

Significant effects for elevated [CO_2] (C), water stress (W) and their interaction (C × W) were determined by two-factor ANOVA (P). Values with the same letter are not significantly different for the interaction [CO_2] × water level. Significant P values are marked in bold ($P < 0.05$).

$\delta^{15}N$ in Regallo, and increased $\delta^{13}C$ in Burgos and Ramirez. Three-factor ANOVA showed significant interactions for $\delta^{15}N$ (Supplementary Table S2). The [CO_2] × genotype interaction mainly showed that $\delta^{15}N$ was higher in Ramirez and Burgos at elevated [CO_2] and in Regallo at both [CO_2], whereas the lowest values were observed in Ramirez at ambient [CO_2]. The water regime × genotype interaction indicated that $\delta^{15}N$ was higher in Mexa and Regallo under water stress than in the other genotypes.

At severe water stress, elevated [CO_2] relative to ambient [CO_2] decreased the N content in Regallo and Burgos and $\delta^{13}C$ regardless of the genotype, while it increased $\delta^{15}N$ in Burgos (**Table 4**). Furthermore, g_s in Burgos and N content in Regallo decreased under severe water stress compared to well-watered conditions. In addition, under well-watered conditions g_s was higher in Burgos than in other genotypes (Supplementary Table S2). Chlorophyll content was lower in Regallo, whereas $\delta^{13}C$ was higher in Burgos, as compared to other genotypes (Supplementary Table S2).

Effect of [CO_2] and Water Regime on Gene Expression for Each Durum Wheat Genotype

Treatment effects on transcript levels were evaluated for each genotype using nine genes that encode enzymes of primary C and N metabolism and stress-responsive proteins (Supplementary Table S1). Elevated [CO_2] and water stress led to changes in

TABLE 3 | Chlorophyll content, stomatal conductance (g_s), N and C content, and N and C isotope composition ($\delta^{15}N$ and $\delta^{13}C$, respectively) in four durum wheat genotypes grown under ambient or elevated [CO_2] and well-watered or moderate water stress conditions (100 vs. 60% pot capacity).

	Genotype	Ambient [CO_2]		Elevated [CO_2]		P_C	P_W	$P_{C\times W}$
		Well-watered	Water stressed	Well-watered	Water stressed			
Chlorophyll (SPAD units)	Mexa	46.0	51.3	52.3	52.5	**0.049**	0.122	0.156
	Regallo	45.8	52.2	50.5	47.7	0.962	0.472	0.093
	Burgos	45.0	49.4	51.3	50.8	0.178	0.470	0.369
	Ramirez	47.2	41.3	49.3	48.5	0.099	0.216	0.337
g_s(mmol m^{-2} s^{-1})	Mexa	260.1	58.5	246.9	201.2	0.466	0.183	0.384
	Regallo	248.2	100.9	315.0	262.4	**0.003**	**0.006**	0.114
	Burgos	255.1	105.4	180.3	144.8	0.680	0.056	0.206
	Ramirez	248.6	140.0	166.1	253.1	0.772	0.839	0.093
N (%)	Mexa	5.04	4.79	3.85	4.34	**0.016**	0.675	0.212
	Regallo	5.14	4.72	3.81	3.84	**0.007**	0.534	0.477
	Burgos	5.05	5.16	3.63	4.78	0.174	0.323	0.410
	Ramirez	4.92	4.58	4.52	3.63	0.069	0.092	0.413
$\delta^{15}N$ (‰)	Mexa	2.43	3.18	3.81	3.78	**0.024**	0.344	0.301
	Regallo	2.35	4.43	3.15	3.34	0.741	**0.029**	0.058
	Burgos	2.19	2.26	3.92	3.18	**<0.001**	0.152	0.087
	Ramirez	2.01	1.98	3.38	3.56	**0.002**	0.831	0.744
C (%)	Mexa	40.6	41.3	41.5	41.0	0.527	0.842	0.158
	Regallo	38.9	39.3	41.0	40.8	**0.015**	0.821	0.577
	Burgos	40.1	40.1	40.3	45.2	0.229	0.268	0.268
	Ramirez	42.1	40.6	40.3	36.8	0.185	0.227	0.620
$\delta^{13}C$ (‰)	Mexa	−32.8	−29.7	−52.7	−55.5	**<0.001**	0.981	0.483
	Regallo	−33.4	−33.9	−53.9	−60.7	**<0.001**	0.205	0.265
	Burgos	−32.7	−29.0	−57.9	−50.4	**<0.001**	**0.016**	0.337
	Ramirez	−32.5	−29.8	−60.5	−52.8	**<0.001**	**0.047**	0.286

Significant effects for elevated [CO_2] (C), water stress (W) and their interaction (C × W) were determined by two-factor ANOVA (P). Significant P values are marked in bold ($P < 0.05$).

gene expression depending on genotype and the level of water restriction (**Table 5**; Supplementary Figure S1). At moderate water stress, elevated [CO_2] decreased transcript levels of RBCL, RBCS, and GS2 relative to control conditions (ambient [CO_2] and well-watered conditions), particularly in the Mexa and Regallo genotypes. Under water stress the transcript levels for these enzymes markedly increased in Ramirez. Elevated [CO_2] caused a generalized increase in the transcript levels of PEPC and GS1, particularly when it was combined with moderate water stress. Transcript abundances of the dehydrins, DHN11 and DHN16, were generally higher under elevated [CO_2] and well-watered conditions, but lower under ambient [CO_2] and water stress relative to control conditions. However, *DHN11* and *DHN16* showed opposite expression patterns under elevated [CO_2] and water stress. [CO_2] enrichment and moderate water stress decreased transcript levels of CAT and SOD in Mexa, Regallo, and Burgos compared with control conditions, whereas in Ramirez they did not change significantly.

Gene expression analysis indicated greater genotype-specific differences under severe water stress than under moderate water stress (**Table 5**; Supplementary Figure S1). In Mexa under elevated [CO_2] and well-watered conditions there were higher transcript levels of RBCL, RBCS, PEPC, GS1, GS2, and CAT and lower levels of DHN16, relative to control conditions. Severe water stress did not substantially alter gene expression. In Regallo most of the transcripts studied were lower in all treatment combinations than in control conditions. However, DHN16 and SOD transcripts increased under ambient [CO_2] and water stress, and these together with CAT and GS1 also increased under elevated [CO_2] and water stress. In the case of Burgos, elevated [CO_2], water stress and their combination strongly reduced transcript levels in comparison to control conditions, especially for GS1 and DHN16, while SOD transcripts increased under water stress and elevated [CO_2] × water stress as observed in Regallo. In Ramirez elevated [CO_2] led to a reduction in the transcript levels of RBCL, RBCS, and SOD and an increase in PEPC and GS1 compared to control conditions. Water stress increased PEPC and DHN16 transcript levels relative to control conditions, while under the combination of elevated [CO_2] and water stress

TABLE 4 | Chlorophyll content, stomatal conductance (g_s), N and C content, and N and C isotope composition ($\delta^{15}N$ and $\delta^{13}C$, respectively) in four durum wheat genotypes grown under ambient or elevated [CO_2] and well-watered or severe water stress conditions (100 vs. 30% pot capacity).

	Genotype	Ambient [CO_2]		Elevated [CO_2]		P_C	P_W	$P_{C\times W}$
		Well-watered	Water stressed	Well-watered	Water stressed			
Chlorophyll (SPAD units)	Mexa	55.8	55.0	52.7	52.7	0.100	0.790	0.807
	Regallo	50.6	43.1	46.6	45.8	0.877	0.351	0.439
	Burgos	56.6	53.2	53.3	58.4	0.661	0.683	0.076
	Ramirez	52.7	51.6	52.8	49.0	0.532	0.230	0.500
g_s(mmol m^{-2} s^{-1})	Mexa	81.9	55.0	223.5	91.5	0.112	0.150	0.323
	Regallo	101.8	82.3	64.4	32.2	0.265	0.499	0.866
	Burgos	245.0	96.7	247.0	30.7	0.390	**<0.001**	0.362
	Ramirez	44.0	52.5	151.3	57.2	0.160	0.270	0.194
N (%)	Mexa	4.39	4.67	4.10	4.54	0.491	0.257	0.785
	Regallo	4.68	4.33	4.37	3.78	**0.017**	**0.011**	0.424
	Burgos	4.89	4.66	4.15	3.97	**0.013**	0.398	0.918
	Ramirez	3.81	4.22	4.16	4.43	0.521	0.437	0.872
$\delta^{15}N$ (‰)	Mexa	2.71	2.74	3.52	3.59	0.060	0.895	0.950
	Regallo	3.78	3.27	3.55	3.58	0.947	0.685	0.646
	Burgos	2.45	2.67	3.69	3.47	**0.018**	0.997	0.547
	Ramirez	2.69	2.99	3.23	2.73	0.508	0.641	0.084
C (%)	Mexa	41.8	41.8	41.3	41.9	0.490	0.368	0.439
	Regallo	39.9	38.0	40.4	41.1	0.064	0.476	0.165
	Burgos	41.9	41.4	41.8	41.3	0.877	0.590	0.968
	Ramirez	41.4	40.7	40.9	42.1	0.416	0.658	0.115
$\delta^{13}C$ (‰)	Mexa	−32.3	−31.4	−56.9	−56.4	**<0.001**	0.546	0.851
	Regallo	−33.5	−32.2	−54.4	−54.0	**0.001**	0.850	0.927
	Burgos	−32.8	−32.4	−46.1	−46.3	**0.004**	0.979	0.940
	Ramirez	−31.9	−32.6	−59.1	−56.4	**<0.001**	0.752	0.594

Significant effects for elevated [CO_2] (C), water stress (W) and their interaction (C × W) were determined by two-factor ANOVA (P). Significant P values are marked in bold ($P < 0.05$).

greater transcript abundances were observed for most of the genes.

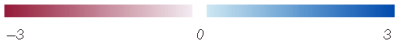
Correlation Network of Physiological Traits and Gene Expression

A Pearson correlation matrix was generated using the mean values for each treatment combination, genotype and sampling date ($n = 32$) of the physiological traits and transcript levels (Supplementary Table S3), excluding NDVI, which was only measured at severe water stress, and $\delta^{13}C$, which was influenced by C composition of the CO_2 bottles used in the elevated [CO_2] chamber (Aljazairi et al., 2015). Of the 190 correlations between parameters, there were 28 positive and 19 negative significant correlations ($P < 0.05$) that are represented in an association network (**Figure 2**). Most of the significant correlations were observed between physiological traits and transcript levels independently. Positive correlations were found among leaf, shoot, and plant dry weights, between the leaf and shoot dry weights with the number of tillers, and between root and plant dry weights. The root/shoot ratio was positively correlated with root dry weight and negatively correlated with leaf and shoot dry weights, the number of tillers and N content. Furthermore, $\delta^{15}N$ was also negatively correlated with N content and the number of tillers. Chlorophyll content was correlated positively with leaf, shoot, root, and plant dry weights, and negatively with g_s. On the other hand, positive correlations were found between N content with leaf and shoot dry weights and the number of tillers, and negative correlations between N content with root dry weight, and between g_s with root and plant dry weights. In the case of transcript levels, RBCL was correlated with RBCS, GS1, GS2, and PEPC, whereas RBCS correlated with GS2 and DHN11, GS2 with DHN11, and PEPC with GS1. Furthermore, some relationships were found between physiological traits and gene expression (**Figure 2**). Positive correlations appeared between DHN16 with plant biomass (leaf, shoot, root, and plant dry weights), CAT with g_s and SOD with C content. Moreover, negative correlations were found between chlorophyll content with RBCL, RBCS, GS2, and CAT, also between root dry weight with RBCL, RBCS, GS2, and DHN11, and finally plant dry weight with RBCL.

TABLE 5 | Transcript changes in four durum wheat genotypes grown under ambient or elevated [CO_2] and well-watered or water stressed conditions: (A) moderate and (B) severe water stress.

Genotype	[CO_2]	Water supply	RBCL	RBCS	PEPC	GS1	GS2	DHN11	DHN16	CAT	SOD
(A) MODERATE WATER STRESS (100 vs. 60% POT CAPACITY)											
Mexa	Ambient [CO_2]	Water stressed	0.15	0.26	−1.01	−0.88	−0.06	−1.02	−2.83	−2.02	−0.33
Mexa	Elevated [CO_2]	Well-watered	−0.96	−4.81	0.76	1.93	−3.43	−0.98	1.79	−1.01	−1.06
Mexa	Elevated [CO_2]	Water stressed	−1.69	−1.66	−2.25	1.65	−1.81	−1.28	−1.02	−2.42	−1.05
Regallo	Ambient [CO_2]	Water stressed	−0.25	−0.62	0.2	−0.53	−0.27	−0.74	−0.66	−2.42	−0.85
Regallo	Elevated [CO_2]	Well-watered	−2.59	−1.71	−1.14	−0.35	−1.26	1.26	1.8	−2.31	−0.42
Regallo	Elevated [CO_2]	Water stressed	−0.59	−0.55	1.52	0.68	−1.29	−0.95	4.81	−1.62	−1.91
Burgos	Ambient [CO_2]	Water stressed	0.06	0.07	0.19	−0.21	0.93	−0.65	3.27	−2.65	−1.99
Burgos	Elevated [CO_2]	Well-watered	−2.45	−1.35	0.1	−0.92	−0.87	0.89	1.8	−3.18	−0.35
Burgos	Elevated [CO_2]	Water stressed	0.47	0.08	2.14	0.67	−0.37	−0.83	2.48	−2.75	−1.76
Ramirez	Ambient [CO_2]	Water stressed	1.7	1.76	0.63	0.34	1.24	−0.03	−2.1	−0.51	0.49
Ramirez	Elevated [CO_2]	Well-watered	−0.42	−0.87	0.67	1.03	−0.7	0.38	0.62	−0.47	−0.68
Ramirez	Elevated [CO_2]	Water stressed	0.56	−1.73	1.99	2.43	−1.21	−1.12	2.49	0.18	0.39
(B) SEVERE WATER STRESS (100 vs. 30% POT CAPACITY)											
Mexa	Ambient [CO_2]	Water stressed	0.57	0.52	0.31	0.19	0.08	−0.37	0.40	0.60	0.25
Mexa	Elevated [CO_2]	Well-watered	1.84	1.49	0.84	1.09	1.27	−0.23	−1.22	1.56	−0.03
Mexa	Elevated [CO_2]	Water stressed	−0.34	0.16	−0.42	−0.29	0.13	−0.58	−0.34	1.30	0.04
Regallo	Ambient [CO_2]	Water stressed	−1.35	−0.59	0.03	−0.19	−0.84	−1.14	1.02	−0.26	0.95
Regallo	Elevated [CO_2]	Well-watered	−1.38	−0.90	−2.38	−0.61	−0.77	−1.35	−1.69	−0.40	−0.17
Regallo	Elevated [CO_2]	Water stressed	−0.30	−0.05	−0.59	2.15	−0.44	0.17	3.31	1.80	0.57
Burgos	Ambient [CO_2]	Water stressed	−0.93	−0.83	−1.98	−3.21	−0.81	−1.35	−6.30	−2.70	0.71
Burgos	Elevated [CO_2]	Well-watered	−2.13	−1.70	−2.18	−2.75	−1.38	0.19	−7.46	−1.85	−0.02
Burgos	Elevated [CO_2]	Water stressed	−1.81	−1.73	−2.08	−2.92	−1.05	−0.46	−5.56	−1.91	0.80
Ramirez	Ambient [CO_2]	Water stressed	−0.44	−0.45	2.34	0.29	0.15	−0.57	1.68	−0.62	0.32
Ramirez	Elevated [CO_2]	Well-watered	−2.71	−2.34	0.55	1.11	−1.00	−0.04	0.04	−0.18	−1.18
Ramirez	Elevated [CO_2]	Water stressed	0.51	0.64	0.98	0.25	0.98	1.01	−0.47	0.81	−0.40

White indicates no change, blue up-regulation, and red down-regulation in each treatment relative to the treatment under ambient [CO_2] and optimal water supply for each genotype, as shown in the color bar for a log_2 scale. RBCL, Rubisco large subunit; RBCS, Rubisco small subunit; PEPC, phosphoenolpyruvate carboxylase; GS1, cytosolic glutamine synthetase; GS2, plastidial glutamine synthetase; DHN11, dehydrin 11; DHN16, dehydrin 16; CAT, catalase; SOD, superoxide dismutase.



DISCUSSION

Although, substantial efforts have been made in recent years to identify traits associated with wheat performance during early growth (Maydup et al., 2012; Rebolledo et al., 2013; Bort et al., 2014; Pang et al., 2014; Wilson et al., 2015), little attention has been paid to the effect of interactions between elevated [CO_2] and water stress in durum wheat. The effects of water restriction on crop growth have been mostly studied with the view of improving drought impacts at late growth stages in Mediterranean environments. However, projections of future climate change in the Iberian Peninsula predict major rainfall limitations and higher evapotranspiration during winter months (Russo et al., 2015) and therefore early-season drought is a matter of concern. In this context, we describe the effects of elevated [CO_2] and water stress during the first part of the growth cycle in four durum wheat genotypes on physiological traits and expression of nine genes that respond to changes in [CO_2] and water levels (Ali-Benali et al., 2005; Budak et al., 2013; Vicente et al., 2015b; Yousfi et al., 2016). The coordination of these parameters under the different combinations of factors is discussed.

Changes in Physiological Traits of Durum Wheat Genotypes under Different Water Regimes and [CO_2] Levels

A moderate water stress in 43-day-old plants did not significantly alter plant growth (**Table 1**). Long-term exposure to elevated [CO_2] led to higher root biomass relative to ambient [CO_2] independently of genotypic variability, in concordance with reports from other crop species (Madhu and Hatfield, 2013). This increment was associated with higher plant growth in Regallo and higher root/shoot ratios in Regallo, Burgos, and Mexa (**Table 1**). In fact, under elevated [CO_2] root growth is often more stimulated than the aerial part of the plant, although it depends on genotype × environment variation (Stitt and Krapp, 1999; Madhu and Hatfield, 2013). A severe water stress

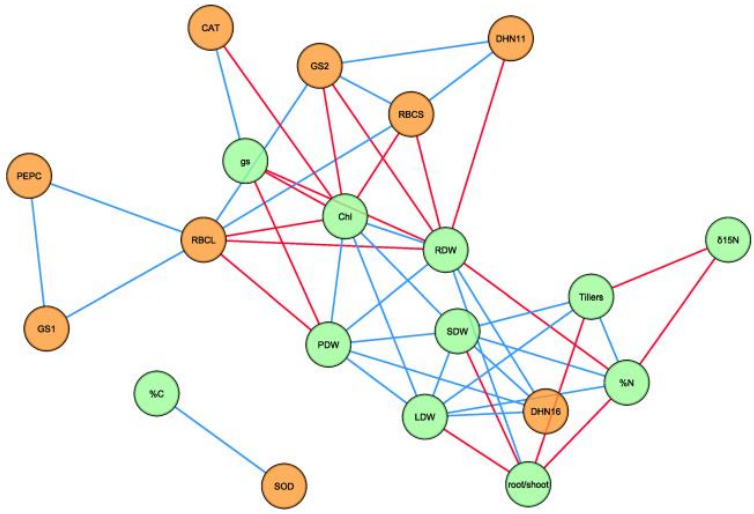


FIGURE 2 | Network analysis of physiological traits and transcript levels under different [CO_2] levels, water regimes and sampling dates in four durum wheat genotypes. The network consists of 20 nodes and 47 edges. Green and orange nodes represent physiological traits and transcript levels, respectively. Blue and red edges represent positive and negative correlations, respectively, based on Pearson's correlation coefficients. For transcript description see the legend of **Table 5**. Chl, chlorophyll; g_s, stomatal conductance; LDW, leaf dry weight; PDW, plant dry weight; SDW, shoot dry weight; RDW, root dry weight; %C, carbon content; %N, nitrogen content.

in 51-day-old plants showed greater effects on plant growth than moderate water stress (**Table 2**). [CO_2] enrichment generally led to an increase in plant biomass by increasing root and shoot biomass and tillering, particularly under optimal water supply. This could be due to the effects of [CO_2] fertilization on the net photosynthetic rate (Long et al., 2006; Vicente et al., 2015b), especially in genotypes with large harvest indices such as post-Green Revolution cultivars (Aranjuelo et al., 2013). It could also be caused by carbohydrate accumulation, which may lead to increases in the number of tillers (Stitt and Krapp, 1999). On the other hand, severe water stress constrained plant growth (dry matter and NDVI), in agreement with earlier studies in durum wheat (Erice et al., 2014; Nakhforoosh et al., 2015; Yousfi et al., 2016), with Ramirez and Burgos being the genotypes most affected. According to Marti et al. (2007), we suggest that progressive water restriction during the vegetative stage constrained the photosynthetic area, which may cause negative effects on final biomass and yield.

Chlorophyll content, g_s, and N and C contents and isotope compositions at moderate and severe water stress did not reveal statistical significance for the interactions [CO_2] × water regime and [CO_2] × water regime × genotype (**Tables 3, 4**; Supplementary Table S2). Stomatal conductance (g_s) generally decreases under elevated [CO_2] and drought stress due to an increase in internal [CO_2] and as a water saving strategy, respectively (Long et al., 2006; Nakhforoosh et al., 2015; Vicente et al., 2015b; Pazzagli et al., 2016). The average g_s values decreased under water restriction at moderate and severe water stress, but it was only significantly decreased in some genotypes (**Tables 3, 4**). On the other hand, elevated [CO_2] did not alter g_s at this growth stage, except for an increase in g_s under moderate water stress in Regallo, which could favor CO_2 assimilation and consequently biomass accumulation under this water regime (**Tables 1, 3**). Earlier studies have shown a decrease in g_s under water stress (Peremarti et al., 2014; Pazzagli et al., 2016), while negligible changes have been reported under elevated [CO_2] in tomato and durum wheat, and increases have even been recorded for Regallo (Vicente et al., 2015a; Pazzagli et al., 2016). Therefore, the growth stage and the severity of the water stress influenced stomatal closure, while elevated [CO_2] had minor effects on g_s during vegetative growth.

Elevated [CO_2] generally decreased N content in the present study (**Tables 3, 4**), which has been observed in C_3 plants through shifts in N uptake and/or assimilation (which agrees with the changes in transcript levels of N-metabolism enzymes; see below) together with other uncertain mechanisms, e.g., the biomass dilution effect, increased N loss, and sink limitation (Stitt and Krapp, 1999; Aranjuelo et al., 2011; Vicente et al., 2015a,b). N content was also diminished by severe water stress in Regallo, in agreement with previous studies in durum wheat (Yousfi et al., 2012, 2016). Chlorophyll content only increased under elevated [CO_2] in Mexa at the first sampling date, but the effect disappeared at the second sampling (**Table 3**). [CO_2] enrichment and water stress did not modify C content in leaves, suggesting that the decrease in N content was not simply due to N dilution caused by rapid growth (Taub and Wang, 2008). Overall, our

data showed that the decrease in N content in plants grown under elevated [CO_2] and water stress during vegetative growth is genotypically dependent.

The $\delta^{13}C$ and $\delta^{15}N$ have been used as potential physiological tracers in plants under elevated [CO_2] and water limitation (Aranjuelo et al., 2011; Yousfi et al., 2012, 2016; Araus et al., 2013; Bort et al., 2014). Elevated [CO_2] and water stress caused an increase in $\delta^{15}N$, although these effects depended on the genotype and were attenuated or disappeared in severe water stress relative to the moderate stress treatment (**Tables 3, 4**; Supplementary Table S2). Variations in $\delta^{15}N$ in response to the growth conditions, together with N content, could indicate shifts in N metabolism (Bort et al., 2014), although $\delta^{15}N$ is determined by many processes that are not completely understood (Ariz et al., 2015). Nevertheless, the higher $\delta^{15}N$ could suggest lower N availability, because N absorption and assimilation cannot fractionate between the ^{14}N and ^{15}N isotopologues under such environmental factors (Lopes and Araus, 2006; Tcherkez, 2011). Additionally, this could reflect a decrease in N translocation from the root to the shoot (Lopes and Araus, 2006). Moreover, $\delta^{13}C$ increased in some genotypes under moderate water stress, regardless of the [CO_2] considered, but this increment, also observed under severe water stress, did not reach statistical significance (**Tables 3, 4**). Elazab et al. (2012) and Bort et al. (2014) also showed a $\delta^{13}C$ increase in flag leaves of different durum wheat genotypes under water stress at later growth stages, which could be associated with higher water-use efficiency (Araus et al., 2008, 2013; Tardieu, 2013; Bort et al., 2014). A stronger water stress does not always lead to larger changes in $\delta^{13}C$, particularly when analyzed in dry matter, as noted in previous studies in rice (Kano-Nakata et al., 2014) and *Pinus tabuliformis* (Ma et al., 2014). In addition, $\delta^{13}C$ was strongly reduced at high [CO_2] because of the very negative $\delta^{13}C$ of the CO_2 used to increase the [CO_2] within the growth chamber (Aljazairi et al., 2015).

Expression of Stress-Responsive Genes in Durum Wheat Genotypes under Different Water Regimes and [CO_2] Levels

Strong differences in gene expression were observed between treatments and among the different genotypes studied (**Table 5**; Supplementary Figure S1). In our study, RBCL and RBCS showed a common expression pattern (**Table 5**), confirming the coordinated expression of both subunits necessary for the assembly of the Rubisco holoenzyme (Suzuki and Makino, 2012). At the first sampling date, gene expression of *RBCL* and *RBCS* was down-regulated in response to elevated [CO_2] no matter which water regime was considered, in agreement with other wheat studies (Aranjuelo et al., 2013; Habash et al., 2014; Vicente et al., 2015b). This down-regulation was associated with lower N content and higher $\delta^{15}N$ in a genotype-dependent manner. The former could be explained by non-selective decreases in N or reallocation of N within the plant under elevated [CO_2] (Aranjuelo et al., 2011; Vicente et al., 2015a). The latter was probably associated with changes in N uptake, assimilation or redistribution within the plant (Araus et al., 2013). At the second sampling date, elevated [CO_2] decreased the N content in Regallo and Burgos, which was related to down-regulation of transcript levels of Rubisco subunits and N-assimilation enzymes (GS1 and GS2), and higher root and plant biomass. These shifts could indicate that plant biomass might increase under elevated [CO_2] in a genotype-dependent manner even when transcript levels of Rubisco subunits decrease during vegetative growth. This could be due to the remobilization of an N over-investment in Rubisco to reuse it in developing new tissues (Richards, 2000; Vicente et al., 2011; Carmo-Silva et al., 2015). However, the decrease in Rubisco transcript levels under water stress did not indicate the greater photosynthetic efficiency that was hypothesized under elevated [CO_2]. Instead it was associated with lower plant biomass, which might suggest an inhibition of CO_2 assimilation and plant growth in concordance with previous studies (Hayano-Kanashiro et al., 2009; Peremarti et al., 2014).

PEPC is a multifaceted key enzyme that in C_3 plants is linked to the provision of Krebs cycle intermediates, and its overexpression in transgenic wheat improved drought tolerance and grain yield (Qin et al., 2015). *PEPC* expression has not been widely studied during early growth in durum wheat plants. In the current work it was induced under the combination of elevated [CO_2] and moderate water stress in most genotypes, whereas at severe water stress genotypic variation determined its expression pattern (**Table 5**). The induction could be related to its major role in providing C skeletons for amino acid and lipid biosynthesis (González et al., 2003). This may be due to an increase in the enzyme's substrates, such as carbohydrates, typically found under elevated [CO_2] and water stress (Khoshro et al., 2013; Vicente et al., 2015b). These results indicate that further work is necessary to broaden our understanding of the biological role of PEPC and its implication in plant growth, especially in genotypes (i.e., Ramirez) with an up-regulation of gene expression under stress conditions.

At moderate and severe water stress, *GS1* and *PEPC* expression was significantly coordinated, as were the expressions of the *GS2* and Rubisco genes (**Table 5**; Supplementary Table S3). Under severe water stress, *GS1* and *GS2* expression was more influenced by genotypic variability than environmental conditions. Yousfi et al. (2016) also reported genotypic differences in the expression of these genes under drought stress, with a general down-regulation under stress conditions. Lower N contents and transcript abundances for RBCL and RBCS under water stress and especially under elevated [CO_2] were associated with higher repression of the *GS2* gene, indicating a coregulation of primary C and N metabolism (Stitt and Krapp, 1999; Vicente et al., 2015b, 2016). In some treatments, mainly at the first sampling date, opposing gene expression patterns were observed between *GS1* and *GS2*. This fact, together with the coordination of *GS1* with *PEPC*, might indicate a predominant remobilization of C and N compounds and an inhibition of primary N assimilation under water stress and elevated [CO_2]. Thus, the results support a significant coordination between C and N metabolism at the transcript level under conditions of elevated [CO_2] and water stress. In addition, the pattern of gene expression for *GS1* and *GS2* supports the use of these genes as indicators of N metabolism under

water stress conditions, as reported previously (Nagy et al., 2013).

DHN11 and DHN16 encode for two dehydrins that belong to group 2 of late embryogenesis abundant (LEA) proteins (Ali-Benali et al., 2005). The up-regulation of dehydrin genes under water restriction is often associated with stress tolerance, although their specific role as osmotically active compounds is still unknown (Kosová et al., 2014). Moderate and severe water stress reduced *DHN11* gene expression regardless of the [CO_2] level compared with control conditions. In the case of the *DHN16* gene, moderate water stress mostly up-regulated its expression, whereas under severe water stress the opposite occurred (**Table 5**). Elevated [CO_2] at the first sampling date mostly enhanced *DHN11* and *DHN16* gene expression, while at the second sampling date its combination with severe water stress led to a wide range of changes in transcript levels in a genotype-dependent manner. Our results showed that the pattern of gene expression could differ between dehydrins, in concordance with previous studies (Ali-Benali et al., 2005; Melloul et al., 2013; Kosová et al., 2014). Additionally, the severity of the water stress, [CO_2] enrichment and the genotype influenced dehydrin transcript levels.

CAT and SOD enzymes form part of the system responsible for lowering ROS and avoiding oxidative stress. In general, gene expression of *CAT* and *SOD* was repressed under moderate water stress regardless of [CO_2] (**Table 5**). Such repression was only maintained for *CAT* at severe water stress in Burgos, while their expression was up-regulated in the other genotypes under elevated [CO_2] × severe water stress. This could suggest a higher demand for ROS control, which would indicate a limitation to the transfer of electrons through photosystems to drive C assimilation (Martins et al., 2016). Enzyme activity and *CAT* gene expression have been reported to decrease under elevated [CO_2] in wheat, possibly due to the inhibition of photorespiration, while they increased only in response to severe drought (Luna et al., 2005; Xu et al., 2010; Vicente et al., 2015b). The available studies reporting changes in *SOD* gene expression and protein content under such conditions are contradictory, reporting different pattern of changes (Kim et al., 2006; Li et al., 2008; Caruso et al., 2009; Xu et al., 2010). Our results highlighted that water regime and genotype were key factors influencing the expression of genes involved in the antioxidant system, indicating a greater need for protection against oxidative damage under severe water stress.

Coordination between Physiological Traits and Transcript Levels in Durum Wheat Grown under Different Environmental Conditions during Vegetative Growth

The different changes in plant growth parameters indicate that the responsiveness to elevated [CO_2] and water stress during early growth depends on (i) the duration of the treatment, because [CO_2] enrichment results in greater increases in plant biomass in older plants; (ii) the severity of the water stress, which is more pronounced under severe water stress; (iii) and the genotypic variability. In general, elevated [CO_2] stimulated plant growth and reduced N content, which at the transcript level was related to a down-regulation of Rubisco and N assimilation genes and up-regulation of genes that take part in C-N remobilization. Moderate water stress did not lead to gross changes in physiological traits, but severe water stress restricted plant growth and N content, while changes in g_s and $\delta^{13}C$ suggested a water-saving strategy relative to well-watered conditions. The transcript profile suggested an inhibition of primary C fixation and N assimilation, differences between dehydrins and a genotypic variation in gene expression under severe water stress, with an induction of genes involved in antioxidant machinery. The stimulation of plant biomass under elevated [CO_2] did not compensate for plant growth limitation under water restriction. Lastly, we observed different genotypic responses to environmental factors, as also reported in barley (Ceccarelli et al., 1991). Regallo showed the lowest plant biomass and chlorophyll and N contents, which was related to a repression of genes for N assimilation and induction for dehydrins, SOD and CAT, while the opposite results were recorded for Burgos (data not shown). Therefore, increased plant growth was linked to up-regulation of N assimilation and down-regulation of stress-responsive genes, suggesting lower oxidative damage.

Considering different environmental conditions predicted for the future climate scenario and genotypic variations, network analysis was used to identify physiological traits, and transcript levels that are correlated during vegetative growth in durum wheat (**Figure 2**). Early growth is a positive trait for improving plant tolerance in water-limited environments that has the potential for larger final plant biomass and yield (Wilson et al., 2015). Plant growth parameters were positively correlated with each other in most cases, suggesting that early plant growth is driven by all plant fractions and tiller production, as reported in other studies (Rebolledo et al., 2013; Wilson et al., 2015). Regardless of genotype, the positive correlation between root and plant biomass was mainly due to the stimulation of root biomass under elevated [CO_2], in agreement with previous reports (Madhu and Hatfield, 2013; and citations therein). In contrast, water restriction (mainly severe water stress) limited both root and shoot biomass, which are often diminished under severe drought conditions (Nezhadahmadi et al., 2013). Positive effects of elevated [CO_2] on root biomass could mitigate drought effects on plant growth by allowing better exploitation of water and nutrients from deep soil layers (Madhu and Hatfield, 2013).

N content was correlated negatively with the root/shoot ratio and positively with the tillers per plant and shoot biomass, and this was probably due to the typically higher N content observed in shoots relative to roots (Vicente et al., 2015a). Hence, greater vegetative growth in durum wheat requires high amounts of N, which in turn will be conditioned by N availability. $\delta^{15}N$ has been proposed as an indicator of responses to stress, such as water stress, N starvation and salinity (Yousfi et al., 2012, 2016; Bort et al., 2014), although it has had little attention for studies of elevated [CO_2] (Ariz et al., 2015). Here we observed a negative correlation of $\delta^{15}N$ with N content and tillers per plant, with elevated [CO_2] being the main factor that increased $\delta^{15}N$ in our experiment. Nevertheless, the fractionating processes of N metabolism affecting $\delta^{15}N$ under elevated [CO_2] and water stress are not fully understood (Tcherkez, 2011).

Leaf chlorophyll content has been extensively used as an indicator of different physiological and agronomical components, particularly at later growth stages (Araus et al., 2008). The network analysis confirmed that chlorophyll content is a positive trait for vegetative growth in durum wheat, and this can be easily implemented in most of studies because this measurement is simple, quick, and non-destructive with modern portable devices. Effects of elevated [CO_2] and water stress on g_s have been widely studied (Long et al., 2006; Pazzagli et al., 2016), including the proposal of g_s as a trait indicator of drought stress tolerance (Nagy et al., 2013). In our study g_s was negatively correlated with chlorophyll content and root and plant biomass. This could highlight that increased vegetative growth was related to stomatal closure, maybe as a water saving strategy or as a direct response to elevated [CO_2].

The positive correlations among the transcript levels of the genes encoding RBCL, RBCS, GS1, GS2, and PEPC supported a balanced coordination between C and N metabolism under elevated [CO_2] and water stress. On the other hand, our results underlined the key role of Rubisco and GS in plant responses to environmental conditions (Nagy et al., 2013; Carmo-Silva et al., 2015; Vicente et al., 2015b; Yousfi et al., 2016). We showed negative associations between transcript levels of Rubisco subunits and GS2 with chlorophyll content and plant biomass. This fact could indicate that a stimulation of plant growth may be associated with a lower investment of resources (mainly N) in Rubisco protein, especially under elevated [CO_2], thus leading to a higher nitrogen efficiency (Pang et al., 2014; Carmo-Silva et al., 2015). The negative correlation between transcript levels of CAT and chlorophyll content highlighted that the up-regulation of *CAT* expression was a response to the high H_2O_2 levels generated under stress conditions (Luna et al., 2005), which could promote chlorophyll degradation (Upadhyaya et al., 2007). Interestingly, transcript levels of CAT were positively correlated with g_s, although a negative correlation should be expected since greater g_s leads to lower photorespiration rates and consequently lower H_2O_2 generation (Luna et al., 2005). We found a positive relationship between C content and transcript accumulation for SOD, not previously reported to our knowledge. Higher *SOD* expression might suggest a better ROS control that triggers an efficient electron transfer and C fixation. In our study, DHN11 transcript accumulation was negatively associated with root biomass, while transcripts for DHN16 were positively linked with plant biomass. These results suggest promising functions for DHN16 in stress tolerance during vegetative growth, as Kosová et al. (2014) proposed in a study examining wheat seed development.

In summary, parameters such as chlorophyll and N content, g_s and $\delta^{15}N$, and the expression of *RBCL*, *RBCS*, *GS2*, *DHN11*, and *DHN16* genes were identified as good indicators for the selection of genotypes with better performance during early plant growth under elevated CO_2 and water stress. Additionally, network analysis underlined the relevance of N metabolism-traits such as N content, $\delta^{15}N$, GS1, and GS2, in the genotypic response of durum wheat to future environmental scenarios in the Mediterranean basin.

CONCLUSION

We conclude that [CO_2] effects on plant growth had greater impacts than moderate or severe water stress during vegetative growth of durum wheat. Whereas, elevated [CO_2] generally led to increases in plant growth, water stress had a negative effect, preferentially as the water stress develops over time. In addition, the interactive effects of both [CO_2] and water regime depends on genotypic variability. Gene expression profiles at moderate water stress were mainly affected by environmental conditions among the different genotypes. However, with further water restriction, genotype-specific differences were found to affect gene expression more than environmental conditions. These facts reflect a wide range of adaptation mechanisms in durum wheat under elevated [CO_2] and water stress during vegetative growth, probably due to the complex regulatory network that takes place with both factors. Moreover, our study did not show a clear trend concerning the genetic advance in response to future climate change scenarios. Our results evidenced for durum wheat the need to take into account the genotypic variability for a greater understanding of plant adaptation to climate change. Moreover, the correlation network demonstrated that the combination of phenotyping and gene expression analysis is a useful approach to identify phenotype-genotype relationships and their behavior in response to different environments during vegetative stages.

AUTHOR CONTRIBUTIONS

SM and JLA conceived and designed the experiments. SM, RV, and AA contributed to the experimental work. SM, RV, and JLA analyzed the data and interpreted the results. RV wrote the paper under the supervision of JLA, and SM and AA revised the manuscript. All authors have read and approved the final manuscript.

FUNDING

This study was supported by the Spanish National Programme for Research Aimed at the Challenges of Society of the Ministry of Economy and Competitiveness (grants No. AGL2013-44147-R and AGL2016-76527-R). SM was the recipient of a fellowship "Presidente de la República PRONABEC-III" from Peruvian Government.

ACKNOWLEDGMENTS

We thank the Unitat de Genòmica of the CCiTUB, Josep Matas (Servei de Camps Experimentals), Adrián Gracia of the University of Barcelona, and Marco Betti of the University of Seville for technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01738/full#supplementary-material

REFERENCES

Alexandratos, N., and Bruinsma, J. (2012). *World Agriculture Towards 2030/2050: The 2012 Revision*. ESA Working paper No. 12-03, FAO, Rome.

Ali-Benali, M. A., Alary, R., Joudrier, P., and Gautier, M. F. (2005). Comparative expression of five Lea genes during wheat seed development and in response to abiotic stresses by real-time quantitative RT-PCR. *Biochim. Biophys. Acta* 1730, 56–65. doi: 10.1016/j.bbaexp.2005.05.011

Aljazairi, S., Arias, C., and Nogués, S. (2015). Carbon and nitrogen allocation and partitioning in traditional and modern wheat genotypes under pre-industrial and future CO_2 conditions. *Plant Biol.* 17, 647–659. doi: 10.1111/plb.12280

Aprile, A., Havlickova, L., Panna, R., Marè, C., Borrelli, G. M., Marone, D., et al. (2013). Different stress responsive strategies to drought and heat in two durum wheat cultivars with contrasting water use efficiency. *BMC Genomics* 14, 1–18. doi: 10.1186/1471-2164-14-821

Aranjuelo, I., Cabrera-Bosquet, L., Morcuende, R., Avice, J. C., Nogués, S., Araus, J. L., et al. (2011). Does ear C sink strength contribute to overcoming photosynthetic acclimation of wheat plants exposed to elevated CO_2? *J. Exp. Bot.* 62, 3957–3969. doi: 10.1093/jxb/err095

Aranjuelo, I., Sanz-Sáez, Á., Jauregui, I., Irigoyen, J. J., Araus, J. L., Sánchez-Díaz, M., et al. (2013). Harvest index, a parameter conditioning responsiveness of wheat plants to elevated CO_2. *J. Exp. Bot.* 64, 1879–1892. doi: 10.1093/jxb/ert081

Araus, J. L., Cabrera-Bosquet, L., Serret, M. D., Bort, J., and Nieto-Taladriz, M. T. (2013). Comparative performance of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ for phenotyping durum wheat adaptation to a dryland environment. *Funct. Plant Biol.* 40, 595–608. doi: 10.1071/FP12254

Araus, J. L., Slafer, G. A., Reynolds, M. P., and Royo, C. (2002). Plant breeding and drought in C_3 cereals: what should we breed for? *Ann. Bot.* 89, 925–940. doi: 10.1093/aob/mcf049

Araus, J. L., Slafer, G. A., Royo, C., and Serret, M. D. (2008). Breeding for yield potential and stress adaptation in cereals. *Crit. Rev. Plant Sci.* 27, 377–412. doi: 10.1080/07352680802467736

Ariz, I., Cruz, C., Neves, T., Irigoyen, J. J., García, C., Nogués, S., et al. (2015). Leaf $\delta^{15}N$ as a physiological indicator of the responsiveness of N_2-fixing alfalfa plants to elevated $[CO_2]$, temperature and low water availability. *Front. Plant Sci.* 6:574. doi: 10.3389/fpls.2015.00574

Bencze, S., Bamberger, Z., Janda, T., Balla, K., Varga, B., Bedő, Z., et al. (2014). Physiological response of wheat varieties to elevated atmospheric CO_2 and low water supply levels. *Photosynthetica* 52, 71–82. doi: 10.1007/s11099-014-0008-y

Bort, J., Belhaj, M., Latiri, K., Kehel, Z., and Araus, J. L. (2014). Comparative performance of the stable isotope signatures of carbon, nitrogen and oxygen in assessing early vigour and grain yield in durum wheat. *J. Agric. Sci.* 152, 408–426. doi: 10.1017/S0021859613000269

Budak, H., Kantar, M., and Yucebilgili Kurtoglu, K. (2013). Drought tolerance in modern and wild wheat. *Sci. World J.* 2013:548246. doi: 10.1155/2013/548246

Carmo-Silva, E., Scales, J. C., Madgwick, P. J., and Parry, M. A. J. (2015). Optimizing Rubisco and its regulation for greater resource use efficiency. *Plant Cell Environ.* 38, 1817–1832. doi: 10.1111/pce.12425

Caruso, G., Cavaliere, C., Foglia, P., Gubbiotti, R., Samperi, R., and Laganà, A. (2009). Analysis of drought responsive proteins in wheat (*Triticum durum*) by 2D-PAGE and MALDI-TOF mass spectrometry. *Plant Sci.* 177, 570–576. doi: 10.1016/j.plantsci.2009.08.007

Ceccarelli, S., Acevedo, E., and Grando, S. (1991). Breeding for yield stability in unpredictable environments: single traits, interaction between traits, and architecture of genotypes. *Euphytica* 56, 169–185. doi: 10.1007/BF00042061

De Leonardis, A. M., Marone, D., Mazzucotelli, E., Neffar, F., Rizza, F., Di Fonzo, N., et al. (2007). Durum wheat genes up-regulated in the early phases of cold stress are modulated by drought in a developmental and genotype dependent manner. *Plant Sci.* 172, 1005–1016. doi: 10.1016/j.plantsci.2007.02.002

Elazab, A., Molero, G., Serret, M. D., and Araus, J. L. (2012). Root traits and $\delta^{13}C$ and $\delta^{18}O$ of durum wheat under different water regimes. *Funct. Plant Biol.* 39, 379–393. doi: 10.1071/FP11237

Erice, G., Sanz-Sáez, A., Urdiain, A., Araus, J. L., Irigoyen, J. J., and Aranjuelo, I. (2014). Harvest index combined with impaired N availability constrains the responsiveness of durum wheat to elevated CO_2 concentration and terminal water stress. *Funct. Plant Biol.* 41, 1138–1147. doi: 10.1071/FP14045

FAO (2013). *Food and Agriculture Organization of the United Nations, Statistics Division*. Available online at: http://faostat3.fao.org (accessed April 10, 2016)

Galmes, J., Medrano, H., and Flexas, J. (2007). Photosynthetic limitations in response to water stress and recovery in Mediterranean plants with different growth forms. *New Phytol.* 175, 81–93. doi: 10.1111/j.1469-8137.2007.02087.x

González, M. C., Sánchez, R., and Cejudo, F. J. (2003). Abiotic stresses affecting water balance induce phosphoenolpyruvate carboxylase expression in roots of wheat seedlings. *Planta* 216, 985–992. doi: 10.1007/s00425-002-0951-x

Guo, Z., Slafer, G. A., and Schnurbusch, T. (2016). Genotypic variation in spike fertility traits and ovary size as determinants of floret and grain survival rate in wheat. *J. Exp. Bot.* 67, 4221–4230. doi: 10.1093/jxb/erw200

Habash, D. Z., Baudo, M., Hindle, M., Powers, S. J., Defoin-Platel, M., Mitchell, R., et al. (2014). Systems responses to progressive water stress in durum wheat. *PLoS ONE* 9:e108431. doi: 10.1371/journal.pone.0108431

Habash, D. Z., Kehel, Z., and Nachit, M. (2009). Genomic approaches for designing durum wheat ready for climate change with a focus on drought. *J. Exp. Bot.* 60, 2805–2815. doi: 10.1093/jxb/erp211

Harnos, N., Bencze, S., Janda, T., Juhász, A., and Veisz, O. (2002). Interactions between elevated CO_2 and water stress in two winter wheat cultivars differing in drought resistance. *Cereal Res. Commun.* 30, 359–366.

Hayano-Kanashiro, C., Calderón-Vázquez, C., Ibarra-Laclette, E., Herrera-Estrella, L., and Simpson, J. (2009). Analysis of gene expression and physiological responses in three Mexican maize landraces under drought stress and recovery irrigation. *PLoS ONE* 4:e7531. doi: 10.1371/journal.pone.0007531

Hudak, C., Bender, J., Weigel, H. J., and Miller, J. (1999). Interactive effects of elevated CO_2, O_3, and soil water deficit on spring wheat (*Triticum aestivum* L. cv. Nandu). *Agronomie* 19, 677–687. doi: 10.1051/agro:19990803

Huseynova, I. M., Aliyeva, D. R., and Aliyev, J. A. (2014). Subcellular localization and responses of superoxide dismutase isoforms in local wheat varieties subjected to continuous soil drought. *Plant Physiol. Bioch.* 81, 54–60. doi: 10.1016/j.plaphy.2014.01.018

IPCC (2013). *Climate Change 2013: The Physical Science Basis*. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, New York, NY.

Kano-Nakata, M., Tatsumi, J., Inukai, Y., Asanuma, S., and Yamauchi, A. (2014). Effect of various intensities of drought stress on $\delta^{13}C$ variation among plant organs in rice: comparison of two cultivars. *Am. J. Plant Sci.* 5, 1686–1693. doi: 10.4236/ajps.2014.511183

Khoshro, H. H., Taleei, A., Bihamta, M. R., Shahbazi, M., and Abbasi, A. (2013). Expression analysis of the genes involved in osmotic adjustment in bread wheat (*Triticum aestivum* L.) cultivars under terminal drought stress conditions. *J. Crop Sci. Biotechnol.* 16, 173–181. doi: 10.1007/s12892-013-0040-7

Kim, S. H., Sicher, R. C., Bae, H., Gitz, D. C., Baker, J. T., Timlin, D. J., et al. (2006). Canopy photosynthesis, evapotranspiration, leaf nitrogen, and transcription profiles of maize in response to CO_2 enrichment. *Global Change Biol.* 12, 588–600. doi: 10.1111/j.1365-2486.2006.01110.x

Kosová, K., Vítámvás, P., and Prášil, I. T. (2014). Wheat and barley dehydrins under cold, drought, and salinity – what can LEA-II proteins tell us about plant stress response? *Front. Plant Sci.* 5:343. doi: 10.3389/fpls.2014.00343

Li, P., Ainsworth, E. A., Leakey, A. D. B., Ulanov, A., Lozovaya, V., Ort, D. R., et al. (2008). *Arabidopsis* transcript and metabolite profiles: ecotype-specific responses to open-air elevated CO_2. *Plant Cell Environ.* 31, 1673–1687. doi: 10.1111/j.1365-3040.2008.01874.x

Liu, E. K., Mei, X. R., Yan, C. R., Gong, D. Z., and Zhang, Y. Q. (2016). Effects of water stress on photosynthetic characteristics, dry matter translocation and WUE in two winter wheat genotypes. *Agr. Water Manage.* 167, 75–85. doi: 10.1016/j.agwat.2015.12.026

Long, S. P., Ainsworth, E. A., Leakey, A. D. B., Nösberger, J., and Ort, D. R. (2006). Food for thought: lower-than-expected crop yield stimulation with rising CO_2 concentrations. *Science* 312, 1918–1921. doi: 10.1126/science.1114722

Lopes, M. S., and Araus, J. L. (2006). Nitrogen source and water regime effects on durum wheat photosynthesis and stable carbon and nitrogen isotope composition. *Physiol. Plantarum* 126, 435–445. doi: 10.1111/j.1399-3054.2006.00595.x

Luna, C. M., Pastori, G. M., Driscoll, S., Groten, K., Bernard, S., and Foyer, C. H. (2005). Drought controls on H_2O_2 accumulation, catalase (CAT) activity and *CAT* gene expression in wheat. *J. Exp. Bot.* 56, 417–423. doi: 10.1093/jxb/eri039

Ma, F., Xu, T. T., Ji, M. F., and Zhao, C. M. (2014). Differential drought tolerance in tree populations from contrasting elevations. *AoB Plants* 6:plu069. doi: 10.1093/aobpla/plu069

Madhu, M., and Hatfield, J. L. (2013). Dynamics of plant root growth under increased atmospheric carbon dioxide. *Agron. J.* 105, 657–669. doi: 10.2134/agronj2013.0018

Marti, J., Bort, J., Slafer, G. A., and Araus, J. L. (2007). Can wheat yield be assessed by early measurements of Normalized Difference Vegetation Index? *Ann. Appl. Biol.* 150, 253–257. doi: 10.1111/j.1744-7348.2007.00126.x

Martins, M. Q., Rodrigues, W. P., Fortunato, A. S., Leitão, A. E., Rodrigues, A. P., Pais, I. P., et al. (2016). Protective response mechanisms to heat stress in interaction with high [CO_2] conditions in *Coffea* spp. *Front. Plant Sci.* 7:947. doi: 10.3389/fpls.2016.00947

Maydup, M. L., Graciano, C., Guiamet, J. J., and Tambussi, E. A. (2012). Analysis of early vigour in twenty modern cultivars of bread wheat (*Triticum aestivum* L.). *Crop Pasture Sci.* 63, 987–996. doi: 10.1071/CP12169

McKersie, B. (2015). Planning for food security in a changing climate. *J. Exp. Bot.* 66, 3435–3450. doi: 10.1093/jxb/eru547

Melloul, M., Iraqi, D., Udupa, S. M., Erba, G., Alaoui, M. A. E., Ibriz, M., et al. (2013). Analysis of mRNA levels of ten genes under water stress in *Triticum turgidum* subsp. durum. *J. Plant Stud.* 3, 65–79. doi: 10.5539/jps.v3n1p65

Nagy, Z., Nemeth, E., Guoth, A., Bona, L., Wodala, B., and Pecsvaradi, A. (2013). Metabolic indicators of drought stress tolerance in wheat: glutamine synthetase isoenzymes and Rubisco. *Plant Physiol. Biochem.* 67, 48–54. doi: 10.1016/j.plaphy.2013.03.001

Nakhforoosh, A., Grausgruber, H., Kaul, H. P., and Bodner, G. (2015). Dissection of drought response of modern and underutilized wheat varieties according to Passioura's yield-water framework. *Front. Plant Sci.* 6:570. doi: 10.3389/fpls.2015.00570

Nezhadahmadi, A., Prodhan, Z. H., and Faruq, G. (2013). Drought tolerance in wheat. *Sci. World J.* 2013:610721. doi: 10.1155/2013/610721

Pang, J., Palta, J. A., Rebetzke, G. J., and Milroy, S. P. (2014). Wheat genotypes with high early vigour accumulate more nitrogen and have higher photosynthetic nitrogen use efficiency during early growth. *Funct. Plant Biol.* 41, 215–222. doi: 10.1071/FP13143

Pazzagli, P. T., Weiner, J., and Liu, F. (2016). Effects of CO_2 elevation and irrigation regimes on leaf gas exchange, plant water relations, and water use efficiency of two tomato cultivars. *Agr. Water Manage.* 169, 26–33. doi: 10.1016/j.agwat.2016.02.015

Peremarti, A., Mare, C., Aprile, A., Roncaglia, E., Cattivelli, L., Villegas, D., et al. (2014). Transcriptomic and proteomic analyses of a pale-green durum wheat mutant shows variations in photosystem components and metabolic deficiencies under drought stress. *BMC Genomics* 15:125. doi: 10.1186/1471-2164-15-125

Pleijel, H., Gelang, J., Sild, E., Danielsson, H., Younis, S., Karlsson, P. E., et al. (2000). Effects of elevated carbon dioxide, ozone and water availability on spring wheat growth and yield. *Physiol. Plantarum* 108, 61–70. doi: 10.1034/j.1399-3054.2000.108001061.x

Qin, N., Xu, W., Hu, L., Li, Y., Wang, H., Qi, X., et al. (2015). Drought tolerance and proteomics studies of transgenic wheat containing the maize C4 phosphoenolpyruvate carboxylase (PEPC) gene. *Protoplasma* 253, 1503–1512. doi: 10.1007/s00709-015-0906-2

R Development Core Team (2008). *R: A Language and Environment for Statistical Computing*. Viena: R Foundation for Statistical Computing.

Rebolledo, M. C., Luquet, D., Courtois, B., Henry, A., Soulié, J. C., Rouan, L., et al. (2013). Can early vigour occur in combination with drought tolerance and efficient water use in rice genotypes? *Funct. Plant Biol.* 40, 582–594. doi: 10.1071/FP12312

Richards, R. A. (2000). Selectable traits to increase crop photosynthesis and yield of grain crops. *J. Exp. Bot.* 51, 447–458. doi: 10.1093/jexbot/51.suppl_1.447

Robredo, A., Pérez-López, U., Miranda-Apodaca, J., Lacuesta, M., Mena-Petite, A., and Muñoz-Rueda, A. (2011). Elevated CO_2 reduces the drought effect on nitrogen metabolism in barley plants during drought and subsequent recovery. *Environ. Exp. Bot.* 71, 399–408. doi: 10.1016/j.envexpbot.2011.02.011

Russo, A. C., Gouveia, C. M., Trigo, R. M., Liberato, M. L. R., and DaCamara, C. (2015). The influence of circulation weather patterns at different spatial scales on drought variability in the Iberian Peninsula. *Front. Environ. Sci.* 3:1. doi: 10.3389/fenvs.2015.00001

Sanchez-Garcia, M., Royo, C., Aparicio, N., Martin-Sanchez, J. A., and Alvaro, F. (2013). Genetic improvement of bread wheat yield and associated traits in Spain during the 20th century. *J. Agric. Sci.* 151, 105–118. doi: 10.1017/S0021859612000330

Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C_T method. *Nat. Protoc.* 3, 1101–1108. doi: 10.1038/nprot.2008.73

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13, 2498–2504. doi: 10.1101/gr.1239303

Soriano, J. M., Villegas, D., Aranzana, M. J., García del Moral, L. F., and Royo, C. (2016). Genetic structure of modern durum wheat cultivars and mediterranean landraces matches with their agronomic performance. *PLoS ONE* 11:e0160983. doi: 10.1371/journal.pone.0160983

Stitt, M., and Krapp, A. (1999). The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. *Plant Cell Environ.* 22, 583–621. doi: 10.1046/j.1365-3040.1999.00386.x

Suzuki, Y., and Makino, A. (2012). Availability of Rubisco small subunit up-regulates the transcript levels of large subunit for stoichiometric assembly of its holoenzyme in rice. *Plant Physiol.* 160, 533–540. doi: 10.1104/pp.112.201459

Tardieu, F. (2013). Plant response to environmental conditions: assessing potential production, water demand, and negative effects of water deficit. *Front. Plant Sci.* 4:17. doi: 10.3389/fphys.2013.00017

Taub, D. R., and Wang, X. (2008). Why are nitrogen concentrations in plant tissues lower under elevated CO_2? A critical examination of the hypotheses. *J. Integr. Plant Biol.* 50, 1365–1374. doi: 10.1111/j.1744-7909.2008.00754.x

Tcherkez, G. (2011). Natural $^{15}N/^{14}N$ isotope composition in C_3 leaves: are enzymatic isotope effects informative for predicting the ^{15}N-abundance in key metabolites? *Funct. Plant Biol.* 38, 1–12. doi: 10.1071/FP10091

Trnka, M., Rotter, R. P., Ruiz-Ramos, M., Kersebaum, K. C., Olesen, J. E., Zalud, Z., et al. (2014). Adverse weather conditions for European wheat production will become more frequent with climate change. *Nat. Clim. Change* 4, 637–643. doi: 10.1038/nclimate2242

Upadhyaya, H., Khan, M. H., and Panda, S. K. (2007). Hydrogen peroxide induces oxidative stress in detached leaves or *Oryza sativa* L. *Gen. Appl. Plant Physiol.* 33, 83–95.

Vicente, R., Martínez-Carrasco, R., Pérez, P., and Morcuende, R. (2016). An association network reveals co-regulation of carbon and nitrogen metabolism-related parameters in durum wheat grown under different environmental conditions. *New Biotechnol.* 33, 414. doi: 10.1016/j.nbt.2015.10.079

Vicente, R., Morcuende, R., and Babiano, J. (2011). Differences in Rubisco and chlorophyll content among tissues and growth stages in two tomato (*Lycopersicon esculentum* Mill.) varieties. *Agron. Res.* 9, 501–507.

Vicente, R., Pérez, P., Martínez-Carrasco, R., Gutiérrez, E., and Morcuende, R. (2015a). Nitrate supply and plant development influence nitrogen uptake and allocation under elevated CO_2 in durum wheat grown hydroponically. *Acta Physiol. Plant.* 37, 114. doi: 10.1007/s11738-015-1867-y

Vicente, R., Pérez, P., Martínez-Carrasco, R., Usadel, B., Kostadinova, S., and Morcuende, R. (2015b). Quantitative RT-PCR platform to measure transcript levels of C and N metabolism-related genes in durum wheat: transcript profiles in elevated [CO_2] and high temperature at different nitrogen supplies. *Plant Cell Physiol.* 56, 1556–1573. doi: 10.1093/pcp/pcv079

Wall, G. W., Garcia, R. L., Kimball, B. A., Hunsaker, D. J., Pinter, P. J., Long, S. P., et al. (2006). Interactive effects of elevated carbon dioxide and drought on wheat. *Agron. J.* 98, 354–381. doi: 10.2134/agronj2004.0089

Wilson, P. B., Rebetzke, G. J., and Condon, A. G. (2015). Of growing importance: combining greater early vigour and transpiration efficiency for wheat in variable rainfed environments. *Funct. Plant Biol.* 42, 1107–1115. doi: 10.1071/fp15228

Xu, Z., Zhou, G., and Shimizu, H. (2010). Plant responses to drought and rewatering. *Plant Signal. Behav.* 5, 649–654. doi: 10.4161/psb.5.6.11398

Yousfi, S., Márquez, A. J., Betti, M., Araus, J. L., and Serret, M. D. (2016). Gene expression and physiological responses to salinity and water stress of contrasting durum wheat genotypes. *J. Integr. Plant Biol.* 58, 48–66. doi: 10.1111/jipb.12359

Yousfi, S., Serret, M. D., Márquez, A. J., Voltas, J., and Araus, J. L. (2012). Combined use of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ tracks nitrogen metabolism and genotypic adaptation of durum wheat to salinity and water deficit. *New Phytol.* 194, 230–244. doi: 10.1111/j.1469-8137.2011.04036.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter 3

Plant-transpiration response to VPD is associated to differential yield performance and gene expression in durum wheat

La respuesta transpirativa a la VPD de la planta está associada a las difernecias de rendimiento y expresión génica en trigo duro

Susan Medina[1,2], Rubén Vicente[1], Maria Teresa Nieto-Taladriz[3], Nieves Aparicio[4], Fadia Chairi[1], Omar Vergara-Diaz[1] and José Luis Araus[1]

[1]Integrative Crop Ecophysiology Group, Plant Physiology Section, Faculty of Biology, University of Barcelona (UB), Barcelona, Spain.

[2]Crop Physiology Laboratory, International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, India.

[3]National Institute for Agricultural and Food Research and Technology (INIA), Madrid, Spain

[4]Agricultural Technology Institute of Castilla and León (ITACYL), Valladolid, Spain

Article in preparation for further publication / Artículo en preparación para su publicación

Plant-transpiration response to VPD is associated to differential yield performance and gene expression in durum wheat

Susan Medina[1,2], Rubén Vicente[1], Maria Teresa Nieto-Taladriz[3], Nieves Aparicio[4], Fadia Chairi[1], Omar Vergara-Diaz[1] and José Luis Araus[1]

[1]Integrative Crop Ecophysiology Group, Plant Physiology Section, Faculty of Biology, University of Barcelona (UB), Barcelona, Spain.

[2]Crop Physiology Laboratory, International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, India.

[3]National Institute for Agricultural and Food Research and Technology (INIA), Madrid, Spain

[4]Agricultural Technology Institute of Castilla and León (ITACYL), Valladolid, Spain

ABSTRACT

The regulation of plant transpiration has been proposed as a key factor affecting transpiration efficiency and agronomical adaption of wheat to water-limited Mediterranean environments. However to date no studies have related this trait with the actual performance under field conditions. In this study, the transpiration response to increasing *vapour pressure deficit* (VPD) of 20 modern semi-dwarf durum wheat cultivars, released during the past four decades in Spain, was evaluated under controlled conditions. The same set of lines was evaluated in the field under a wide range of growing conditions in the Mediterranean, from water stressed environments to good agronomical conditions. The group of non-restrictive (NR) lines to plant transpiration exhibited a better performance in terms of grain yield and biomass compared with the restrictive (R) lines, particularly in the wetter environments, whereas the reverse occurred only in the most stressed trial. Except for this trial, in general NR lines exhibited better water status (stomatal conductance)

and larger green biomass (inferred through vegetation indices) during the reproductive stage than the R lines. In both categories of genotypes, the response to growing conditions were associated with the expression levels of dehydration responsive transcription factors (*DREB*) leading to complex and different performances of primary metabolism related enzymes. Thus the response of NR genotypes under fairly good to good conditions was associated with higher transcript abundances for genes involved in nitrogen (*GS1* and *GOGAT*) and carbon (Rubisco large subunit) metabolism, as well as in water transport (*TIP1.1* aquaporin). In conclusion, modern durum wheat lines varied in their response to water loss where, except for very harsh drought conditions, less restrictive transpiration lines seem to favor the uptake and transport of water and nutrients, the photosynthetic gas exchange, and thus a higher grain yield. This plant transpiration response to VPD may be a trait to further explore when selecting of genotypes best adapted to specific water conditions.

Keywords: aquaporin, durum wheat, gene regulation, drought, transcription factors, transpiration restriction, vapour pressure deficit, vegetation indices, yield.

Abbreviations: Water use; TE, transpiration efficiency; VPD, Vapour pressure deficit; RH relative humidity; DAS, days after sowing; OM, organic matter; NDVI, normalized vegetation index; TR, transpiration rate, $\delta^{13}C$ carbon isotope composition; Yield.

1 Introduction

Agriculture is highly vulnerable to climate change, which is expected will modify crop productivity. A predicted rise of ambient temperatures, together with a decrease in precipitation will likely increase the severity and the frequency of drought stresses in the Mediterranean basin, which will negatively affect crop performance (Li *et al.*, 2009; Ceccarelli *et al.*, 2010). Durum wheat is one of the most important crops in the Mediterranean countries due to its use as staple food (IGC, 2017 http://www.igc.int/es/). Additional efforts have to be done to increase yield gains for the next decades by selecting traits for higher productivity under high temperatures and water limitation (Robertson *et al.*, 2016). In that sense a combination of classical and novel breeding approaches together with the choice of the proper phenotyping traits and a better understanding of the complex metabolic mechanisms operating under abiotic stresses may contribute to that aim (Araus *et al.*, 2008; Tardieu *et al.*, 2011; Mwadzingeni *et al.*, 2016). Most of the traits with agronomic significance are complex traits controlled by multiple genes and environmental signals which determine plant phenotype (Ficklin and Feltus, 2013). Therefore, the improvement of yield production under stress (e.g. drought) conditions may benefit from an integrative approach, combining different levels (organ, individual plants, crop) of phenotyping together with a molecular characterization (Liu *et al.*, 2017).

Mediterranean environments are characterized by water scarcity usually developing during spring which, in the case of durum wheat (and other small grain cereals), coincides with the grain-filling period. Therefore an increment in grain yield, requires crops not necessarily with a higher water use efficiency but instead with a more effective use of water (Blum, 2009). This concept refers not only to the photosynthetic activity of the plant but also to its capacity to manage the amount of water which is available in the soil, in order to sustain the plant transpiration, particularly under water limited environments (Lopes *et al.*, 2011). The transpiration rate of the plant is driven by changes in vapour pressure deficit (VPD), which is a

combined function of air temperature and relative humidity (Kholová *et al.*, 2012; Belko *et al.*, 2013). Recently, Lobell *et al.* (2014) have reported for maize that atmospheric VPD, also termed 'atmospheric drought' has a much stronger effect on current and future yields than previously thought. Moreover transpiration responses to increasing VPD have been linked both theoretically and experimentally to yield under terminal water deficit regimes (Vadez *et al.*, 2014). This is fully relevant for cereals under Mediterranean conditions, which are exposed to terminal (i.e. during grain filling) droughts. Furthermore, the increase in the frequency of heat and drought events in the Mediterranean, driven by climate change, will result in higher VPD conditions.

In wheat large genetic variability has been reported in transpiration sensitivities to evaporative demand and leaf areas (Schoppach and Sadok, 2013). Recently Schoppach *et al.* (2017) have shown that limited whole-plant transpiration under high atmospheric VPD has resulted in advantageous water conservation and crop yield increase under south Australian conditions. Thus, selection over 120 years by breeders for yield increase unconsciously resulted in genotype selection for the expression of the limited-transpiration trait. Moreover changes in transpiration rates were independent of plant leaf area and only marginally correlated with phenology (Schoppach *et al.,* 2017). However, other evidences on wheat (Schoppach and Sadok, 2013; Schoppach *et al.*, 2016) and soybean (Devi *et al.*, 2016) suggest that transpiration rates and leaf area responses to VPD are coupled, such that increases in transpiration under high VPD are 'compensated' by decreases in leaf area. This suggest the existence of a trade-off between both traits that may eventually diminish or even offset the potential usefulness when breeding for this trait. In any case studies in species other than wheat also suggest that limited transpiration at high VPD in water-limited environments resulted in the increment of yield (Gholipoor *et al.*, 2010). However the environments typical of the Mediterranean climate

conditions of Australia are drought prone, with wheat yields usually below the 3-4 tons per hectare.

Besides the need to investigate the potential consequences at the agronomical level of the genotypic differences in transpiration response to VPD, more efforts have to be placed to understand the mechanisms underlying the genotypic responses in this trait (Vadez *et al.*, 2014). A recent study in bread wheat using a mapping population composed of 143 DH lines growing in greenhouse conditions had identified six QTL for the transpiration response to VPD, with one major QTL harbouring several genes previously reported to be involved in ABA signalling, interaction with *DREB2A* and root hydraulics (Schoppach *et al.*, 2016). Genetic differences in the response of transpiration appear to have also a hydraulic basis, in which aquaporins might play a role (Vadez *et al.*, 2014). In the same sense for pearl millet, a limitation in transpiration demand in a high VPD environment was genotype-specific, linked to drought adaptation mechanisms involving abscisic acid and hydraulic signals (Kholová *et al.*, 2010*a*; Kholová and Vadez, 2013). However, to the best of our knowledge there are not studies in wheat relating the phenotyping characteristics of plant transpiration to increasing VPD with the agronomical and physiological performance and the gene expression under field conditions of the same genotypes.

On the other hand many transcription factors and stress-inducible genes have been identified under drought conditions. The dehydration-responsive element-binding proteins, *DREB1* and *DREB2*, are transcription factors that play a key role as regulators of several developmental mechanisms of response to stress, including drought, reported in wheat and other plants (Salekdeh *et al.*, 2009; Gahlaut *et al.*, 2016; Yousfi *et al.*, 2016). Other drought-inducible genes are dehydrins with protective functions to stress conditions (e.g. drought stress) as reported in many plants (Tsvetanov *et al.*, 2000; Kosová *et al.*, 2014*a*). The actin-binding protein *Wcor719* is a cold-responsive dehydrin that have been also upregulated under water

and cold stress (Danyluk *et al.*, 1996; Tsvetanov *et al.*, 2000; Talamè *et al.*, 2007). In addition, to counter-act the increased levels of reactive oxygen species under water stress, genes related to protective functions are generally overexpressed. In this regard, superoxide dismutase (*SOD*) enzyme plays a key role in the elimination of superoxide and prevents cell damage (Huseynova *et al.*, 2015), and ATP synthase (*ATPase*) in the synthesis of ATP which provides energy to the metabolism processes (Zhang *et al.*, 2008; Cheng *et al.*, 2016). Also relevant under water stress is the movement of water and other small solutes such as CO_2, ammonia and urea, which is mediated by the water channel proteins known as aquaporins that belong to major intrinsic protein superfamily (Forrest and Bhave, 2007; Hove *et al.*, 2015)

Moreover in durum wheat, as is the case for other crop species, water stress affects the main metabolic pathways and regulatory mechanisms, leading to a downregulation of genes involved in photosynthesis, N uptake and assimilation, amino acid synthesis, and upregulation in energy provision genes, also in those involved in remobilization and protective functions (Habash *et al.*, 2014; Vicente *et al.*, 2015; Cheng *et al.*, 2016; Medina *et al.*, 2016; Yousfi *et al.*, 2016). Those plants strategies in response to environmental conditions will led a interplay of the main metabolism networks (Hu and Xiong, 2014; Langridge and Reynolds, 2015). Among the genes involved in energy and biosynthesis it is worth to mention the ATPase, involved in the synthesis of ATP for the provision of energy; the ribulose bisphosphate carboxylase oxygenase (Rubisco), which catalyses the first step of CO_2 fixation and photorespiration; the phosphoenolpyruvate carboxylase (*PEPC*) and the pyruvate kinase (*PK*), which participate in the provision of C skeletons for the biosynthesis of organic and amino acids; and the chloroplastic (*GS2*) and cytosolic (*GS1*) glutamine synthetase isoenzymes and the glutamate synthase (*GOGAT*) that catalyse the biosynthesis of such products.

The aim of this study was to assess the differences in at the whole-plant transpiration response to VPD in a set of 20 modern (semi dwarf durum) wheat cultivars widely grown in Spain during the past four decades. Different categories of restrictive and non-restrictive to water loss genotypes were determined. Further, the translation of the transpiration response in terms of grain yield and physiological characteristics was evaluated under a wide range of environmental conditions provided by different locations, years and water regimes (rainfed and support irrigation). Finally differences in the pattern of gene expression during grain filling were investigated in the same set of genotypes. Thus differences in transcript profiles, for a wide range of genes involved in assimilatory metabolism and defence mechanisms, between groups of transpiration-restrictive and non-restrictive cultivars were evaluated under contrasting water regime (rainfed versus irrigation). The results obtained may pinpoint future research directions to speed breeding programs aiming to select genotypes better adapted to future climate scenarios.

2 Materials and methods

2.1 Experimental setup

Two different groups of experiments were carried out. One under controlled conditions, to study differences among genotypes in transpiration pattern to increasing VPD. Another in field conditions, during two consecutive crop seasons, to assess genotypic variability in grain yield and physiological-related parameters in a wide range of environmental conditions, including different water regimes (rainfed versus support irrigation) and locations (with different temperatures and evaporative demand) in Spain. The experiments were conducted with a collection of 20 semi-dwarf durum wheat [*Triticum turgidum* L. ssp. *durum* (Desf.)] commercial varieties released in Spain during the last four decades (i.e. after Post-Green Revolution): Mexa (1977), Vitron (1981), Simeto (1988), Regallo (1988), Gallareta (1990), Bolo

(1991), Don Pedro (1991), Sula (1991), Bólido (1993), Iride (1996), Dorondón (1996) Burgos (1997), Claudio (1998), Amilcar (1999), Pelayo (2000), Avispa (2001), Don Sebastián (2001), Don Ricardo (2005), Kiko Nick (2006) and Ramirez (2006). The year of release in Spain is indicated between brackets, except for the genotypes Simeto, Iride and Claudio, which the year corresponds to their release in Italy. These cultivars represent high yielding genotypes at the time they were released and some of them are still cultivated across the Mediterranean basin.

2.2. Transpiration response to vapour pressure deficit

The experiment was conducted from August to October 2015 at the Experimental Facilities of the Faculty of Biology at the University of Barcelona. For each line ten plants were grown in a greenhouse; each two plants were sowed in 2 L pots containing a mixture (1:1, v/v) of standard substrate and perlite. Photosynthetic photon flux density (PPFD) at midday of a sunny day inside the greenhouse was 800 µmol m^{-2} s^{-1}, the average day/night temperature 25/17°C and the relative humidity (RH) 50%. The plants were uniformly irrigated every two days with 50% Hoagland's nutrient solution (Hoagland and Arnon, 1950). At 36 days after sowing, corresponding to Zadoks stage 23-25 (Zadoks *et al.*, 1974), plants were fully irrigated to reach 100% pot capacity and drained overnight. During the afternoon of the next day, all pot surfaces were completely covered with a layer of aluminium foil to avoid evaporation, and transferred to controlled environment chambers (Conviron E15; Controlled Environments, Winnipeg, MB, Canada) for acclimatization with a night temperature of 15°C and 70% RH (with a night VPD of 0.51 kPa). The following day the transpiration response to changes in VPD was performed by exposing the plants, organized in a complete randomized design, to a controlled VPD ladder from 0.6 to 4.1 kPa, applied by changing both temperature and humidity every hour from 8 am (19°C and 70% RH), after 80 min. of light adaptation, to 5 pm (38°C and 40% RH), and maintained at a constant PPFD of ~400 µmol m^{-2} s^{-1} during the entire experiment. The RH and temperature were recorded by two external sensors (DO9847, Delta

Ohm, Caselle di Selvazzano, Italy) placed inside the chamber. Meanwhile the plant transpiration was recorded by weighing each pot every hour in a bench electronic 10 Kg balance with a resolution of 0.1g (KB Kern 573, Kern & Sohn GmbH, Balingen, Germany); then we recorded one transpiration value per pot at each VPD point based in the loss of pot mass. Further, the plants were harvested by cutting the stem above 1 cm of the soil level, immediately the leaf area was measured by scanning each leaf (HP Scanjet 200, Hewlett-Packard, California, US) and processing the image with Image J software (https://imagej.nih.gov/ij/). To rule out the effect of plant size variation, for each plant the transpiration was normalized by it correspondent leaf area.

2.3. Field trials

The field experiments were carried out from November to June during two consecutive campaigns, 2013-2014 and 2014-2015, at three experimental field locations placed in the north, central and south parts of Spain; for growing season details see Table 1. Two water regimes were imposed in Aranjuez and Valladolid trials (rainfed and supported irrigation), whereas in Sevilla plants were evaluated, only during the second crop season, under rainfed conditions due to the shallow water table by its proximity of the Guadalquivir River to the trial (~0.5 km). Therefore, nine field trials considering location, water regime and crop season were conducted with a complete randomized split plot model with three sets of plot replications. Each plot consisted in six rows 7 m long and 0.2 m apart, with a planting density of 250 seeds m^{-2}. During both campaigns the fertilization was applied in two steps, a first basal application and then a second top dressing application (Table 1). All trials were carried free of weeds, insect pests and diseases by recommended chemical doses (Sanchez-Bragado *et al.*, 2014). Plants were harvested mechanically at maturity and grain yield assessed.

Table 1. Field experimental trial conditions.

	Instituto Tecnológico Agrario de Castilla y León (ITACYL)	*Instituto Nacional de Investigación Agraria y Alimentaria (INIA)*	
Station	**Valladolid**	**Aranjuez**	**Sevilla**
Location	Zamadueñas	Colmenar de Oreja	Coria del rio
Latitude	41°41´N, 04°42´W	40°04´N, 3°31´W.	37°14´N, 06°03´W.
Altitude	700 m a.s.l	590 m a.s.l.	5 m a.s.l
Soil (Organic matter)	Loam (0.8%)	Clay-loam (0.5%)	Loam (0.9%)
1st Crop season	2013-2014	2013-2014	
Sowing date	November 25th, 2013	November 22nd, 2013	
Harvesting date	July 22th, 2014	July 9th, 2014	
Conditions	-2-26°C/ 34-99 RH%	0-25°C/ 31-95% RH	
Rainfall	212 mm	203 mm	
Suplemented irrigation	125 mm	180 mm	
1st Fertilization: Prior sowing	300 kg.ha^{-1} 8:15:15 NPK	400 kg.ha^{-1} 15:15:15 NPK	
2nd Fertilization: Top dressing	300 kg.ha^{-1} Calcium ammonium nitrate	150 kg ha^{-1} diluted urea (46%)	
Sampling date	May 14th	May 12th	
2nd Crop season	2014-2015	2014-2015	2014-2015
Sowing date	November 24th, 2014	November 20th, 2014	December 1st, 2015
Harvesting date	July 10th, 2015	July 22nd ,2015	July 10th, 2015
Conditions	4-17°C/ 53-100 RH%	5-21°C/ 27-100 RH%	4-28°C/ 34-99 RH%
Rainfall	258 mm	206 mm	162 mm
Suplemented irrigation	125 mm	180 mm	-
1st Fertilization: Prior sowing	300 kg.ha^{-1} 8:15:15 NPK	400 kg.ha^{-1} 15:15:15 NPK	400 kg.ha^{-1} 15:15:15 NPK
2nd Fertilization: Top dressing	300 kg.ha^{-1} Calcium ammonium nitrate	150 kg ha^{-1} diluted urea (46%)	150 kg ha^{-1} diluted urea (46%)
Sampling date	May 15th	May 13th	April 17th

2.4 Field measurements, sampling and stable isotope signatures

Field measurements and sampling of flag leaves were performed for all the trials at post-anthesis (Zadok stage 72-73) on sunny days at mid-day (10 am-2 pm). Pools of five flag leaves per plot were frozen into liquid nitrogen and stored at -80 °C for laboratory analysis during the campaign 2013-2014 (sampling dates are described in Table 1). The normalized difference vegetation index (NDVI) was estimated in each plot using a hand-held portable spectroradiometer (GreenSeeker, NTech Industries, Ukiah, CA, USA), scanning with the sensor perpendicularly to the canopy and 0.5-0.6 m above. The relative chlorophyll content was measured with a Minolta SPAD-502 chlorophyll meter (Spectrum Technologies, Plainfield, IL, USA) in the adaxial surface of the central segment of the flag leaf blades, recording five flag leaves per plot and then averaged. Similarly, stomatal conductance (g_s) was measured in two flag leaves per plot using a Decagon SC-1 Leaf Porometer (Decagon Device, Inc., Pullman, WA, USA). The canopy temperature of each plot was measured with an infrared thermometer (PhotoTemp^{TM} MX6^{TM}TM, Raytek Corporation, Santa Cruz, USA). Ambient temperature was measured simultaneously above each plot using a thermo-hygrometer (Testo 177-H1 Logger, Germany). Canopy temperature depression (CTD) was then calculated as the difference between canopy temperature and air temperature. The vegetation indices were estimated using digital RGB (red-green-blue) pictures taken above the plot, holding the camera at 0.8–1.0 m above plant canopy in zenithal plane and focusing near the centre of each plot. Pictures were taken with an Olympus EM-10 and Nikon D90 digital cameras, with a focal length of 18 mm and 14mm, and fields of view (FOV) of 66° 43′ and 46° 51′, during 2013-2014 and 2014-2015 crop seasons, respectively, with a shutter speed of 1/125 for both cameras. No flash was used and the aperture remained in automatic mode. Photographs were saved in JPEG format with a size of 4608 × 3456 pixels and 4288 × 2848 pixels respectively. Subsequently, pictures were analysed with open source Breedpix 0.2 software (Casadessús *et al.*, 2007) designed for digital photograph processing that determines the RGB vegetation indices from the

different properties of colour (Hue, intensity, saturation, lightness, a*, b*, u*, v*, and GA as green area), according to Vergara-díaz *et al.* (2016).

At the end of the season the grain was harvested and the yield recorded. A representative part of the grain pool in every plot was dried in an oven for 48 h at 70 ºC, and finely powdered. Then 1 mg was weighed in tin capsules for the measurements of the stable C ($^{13}C/^{12}C$) ratio together with the total C and N content. Measurements were carried out in an elemental analyser (Flash 1112 EA; ThermoFinnigan, Bremen, Germany) coupled with an isotope ratio mass spectrometer (Delta C IRMS; ThermoFinnigan), operated in continuous flow mode, at the Scientific Facilities of the University of Barcelona as described elsewhere (Bort *et al.,* 2014).

2.5 Quantitative reverse transcriptase PCR amplification

The frozen flag leaf samples were ground with liquid nitrogen and subsequently RNA was isolated from 100 mg of this material using Ribozol RNA Extraction Reagents (Amresco, Solon, OH, USA) as described in Medina et al. (2016). RNA quantity was measured by Qbit fluorometric quantification (Qubit™ 3.0 Fluorometer, Thermo Fisher Scientific, Waltham, MA, USA) while RNA integrity was assessed with an RNA bioanalizer (Agilent 2100 Bioanalyzer, Agilent Technologies, Waldbronn, Germany), obtaining RIN (RNA Integrity Number) scores higher than 6.5 for all samples. Total RNA (1 µg) was treated with PerfeCTa DNase I RNase-free (Quanta Biosciences, Gaithersburg, MD, USA) to eliminate residual genomic DNA and cDNA was synthesized using a qScript cDNA Synthesis Kit (Quanta Biosciences) following the manufacturer's instructions. The qRT-PCR assays, thermal profile and primer design were performed according to Medina et al. (2016). Three technical replicates were analysed per biological replicate, while primer efficiency and specificity was checked experimentally. The primers used for gene expression analysis are listed in

Supplementary Table S1. These included the genes encoding the transcription factors *DREB1* and *DREB2*, dehydrins *Td16* (*DHN16*) and *WCOR719* (*WCOR*), the superoxide dismutase (*SOD*), chloroplastic ATP synthase β-subunit (*ATPase*), cytosolic (*GS1*) and chloroplastic (*GS2*) glutamine synthetases, ferredoxin-dependent glutamate synthase (*GOGAT*), phosphoenolpyruvate carboxylase (*PEPC*), pyruvate kinase (*PK*), Rubisco large subunit (*RBCL*), and aquaporin *TIP1.1*. The internal control genes encoding the ubiquitin and 18S ribosomal subunit were used to normalize qRT-PCR results, which were widely used in previous reports (Vicente *et al.*, 2015; Yousfi *et al.*, 2016). The relative expression was analysed using the comparative C_t method (Schmittgen and Livak, 2008) as the changes between the expression of the target and reference genes (ΔC_t) using fold expression $E^{-\Delta Ct}$, where E is corrected efficiency of each primer. For the comparison within categories of genotypes or environments gene expression was described as $E^{-\Delta\Delta Ct}$ values.

2.6 Data analysis

To fit the data collected for transpiration rate (TR) and VPD levels, we applied a segmented linear regression (model Y1=Slope1.X + Intercept1 and Y2= Slope2.X + Intercept2) or alternatively a linear regression (model Y1=Slope1.X + Intercept1) with 1000 interactions; these algorithms fitted the better model depending of the data accounting a 95% of confidence interval and a significance $p > 0.05$ and the slopes were compared. This analysis was performed with GraphPad Prism software (Graph Pad Software Inc, La Jolla, USA). The slope variation (Δslope) was calculated and used to classify the genotypes according to its sensitiveness to increasing VPD. In addition the slope of the linear increase in transpiration as VPD augmented from around 1 to 4 kPa was also calculated to compare with the range of VPD values usually tested in wheat (Schoppach *et al.,* 2016, 2017). In that case the starting point corresponded to the second measurement, 140 minutes after the light period started, when VPD reached a value of 1.07 kPa..

The effects of the transpiration response and growing conditions on agronomical, physiological and gene expression were evaluated through analysis of variance (ANOVA) and linear model comparisons (p<0.001). Particularly, for gene expression data, a $\log_2$ transformation was needed. When the differences between treatments were significant (p < 0.05), the mean comparison was assessed by LSD (Least significant differences). The correlation analysis was performed with Pearson method (p<0.001). All tests were performed with R package for statistical computing (R Foundation for Statistical Computing: Vienna, Austria). Heat maps of relative gene expression were performed using a log transformation of the real-time PCR data presented as ΔC_T ($C_{T\ mRNA} - C_{T\ 18SrRNA,\ UBImRNA}$) with GraphPad Prism version 7.00 (GraphPad Software, La Jolla California USA) The network analyses for all traits were carried out using significant correlations (p<0.001) with higher Pearson´s coefficients (r>0.8 and r<-0.8) , then the representation was performed into Cytoscape v3.4.0 (Shannon *et al.*, 2003).

3 Results

3.1 Transpiration response of wheat lines to changes in vapour pressure deficit

The transpiration response to increasing VPD under controlled conditions showed significant differences in the slopes of the 20 durum wheat lines of this assay (Table 2 and Fig. 1). The significant variation in the slopes classified the 20 lines in two main groups: the non-restrictive to water lose (NR), which was not sensitive to VPD changes, and restrictive to water lose (R), which included lines sensitive to the increment of VPD with two different subgroups, less restrictive (R-) and very restrictive (R+). The NR group included six lines (Burgos, Claudio, Dorondón, Pelayo, Ramirez and Regallo) that fitted better in a linear regression and did not show a consistent VPD threshold (X_0) neither a higher significant slope variation. The R-

group included the lines Amilcar, Bólido, Don Ricardo, Don Pedro, Don Sebastián, Iride, Kiko Nick and Vitron and the R+ group the lines Avispa, Bolo, Gallareta, Mexa, Simeto and Sula. The slope variation (Δslope) within these three subgroups was significantly different, with values close to cero in NR lines, and decreasing progressively for R- and R+ genotypes (Table 2). Furthermore, both restrictive subgroups started to decrease the transpiration at similar VPD break points (R+: 1.063 and R-: 1.072 kPa), but differed significantly in their Δslope (R- = -47.2 and R+ = -62.5 $mg_{H2O}\, m^{-2}\, s^{-1}$) (Fig. 1). The NR lines showed significant lower mean slopes under low VPD (Slope1) but higher mean slopes under high VPD (Slope2) compared to the R group, where R+ lines showed the highest mean slope value under low VPD and the lowest value under high VPD. The R- genotypes showed values between NR and R+ ones. Marginal significant negative correlations were found between the whole plant area and transpiration rate at 1 kPa (r= -0.24) and 4 kPa (r=-026) (Supplementary Fig. S1).

In addition, the transpiration response from 1.07 to 4.10 kPa was also calculated (Supplementary Fig. S2 and Table S2). For all the 20 genotypes relationships between transpiration and VPD were clearly linear and no breakpoint pattern in transpiration as VPD increased was identified. Except Burgos and Regallo, all the genotypes termed above as a NR also were placed among the lowest in transpiration rates at 1.07 kPa, while their slope of increase in transpiration as response to increasing VPD were among the highest. However, Don Sebastián, Iride (classified above as R- genotypes) and Gallareta (R+ genotype) also were among the genotypes exhibiting the highest slopes, whereas Amilcar, Bólido, Don Ricardo and Kiko Nick (R- genotypes) exhibited relatively low transpiration values at 1.07 kPa.

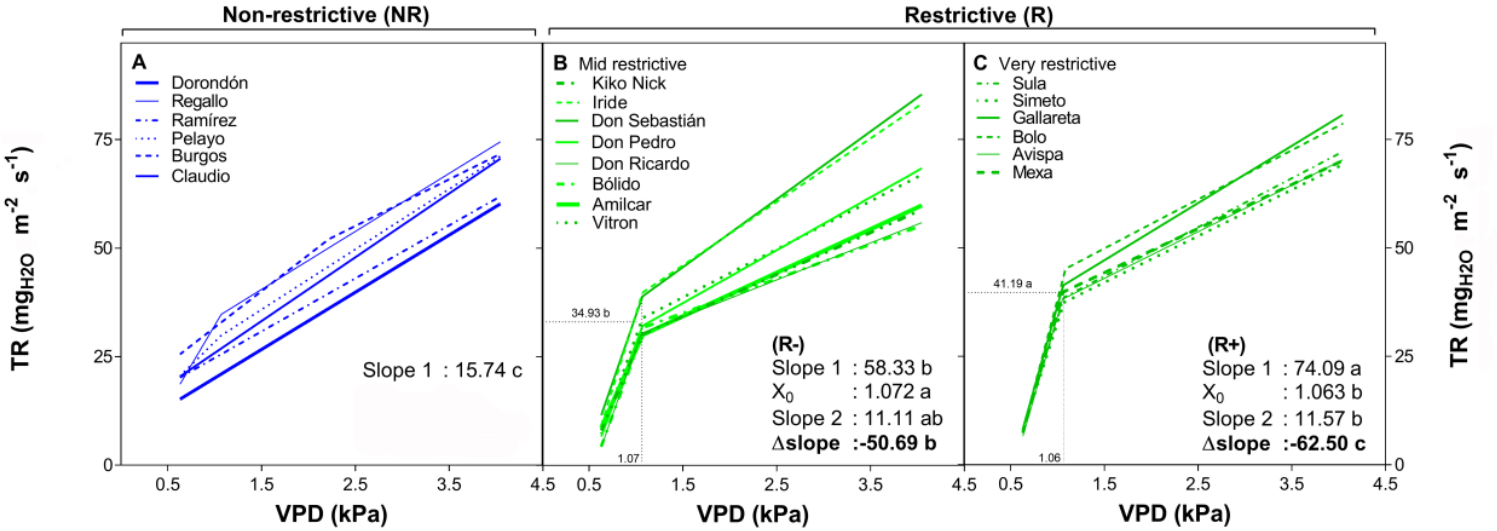


Figure 1. Transpiration rate (TR) of 20 durum wheat lines exposed to increasing VPD regimes as described in Table 1. Each curve expresses the mean TR values across low to high VPD of a particular line. Plants were tested at the vegetative stage and values represent the mean of five plants per line. (A) The linear regressions of non-restrictive lines (NR), and (B and C) the segmented regressions of the restrictive [R: mid restrictive (R-) and very restrictive (R+)] lines are shown. All panels show the comparison according LSD test (p<0.05) indicating the mean slope of the TR response before and after (Slope 1 and 2) putative VPD breakpoints (X_0) and their slope variation (Δslope).

Table 2. Transpiration response to a variation in vapor pressure deficit (VPD) of 20 durum wheat lines. The lines were grouped in non-restrictive (NR) and restrictive [R; including mid restrictive (R-) and very restrictive (R+)] transpiration to water lose based on the fit parameters of segmented and linear regressions (p<0.001) for the transpiration response to a changes in VPD between 0.5 and 4.5 kPa. The values represent the mean of five biological replications. The parameters evaluated are the Intercept, the TR response at low and high VPD (Slope 1 and 2 respectively), the VPD breakpoint (X_0), and the slope variation between high and low VPD (Δslope), and the R^2 of the fitting curve. In the bottom is shown the average comparison between non-restrictive (NR) and restrictive (R) lines according LSD test (p<0.05). The intercept and Δslope are expressed in mg_{H2O}. m^{-2} s^{-1}, the X_0 in kPa and the slopes 1 and 2 in mg_{H2O}. m^{-2} s^{-1} kPa^{-1}.

	Class	*Line*	*Intercept*	*Slope 1*	*X_0*	*Slope 2*	*Δslope*	*R^2*
Non-restrictive (NR)	NR	Burgos	13.89	13.90	-	13.89	**0**	0.604
	NR	Claudio	11.11	13.89	-	13.89	**0**	0.565
	NR	Dorondón	5.55	11.11	-	11.11	**0**	0.576
	NR	Pelayo	8.33	13.89	-	13.89	**0**	0.718
	NR	Ramírez	11.11	11.11	-	11.11	**0**	0.575
	NR	Regallo	5.55	30.56	1.071	13.89	**-16.67**	0.403
Restrictive (R)	R -	Amilcar	-22.22	47.22	1.070	11.11	**-36.11**	0.814
	R -	Bólido	-33.33	58.33	1.095	8.33	**-50.00**	0.881
	R -	Don Ricardo	-33.33	61.12	1.058	8.33	**-52.78**	0.811
	R -	Don Pedro	-30.55	58.33	1.070	11.11	**-47.22**	0.855
	R -	Don Sebastián	-27.78	61.11	1.070	16.67	**-44.44**	0.718
	R -	Iride	-33.33	69.44	1.070	13.89	**-55.56**	0.872
	R -	Kiko Nick	-22.22	50.00	1.070	8.33	**-41.67**	0.814
	R -	Vitron	-30.55	61.11	1.070	11.11	**-50.00**	0.669
	R +	Avispa	-41.67	75.00	1.054	11.11	**-63.89**	0.771
	R +	Bolo	-44.44	80.56	1.086	11.11	**-69.44**	0.685
	R +	Gallareta	-38.89	77.78	1.060	13.89	**-63.89**	0.614
	R +	Mexa	-41.67	75.00	1.058	11.11	**-63.89**	0.620
	R +	Simeto	-33.33	66.67	1.070	11.11	**-55.56**	0.773
	R +	Sula	-36.11	69.44	1.070	11.11	**-58.33**	0.676
Non-restrictive $_{average}$			**9.26 a**	**15.74 b**	**-**	**12.96 a**	**-**	
Restrictive $_{average}$			**-33.53 b**	**65.08 a**	**1.069**	**11.31 b**	**-53.77**	

3.2 Effect of water regime on plant growth and yield in field conditions at different locations and crop seasons.

As expected, lower grain yields for the 20 wheat genotypes were observed in rainfed relative to supported irrigation trials (Table 3). In general, vegetation indices such as NDVI, Hue, lightness, v*, GA and chlorophyll content were increased as agronomical conditions improved, while saturation, a* and u* decreased and intensity and b* were not affected by growing conditions (Table 3). Grain $\delta^{13}C$ decreased and CTD and g_s increased as growing conditions improved, while the N content in grains decreased.

Table 3. Mean values of Grain yield, vegetation indices and water status parameters measured in a set 20 commercial durum wheat lines (as detailed in Table 1) grown under nine different growing conditions depending on field site (Sevilla, Aranjuez and Valladolid), Two water regimes (rainfed and support irrigation), and crop campaign (2013-2014 and 2014-2015). The right side displays the mean comparison of the values for each trait under stress and optimal conditions, including in the first category the three trials with lower yield and in the second the rest of the trials. Means exhibiting different letter are significantly different (p <0.05) by LSD test.

Trait	*Valladolid 2014 Rainfed*	*Valladolid 2015 Rainfed*	*Aranjuez 2015 Rainfed*	*Aranjuez 2015 Irrigation*	*Aranjuez 2014 Rainfed*	*Sevilla 2015*	*Valladolid 2014 Irrigation*	*Aranjuez 2014 Irrigation*	*Valladolid 2015 Irrigation*	*Conditions*	
										Low yield	*High yield*
Yield	2816 f	3789 e	4661 d	5078 cd	5532 c	6494 b	6519 b	6857 ab	7194 a	4199 b	6429 a
Int.	0.30 c	0.30 c	0.32 b	0.32 ab	0.27 e	0.31 c	0.29 d	0.25 f	0.33 a	0.30 a	0.30 a
Hue	71.92 d	77.60 d	84.94 c	86.39 c	109.19 a	89.72 c	96.06 b	106.12 a	86.38 c	85.92 b	92.94 a
Sat.	0.31 a	0.29 ab	0.26 c	0.27 bc	0.11 f	0.30 a	0.19 d	0.15 e	0.26 c	0.25 a	0.24 b
Light.	39.56 d	39.78 d	42.60 bc	43.33 ab	34.44 f	42.29 c	38.01 e	33.28 g	43.92 a	39.09 b	40.17 a
a*	-10.67 a	-15.20 bc	-17.83 d	-19.22 e	-11.79 a	-21.26 f	-16.28 c	-14.69 b	-18.81 de	-13.87 a	-18.05 b
b*	26.98 bc	26.52 c	26.83 bc	28.16 ab	13.71 f	29.40 a	20.71 d	16.58 e	27.60 bc	23.51 a	24.49 a
u*	-2.47 a	-8.62 b	-11.94 cd	-13.34 e	-8.40 b	-15.54 f	-11.67 cd	-10.74 c	-13.00 de	-7.86 a	-12.86 b
v*	28.50 c	29.39 c	30.99 b	32.55 a	16.29 f	33.56 a	24.22 d	19.19 e	32.17 ab	26.29 b	28.34 a
GA	0.63 f	0.78 e	0.98 a	0.98 a	0.83 d	0.99 a	0.90 c	0.93 bc	0.97 ab	0.81 b	0.96 a
SPAD	54.16 c	52.13 c	58.18 ab	58.90 a	57.96 ab	53.87 c	56.44 b	58.19 ab	52.03 c	55.61 b	55.89 a
NDVI	0.53 f	0.65 d	0.76 a	0.78 a	0.62 e	0.76 a	0.68 c	0.72 b	0.73 b	0.64 b	0.74 a
C%	41.14 f	43.02 e	42.74 d	41.33 cd	41.65 c	40.92 b	42.70 b	41.47 ab	42.25 a	42.14 a	41.73 b
$\delta^{13}C$	-23.74 a	-24.22 b	-26.46 e	-26.44 e	-25.81 d	-27.61 f	-24.73 c	-26.32 e	-25.85 d	-25.06 a	-26.19 b
CTD	0.97 e	1.41 de	7.51 b	6.89 b	4.78 c	18.43 a	2.53 d	4.58 c	4.95 c	3.67 b	7.47 a
g_s	171.9 c	-	-	-	392.6 b	-	250.3 bc	439.7 a	-	282.3 b	345.0 a
N%	2.31 cde	2.87 a	2.39 cde	2.46 c	2.29 cde	2.42 cd	2.65 b	2.20 e	2.26 de	2.47 a	2.40 b

Grain yield (Yield) in kg. ha^{-1}; Vegetation indices: Intensity (Int.), Hue, Saturation (Sat.), Lightness (Light.), a*, b*, u*, v*, GA, SPAD and NDVI; Water status traits: Carbon content (C%) in percentage of dry mass to charge ratio, Carbon isotope composition ($\delta^{13}C$) in δ‰, Canopy temperature depression (CTD) in C°; and nitrogen traits: Nitrogen content (N%) in percentage of dry mass.

3.3. Effect of genotypic variability on plant growth and yield associated with the capacity to manage water loses.

We observed differences on grain yield and physiological traits between groups according to their capacity to manage water loss (Table 4) evaluated as whole-plant transpiration response to increasing VPD under controlled conditions (Table 2). Except for the trial with the lowest average grain yield (Valladolid rainfed 2014) where R lines exhibited higher yield than NR lines, and the second trial with the lowest yield (Valladolid rainfed 2015) where no differences between R and NR lines were recorded, in the other seven trials the NR lines showed higher yield than R lines. Good linear fits for grain yield of each group against average grain yield in every growing condition were achieved for each group (R^2_{NR}: 0.995 and R^2_R: 0.999; Fig. 2). Highly significant differences (p<0.001) between NR and R fitting lines were observed, especially under high yield conditions. Moreover when considered the whole set of trials, NR lines exhibited higher values of NDVI and of some RGB indices (saturation, b*, v*) and lower values of other indices (hue, a*) as well as leaf chlorophyll content and carbon content than R lines (Table 4). Considering the two sub-groups in R lines (R- and R+) relative to NR lines, the R+ lines showed lower values for saturation, b* and v* and higher values for hue than R- and NR lines. The NR lines also exhibited slightly higher g_s than the R lines, whereas no clear differences in CTD and $\delta^{13}C$ emerged for the whole set of trials. However significant differences were observed in grain $\delta^{13}C$ between NR and R groups within some of the trials. Particularly in the two extreme trials in terms of grain yield NR cultivars exhibited more negative $\delta^{13}C$ compared with R cultivars (Table 4). Further the water status represented as the mean grain $\delta^{13}C$ of the most contrasting groups of genotypes (R+ and NR) against the average $\delta^{13}C$ value for the whole set of 20 lines in each field trial were compared (supplementary Fig. S3). Both regression curves for NR and R+ were significantly different (p<0.036), highlighting that higher (less negative) $\delta^{13}C$ values were achieved for the R+ compared to NR group of genotypes for an environment (trial) with the highest averaged $\delta^{13}C$ values (around -24 ‰),

whereas in the trial with the lowest (i.e. more negative) averaged $\delta^{13}C$ (near -28 ‰) differences between the two categories of genotypes were absent.

On the other hand, we did not found consistent differences across the 9 growing conditions in grain yield between the subgroups of genotypes when selection was based in the slope of transpiration response to increasing VPD between 1.07 and 4.1 kPa.

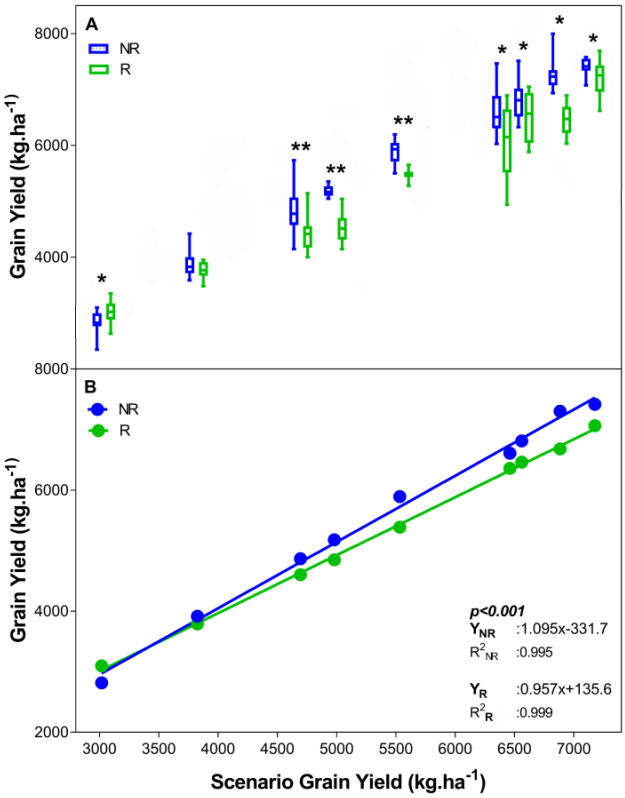


Figure 2. Grain yield differences between the restrictive (R: including mid restrictive and very restrictive) and non-restrictive (NR) lines across nine different growing conditions in the field. In (A) the box-and-whisker plots represents the grain yield of R and NR groups within each growing condition whereas the boundary of the box closest to zero indicates the 25th percentile, the line within the box marks the median and the boundary of the box farthest from zero indicates the 75th percentile). Asterisks indicate significant differences between NR and R groups performed by ANOVA (*, p<0.05, **, p<0.01). In (B) the linear regressions represents the average grain yield of the complete set of R and NR groups against the average yield for the complete set of 20 lines tested in each of the nine growing conditions. Level of significance (p), between fitting lines as well as the determination coefficient (R^2) and the equation of each line are also indicated.

Table 4. Differences in grain yield, vegetation indices and water regime parameters between the non-restrictive (NR) and restrictive (R) groups across nine field growth conditions. The right side of the table shows the mean average within all field trials of R (including mid restrictive (R-) and very restrictive (R+) lines) and NR lines. Traits and growing conditions as detailed in Table 2. Means exhibiting different letters area significantly different (p <0.05) by ANOVA and LSD test. For trait abbreviations and units see Table 2.

	Valladolid 2014 Rainfed		*Valladolid 2015 Rainfed*		*Aranjuez 2015 Rainfed*		*Aranjuez 2015 Irrigation*		*Aranjuez 2014 Rainfed*		*Sevilla 2015*		*Valladolid 2014 Irrigation*		*Aranjuez 2014 Irrigation*	*Valladolid 2015 Irrigation*		*NR*	*R*		
Class	***NR***	***R***	***NR***	***R***	***NR***	***R***	***NR***	***R***	***NR***	***R***	***NR***	***R***	***NR***	***R***	***NR***	***R***	***NR***	***R***		***R+***	***R-***
Yield	**2668b**	**2851a**	3816	3788	**4759a**	**4621b**	**5127a**	**5019b**	**5893a**	**5378b**	**6470a**	**6436b**	**6656a**	**6432b**	**7215a**	**6704b**	**7417a**	**7124b**	**5540a**	**5346b**	**5430b**
Int.	0.30	0.31	0.30	0.30	0.32	0.32	0.33	0.33	0.28	0.28	0.31	0.31	0.29	0.29	0.27	0.26	**0.33b**	**0.34a**	0.30	0.30	0.30
Hue	70.9	72.7	77.8	77.6	85.1	84.9	86.9	86.2	109.2	110.3	89.7	89.9	98.2	94.7	109.1	104.4	86.3	86.4	**89.7b**	**91.5a**	**88.6b**
Sat.	0.32	0.31	0.30	0.29	0.27	0.26	0.28	0.28	0.12	0.11	0.3	0.29	0.19	0.19	0.14	0.16	0.28	0.26	**0.25a**	**0.23b**	**0.24ab**
Light.	39.5	39.5	39.8	39.8	42.6	42.6	43.3	43.3	34.8	34.3	42.3	42.2	38.1	37.9	**34.2a**	**32.9b**	**43.5b**	**44.1a**	39.8	39.5	39.5
a*	-10.4	-10.9	-15.5	-15.0	-17.9	-17.7	-19.5	-19.1	-12.2	-11.7	-21.4	-21.1	-16.5	-16.2	**-15.5b**	**-14.4a**	-19.3	-18.6	**-16.5b**	**-15.9a**	**-16.1a**
b*	27.1	26.9	26.9	26.3	26.9	26.7	28.3	28.0	14.1	13.4	29.6	29.1	20.4	20.9	16.1	16.9	28.3	27.2	**24.4a**	**23.4b**	**24.3ab**
u*	-2.1	-2.8	-8.9	-8.5	-12.1	-11.9	-13.7	-13.2	-8.7	-8.3	-15.7	-15.4	-12.0	-11.5	**-11.5b**	**-10.4a**	-13.4	-12.8	-10.9	-10.6	-10.5
v*	28.5	28.5	29.8	29.2	31.1	30.9	32.7	32.4	16.8	15.9	33.7	33.3	23.9	24.4	18.8	19.5	32.8	31.9	**27.8a**	**26.7b**	**27.6a**
GA	0.62	0.64	0.8	0.78	0.98	0.98	0.99	0.99	0.86	0.83	0.99	0.99	0.91	0.9	0.92	0.94	0.98	0.97	0.90	0.88	0.89
SPAD	52.3	55.3	51.9	52.4	58.1	58.2	**57.6a**	**59.6b**	**57.1b**	**58.5a**	53.8	54.1	57.1	55.8	58.6	57.9	51.1	52.5	**55.1b**	**57.0a**	**55.3ab**
NDVI	0.53	0.54	0.66	0.65	0.76	0.76	0.78	0.78	**0.64a**	**0.62b**	0.77	0.76	0.68	0.69	**0.74a**	**0.72b**	0.74	0.73	**0.70a**	**0.69b**	**0.69b**
C%	**36.5b**	**43.1a**	43.1	42.9	42.6	42.7	41.3	41.3	41.3	41.7	40.7	40. 9	42.1	42.9	41.8	41.3	42.7	41.9	**41.4b**	**41.7a**	**42.5a**
$\delta^{13}C$	**-23.9b**	**-23.6a**	-24.1	-24.3	**-26.3a**	**-26.5b**	-26.4	-26.5	-25.9	-25.8	-27.6	-27.6	-24.8	-24.7	-26.4	-26.3	**-26.0b**	**-25.8a**	-25.7	-25.7	-25.6
CTD	0.94	1.13	1.52	1.33	7.44	7.54	7.12	6.83	4.74	4.81	5.37	5.44	2.47	2.55	**4.83a**	**4.44b**	4.92	4.96	5.80	6.05	5.60
g_s	190	153	-	-	**-**	**-**	-	-	**402a**	**382b**	-	-	242	258	455	424	-	-	**322a**	**306b**	**311b**
N%	**2.2b**	**2.4a**	2.9	2.8	2.4	2.3	2.5	2.5	2.3	2.3	2.5	2.4	2.6	2.7	2.2	2.2	2.3	2.3	2.4	2.4	2.5

3.4 Changes in gene expression between wheat lines with different transpiration response patterns respect to grain yield productivity.

The transcript profiles of 13 genes involved in C and N metabolism and stress response were studied in the 20 wheat lines collected from two trials exhibiting strong differences in gran yield associated to the water regime: LY (Valladolid 2014 under rainfed conditions) and HY (Valladolid and Aranjuez 2014 under irrigated conditions; Fig. 3, Table 5). Gene expression analysis indicated significant changes in transcript levels between low and high yield scenarios, NR and R groups and their interaction. In general terms and compared to the transcript abundance of the housekeeping genes, the transcript abundance of *RBCL* and *ATPase* genes were higher, while for the rest of genes, particularly *DREB1*, *DREB2*, *WCOR*, *PEPC*, *SOD* and *PK*, they were lower (Table 5). Furthermore, NR and R+ lines showed different profiles within HY and LY while R- group showed a gene expression pattern between the other two groups.

Comparing low yield (LY) with regard to high yield (HY) conditions, dehydrins genes (*DNH16* and *WCOR*) were downregulated whereas *GS2* and *TIP1.1* genes were overexpressed (Table 5). Considering all growing conditions R+ lines overexpressed *DREB2*, *GS2* and *RCBL* genes and underexpressed *GS1*, *GOGAT* and *TIP1.1* genes compared to NR group. The expression for the rest of the genes did not reach statistically significant differences between neither subgroups of genotypes nor yielding scenarios. Furthermore, the interaction was significant for *DREB1*, *GOGAT* and *TIP1.1*. Comparing R+ relative to NR under LY conditions, *DREB2* was significantly overexpressed, whereas *GOGAT*, *GS1*, *RBCL* and *TIP1.1* were downregulated.

Table 5. Comparative gene expression of the very restrict (R+) and non-restrict (NR) genotypes under low yield (LY, < 3000 kg ha^{-1}) and high yield (HY, > 5000 kg ha^{-1}) trials assayed in the crop campaign 2013-2014. The left part shows the fold change in expression relative the groups of lines (R+ respect to NR), environment (LY respect to HY) and their interaction (L x E). The right part shows the fold change relative to reference genes of NR and R+ groups under HY and LY conditions, as well as the fold change of R+ respect to NR lines in each environment. The comparisons were assessed by ANOVA and LSD test using a $\log_2$ transformation of the fold change values. Different letters indicate significant differences (p < 0.05), while asterisks indicate levels of significance (ns, non-significant; *, p<0.05; **, p<0.01; ***, p<0.001). Positive values indicate up-regulation and negative values indicate down-regulation of target genes. For details see Material and Methods.

Fold change	***Target gene***	*Line*	*Environment*	*Interaction*	*Low yield (LY)*			*High yield (HY)*		
		R+:NR	***LY:HY***	***L x E***	***NR***	***R+***	***R+:NR***	***NR***	***R+***	***R+:NR***
Stress response	*DREB1*	-3.9	-1.9	*	-5.42a	-7.81ab	-5.2	-5.81a	-7.39b	**-3.0****
	DREB2	**4.8***	-2.1	ns	-8.57b	-8.05a	**1.4***	-8.24	-5.33	7.5
	DNH16	-1.3	**-1.9*****	ns	-4.01	-3.67	1.3	-2.97	-3.90	-2.0
	WCOR	2.3	**-1.8***	ns	-8.01	-7.61	1.3	-7.86	-6.16	3.2
	SOD	1.6	-1.8	ns	-6.03	-5.79	1.2	-6.07	-5.10	2.0
N metabolism	*GOGAT*	**-1.1****	1.2	*	-2.87a	-2.91b	**-1.0****	-3.36c	-3.60c	-1.2
	GS1	**-13.9***	-2.8	ns	-0.66a	-5.40b	**-26.8***	0.47a	-3.04b	**-11.4***
	GS2	1.3*	**2.3****	ns	-2.65	-2.16	1.4	-3.56b	-3.51a	**1.0***
C metabolism	*ATPase*	1.0	1.5	ns	0.60	0.69	1.1	-0.18	-0.13	1.0
	PK	1.4	-1.9	ns	-5.77	-5.66	1.1	-5.56	-4.77	1.7
	PEPC	1.1	-1.6	ns	-6.88	-7.14	0.8	-6.43	-6.13	1.2
	RBCL	**1.1***	1.1	ns	3.63a	2.95b	**-1.6*****	3.11b	3.91a	**1.7***
Aquaporin	*TIP 1.1*	**-1.5****	**3.2****	*	-2.94a	-3.29b	**-1.3***	-4.89c	-7.66d	**-6.8*****

3.5 Interaction network of physiological traits and gene expression

Four correlation matrices, with a total of 30 variables each, were generated for each combination between contrasting groups of genotypes (NR and R+) and yielding scenarios (HY, LY). Network analysis was performed using significant correlations between parameters based on Pearson correlation coefficients (-0.75>*r*>0.75) and *p* values ($p<0.05$) (Fig. 3).

In the NR lines under high-yielding conditions (Fig. 3A), there were positive relationships between the expression of stress responsive genes and other traits: (i) the expression of the transcription factor *DREB1* with the vegetation index (lightness), grain $\delta^{13}C$, and the expression of *GOGAT*, *PK* and *SOD* genes; (ii) the expression of dehydrin *DNH16* with N content of grains and the expression of *PK* and (iii) the expression of *WCOR* with that of *ATPase*. With regard to N metabolism, (i) the *GS1* expression correlated negatively with biomass (NDVI) and (ii) the expression of both *GS2* and GOGAT genes negatively with g_s. Concerning the C metabolism, the expression of *RBCL* correlated positively with the vegetation index u*, as well as the expression of *PEPC* with C content. Vegetation indices also correlated against yield and physiological traits; for example g_s correlated negatively with biomass greenness (GA), while Hue correlated positively against grain yield and negatively with chlorophyll content.

For the R+ lines under high-yielding scenario (Fig. 3B), *DREB1* expression was positively correlated with some vegetation indices (v* and b*), as well as with the expression of *DREB2*, *GOGAT* and *PEPC* genes. Both *GS1* and *GOGAT* expression were negatively associated grain C content, while the expression of *GS2* was positively correlated with that of *RBCL* and *DHN16* genes. *PEPC* expression was positively associated with *DREB2* and *GOGAT* expression as well as with some vegetation indices (v* and b*). *PK* and *PEPC* expressions were positively correlated with $\delta^{13}C$ and

CTD respectively, while the expression of *RBCL* was negatively related with g_s. *TIP1.1* expression was negatively related with GA and positively related with *DREB2* and *WCOR* expressions. Vegetation indices such as NDVI and chlorophyll content exhibited a negative correlation with SOD gene expression.

In NR lines under low-yielding conditions (Fig. 3C), the expression of *DREB2* was positively related with that of the *ATPase* gene, and negatively correlated with *GS1* and *DNH16* expressions. The amount of *WCOR* transcripts was positively correlated with the expression of *PEPC*, *NDVI* and GA, while the vegetation indices intensity and a* were negatively related. The *GS2* expression was negatively related with biomass (NDVI). *PEPC* expression correlated positively with the expression of *RBCL* as well as with the vegetation index lightness, while the expression of *PK* and *SOD* were positively associated each other. Grain $\delta^{13}C$ was negatively associated with yield and positively with TIP1.1 expression, while g_s was positively related to *DREB1* expression. Furthermore chlorophyll content and grain C content showed a positive correlation between them.

Last, for R+ lines under low-yielding conditions (Fig. 3D) grain yield was positively correlated with grain C and N content, and negatively related with the vegetation index intensity and the expression of the *GS2*. *DREB2* expression was positively correlated with g_s and negatively correlated with grain $\delta^{13}C$. *GS2* expression was positively associated with *TIP1.1* expression and negatively correlated with grain N and C content, while *GS1* expression was positively correlated with *PEPC* expression. Moreover PK expression and grain $\delta^{13}C$ were positively correlated.

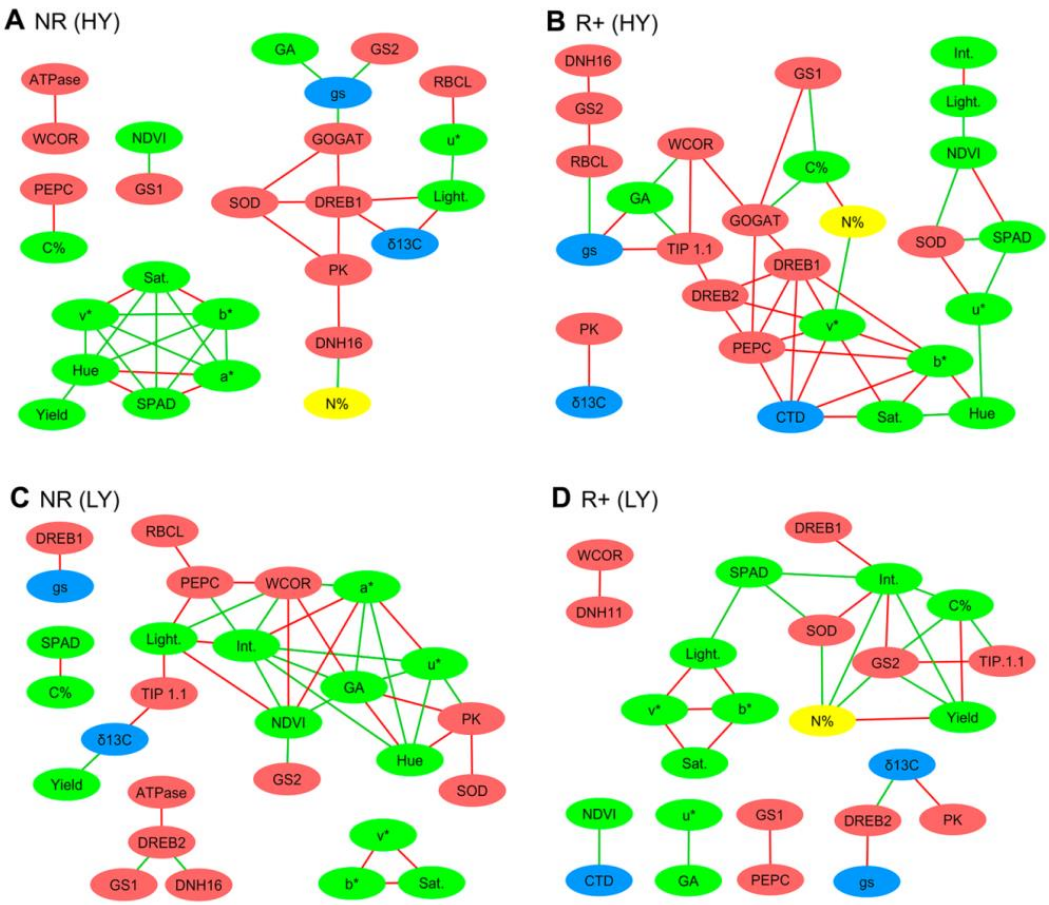


Figure 3. Network analysis of traits and transcript levels of very restrictive (R+) and non-restrictive (NR) durum wheat lines under (A, B) high (HY) and (C, D) low (LY) yield environments. Red nodes represent transcript levels, green nodes vegetation indices and carbon content, blue nodes water status traits and yellow nodes N content. The red and green edges represent significant positive and negative correlations (p<0.05), respectively, based on Pearson's correlation coefficients. For trait and transcript abbreviations see Tables 3 and 4.

4 Discussions

4.1 Transpiration response of wheat lines to increasing VPD

The ability to restrict water loss through the stomata, measured as the transpiration response to a variation in VPD, let us to classify 20 durum wheat lines in three significantly different groups (Table 2, Fig. 1): non-restrictive (NR), mid restrictive (R-) and very restrictive (R+) to water lose. The NR lines did not limit their transpiration as the VPD increased. A linear pattern in transpiration increase as response to rising VPD may be characteristic of elite wheat cultivars (in our case commercial cultivars) which may keep the stomata open as VPD increases (Schoppach and Sadok, 2012). Previous studies in wheat also reported genetic variability in the transpiration response (Schoppach and Sadok, 2012, 2013). However in contrast to these previous studies, differences in transpiration response to increase VPD were identified at relatively low VPD (around 1 kPa). Moreover the groups of genotypes, when classified following the protocol published for wheat (Schoppach and Sadok, 2012), as the slope of the increase in transpiration between around 1 and 4 kPa, did not exhibit any consistent difference in yield across the set of environmental conditions assayed. In addition we failed to find across the 20 commercial lines assayed a break in the linear pattern of increase in transpiration as VPD augmented above 1kPa (Schoppach and Sadok, 2012; Schoppach *et al.*, 2017). However the same authors have reported in other works (Schoppach and Sadok, 2013; Schoppach *et al.*, 2016) a linear pattern of increase in transpiration up to VPD values similar to those (ca. 4 kPa) of our study.

As pointed above in our study R- and R+ groups gattered the restriction at closely but significant different breakpoint values near to 1 KPa. These VPD values are clearly lower than those reported previously in wheat of 2 KPa (Schoppach *et al.*, 2017) or even higher (Schoppach *et al.*, 2012), including for genotypes selected for the Mediterranean conditions of Australia. However there are reports indicating

stomatal closure may already start at mild VPD values below 2 kPa (Choudhary *et al.*, 2013; Gholipoor *et al.*, 2013; Choudhary and Sinclair, 2014). The mechanism that causes stomatal closure at high VPD is not well understood (Streck, 2003).The feedforward hypothesis states that stomatal conductance decreases directly as VPD increases, with abscisic acid (ABA) in the leaves probably triggering the response (Bunce, 1996, 1998). The feedback hypothesis states that stomatal conductance decreases as VPD increases because of an increase in transpiration (E) that lowers the leaf water potential. Available results in wheat are not consistent with stomatal closure at high VPD caused by increased whole leaf transpiration rate or by lower leaf water potential. The lack of response of conductance to VPD in CO_2-free air suggests that ABA may mediate the response (Bunce, 1998). The sensitivity of conductance was approximately linearly related to VPD in wheat and barley (Bunce, 1998).

In wheat previous reports described variable adaptation strategies to maintain a stable photosynthetic surface while adjusting the water in response to transpiration demand (Schoppach and Sadok, 2012). Any negative relationship between transpiration rates and plant leaf area may suggest trade-off between these traits. Previous studies in wheat have reported either no relation (Schoppach *et al.,* 2017) or a negative relation (Schoppach and Sadok, 2013; Schoppach *et al.,* 2016). In our study we found a significant, but marginal, negative relationship between plant leaf area and leaf transpiration (Supplementary Fig. S3).

4.2 Effect of transpiration-response to VPD on crop performance under a range of growing field conditions

The nine growing conditions assayed represented a wide range of grain yields under Mediterranean conditions, going from severely stressed to near optimal conditions (Acreche *et al.*, 2008; Araus *et al.*, 2013). Such differences were associated with

water status as shown by the lower g_s and CTD, together with a higher (less negative) $\delta^{13}C$ of mature grains (Cabrera-Bosquet *et al.*, 2011) of the less productive trials, while the better trials exhibited larger and greener canopies as indicated by the differences in spectroradiometrical and RGB canopy vegetation indices like NDVI, Hue, lightness, a*, u*, v* and GA (Casadessús *et al.*, 2007; Elazab *et al.*, 2015)

The evaluation of the set of 20 modern (i.e. semidwarf) durum wheat cultivars under a wide range of environmental growing conditions in the field confirmed a clear different performance of the NR versus the R groups of genotypes in terms of grain yield. However the relative performance was strongly affected by the growing conditions. Thus in the trial with the lowest yield conditions, which were associated with water scarcity (Valladolid 2014 rainfed; the trial with the highest $\delta^{13}C$), R lines accounted for higher grain yield than NR lines. Moreover vegetation indices (GA, NDVI, SPAD), indicative of photosynthetic biomass and greenness (Casadessús *et al.*, 2007; Robertson *et al.*, 2016) also tended to be higher in R lines (Table 4), while $\delta^{13}C$ was higher (less negative) in R than in NR lines indicating a higher water use efficiency in the former (Farquhar and Richards, 1984). A better plant water status could favour N uptake, assimilation and its remobilization to the grains (Alva *et al.*, 2006; Hirel *et al.*, 2007) which would agree with the greater grain N content of R compared with NR lines. In the seven trials with higher yields, where water availability was greater, the NR lines showed superior yields than the R lines, together with higher green biomass (NDVI, lightness, a*, and u*) (Table 4, Fig. 2). Moreover in the most productive trial (Valladolid 2015 irrigation) $\delta^{13}C$ was more negative in the NR compared with the R group of genotypes. In that environment grain $\delta^{13}C$ correlated negatively with grain yield across the set of 20 genotypes (R^2 = -0.40, p <0.05, data not shown), indicating that the more negative the $\delta^{13}C$ the better the water status of the crop is (Araus *et al.*, 2003). Other studies under relatively good agronomical conditions have reported for wheat negative correlations of $\delta^{13}C$ with g_s and grain yield (Lu *et al.,* 1998; Fischer *et al.,* 1998). In fact it has been

reported that except for very drought prone environments genetic advance in yield of wheat and other species are related with a higher stomatal conductance (Roche, 2015 and references herein). However at the lowest-yield trial of our study (Valladolid 2014 rainfed), the correlation between grain $\delta^{13}C$ and yield was absent (data not shown), which suggest that a more open stomata attitude (and thus a lower $\delta^{13}C$) does not represent a positive trait under low -yield drought stressed scenarios (Araus *et al.*, 2003). Overall, the results show the existence of a genotype x environment interaction between NR and R group of lines. Thus NR lines yield more and exhibit higher biomass and greenness under good to optimal agronomical conditions while the R lines perform better under the most water limiting trial. The restrictive behaviour of R lines due to transpiration sensitivity to VPD fits with previous studies in other crop species about the water saving capacity of crops to enhance yield under severe water stress conditions (Kholová *et al.*, 2010*b*, 2012; Belko *et al.*, 2013; Vadez *et al.*, 2014). By contrast, under adequate water supply conditions, like those for wheat in the Mediterranean yielding five or more tonnes per hectare, lines with a non-restrictive behaviour yield more.

4.3 Integration of physiological traits and gene expression in response to restriction to water loss under contrasting yielding scenarios

Plant phenotype is based on the complex association of physiological responses driven by gene regulation, which also determines the growth and the crop productivity. The integration of transcript profiles for genes involved in the response to stress and C and N metabolism with physiological traits may help to understand the adaptation strategies for a given environment (Kosová *et al.*, 2014*b*). In our study the phenotype-environment interactions (Fig. 3) showed different patterns between HY and LY scenarios, as well as comparing R+ and NR groups, probably driven by the stress-responsive genes which influence the expression of the basal metabolism and water transport genes described in Table 5. Significant differences in transcript expression suggest a major role of transcription factors (*DREB1* and *DREB2*) and

dehydrins (*WCOR, DNH16*), influencing significantly the enzymes related to primary metabolism (*GOGAT, GS1, GS2* and *RBCL*) as well as the tonoplast aquaporins (*TIP 1.1*). *DREB1* and *DREB2* seem to be co-regulated in both groups NR and R+ with the water status traits (g_s, CTD and $\delta^{13}C$) (Fig 3 A, B, D). These two transcription factors seem to be principal cores in the integration of the plant responses to growing-conditions.

Regarding the two groups of genotypes, *DREB1* tended to be downregulated in R+ lines compared to NR, with differences being significant at HY (Table 5). The positive significant correlations of *DREB1* with genes involved in the N metabolism (*GOGAT*) and in the provision of carbon skeletons (*PK* and *PEPC*), and with traits informing on the water status ($\delta^{13}C$ and CTD) as well as the vegetation attributes (lightness and v* indices) (Fig 3 A, B, D), point out that *DREB1* may be a key regulator of metabolic signals in response to environmental conditions. It may drive the regulation of N remobilization and the provision of carbon skeletons for biomass development. Also *DREB1* was overexpressed in NR compared with R+ lines (Table 5). An overexpression of *DREB1* (may gather a protection signal from water scarcity as reported in wheat and other cereals (Zhao *et al.*, 2016), and probably influences positively the regulation of *GS1* as reported in relation to metabolic imbalances (Thomsen *et al.*, 2014).

In the case of *DREB2* it was up-regulated in R+ lines compared to NR, especially under stress conditions (Table 5). Previous studies in durum wheat have reported an increase in *DREB2* as response to water and salinity stresses (Yousfi *et al.*, 2016; Sheshadri *et al.*, 2016). *DREB2* close relationships with water status traits ($\delta^{13}C$ and g_s) and the *DNH16* dehydrin under low yield conditions (Fig. 3 D), support the fact the up-regulation in the R+ lines of this transcription factor helps in the adaptation of these lines to an inherent poorer water status. Such assumption is supported by previous reports of overexpression of *DREB2*-type (*TaDREB2, TaDREB3* and

TaDREB5) genes in low yielding wheat genotypes (Morran *et al.*, 2011; Shavrukov *et al.*, 2016) as well as by reports in soybean about their involvement in the response to drought stress (Engels *et al.*, 2013). Moreover *DREB2* may interact with genes involved in ABA signalling to drive root hydraulics and transpiration response, as identified by a major QTL on wheat (Schoppach *et al.*, 2016).

Dehydrins (*DNH16* and *WCOR*) expression was lower in LY respect to HY conditions, which contrast with previous reports of enhanced dehydrin signal under drought conditions (Rampino *et al.*, 2012). Nevertheless, the aquaporin gene family is large in cereals and show notable differential expression under stress and in different tissues (Hove *et al,* 2015). In HY the expression of DNH16 was positively related with the expression of genes encoding C (*PK*) and N (*GS2*) metabolism enzymes which suggest this dehydrin may be playing a protective role to assure the higher N assimilation (*GS2*) and carbon skeleton transformation (*PK*) (Fig. 3 A and B). Moreover our study suggests that this complex dehydrin response may be also driven by transcription factors; i.e the negative relation between *DNH16* and *DREB2* agrees with previous reports of (Kosová *et al.*, 2014*a*). Similarly WCOR overexpression, which was positively related with the expression of the gene encoding a basal metabolism enzyme (*ATPase*) (Fig. 3 A), agrees with previous reports of overexpression of *WCOR* genes in high yielding wheat and barley genotypes (Tsvetanov *et al.*, 2000), as well as the *WCOR* response to environmental changes involving regulatory function in stomatal opening (Danyluk *et al.*, 1996).

Concerning the N metabolism genes and comparing yield scenarios (Table 5), the transcripts levels of GS2 were significantly greater under LY conditions, reflecting the higher need of N assimilation in this unfavourable environment. Regarding the comparative between groups of genotypes, *GS1* and *GOGAT* genes were down-regulated in R+ compared to NR lines, with differences being more evident under low yielding conditions. This pattern suggests a lower N remobilization in the R+ lines

due to lower yield, in agreement with evidences in wheat reporting an increase of *GS1* transcripts to support N remobilization to the grains (Zhang *et al.*, 2017). Particularly the *GS1* overexpression in the flag leaves of NR lines compared to R+ lines under optimal conditions, it may suggest a better capacity of the former to remobilize N from leaves to the grains. In NR compared with R+ lines this is probably an indicator of a higher grain nitrogen yield (for example 180 versus 174 kg ha^{-1} at Valladolid support irrigation 2014 and 154 versus 144 kg ha^{-1} at Aranjuez support irrigation 2014, respectively) as well as of a higher nitrogen use efficiency as previously reported (Thomsen *et al.*, 2014; Tian *et al.*, 2015). Opposite to the other two genes, GS2 was upregulated in R+ lines, particularly under high yield conditions, which may indicate a need of N assimilation due to a clear inhibition of N remobilization and/or a low content of end-products.

RBCL was significantly and slightly over-expressed in R+ lines compared with NR lines under high yielding conditions, while the opposite occurred under low yielding conditions. The good agronomical conditions, which favour the NR compared with the R+ lines in terms of yield, may imply less need to increase the capacity for photosynthetic CO_2 fixation of the former and could benefit plant growth by diversifying the high amount of N invested in Rubisco. In that sense Rubisco upregulation in R+ lines was accompanied by overexpression of *GS2* as well as the downregulation of *GS1* and *GOGAT*, probably as a response to a higher demand of N supply to synthetize more Rubisco enzyme, which agrees previous reports about co-ordinated regulation of CO_2 fixation and N assimilation during grain filling in wheat (Nagy *et al.*, 2013; Komatsu *et al.*, 2014) and specially in durum wheat (Vicente *et al.*, 2015).

By other side, under low yield environments, the downregulation of the *RBCL* gene in R+ lines respect to NR, may be just the consequence of the better growing conditions of the former. A small decrease in Rubisco expression can lead to an improvement of

biomass and grain yield due to lower N allocation in Rubisco synthesis, and greater investment in other limiting processes, as described for rice (Kanno *et al.*, 2017). As a consequence nitrogen grain yield will probably also increase. This may be the case in R+ compared with NR lines (eg. 82 kg ha^{-1} versus 74 kg ha^{-1}, respectively, in Valladolid 2014 rainfed, the most stressed trial assayed). In the case of the NR lines, the upregulation of the large Rubisco subunit may be associated to a water scarcity signal. These results agree with studies in wheat leaves where ABA signal is gattered by the stress responsive genes resulting in higher Rubisco transcripts under water stress (Ashgari and Ebrahimzadeh, 2006; Budak *et al.*, 2013).

The aquaporin *TIP1.1* was significantly upregulated in LY compared to HY scenarios (Table 5), it agrees with the role of TIP1.1 favouring water channel activity under low water availability conditions (Tardieu *et al.*, 2014) and therefore cell rehydration (Willigen *et al.*, 2004). This aquaporin expression pattern, together with the upregulation of *GS2*, which also increases under LY compared with HY conditions (Table 5), may also favour the N assimilation.

After blast analysis, *TIP1.1* gene sequence in durum wheat was homologous to the same gene in barley, which has been experimentally tested to transport water, together with other potentially substrates such as urea and hydrogen peroxide (H_2O_2) (Hove *et al.*, 2015). Regardless the yield environment, *TIP1.1* gene was underexpressed in R+ compared to NR lines (Table 5), which supports a different mechanism of water transport between both groups of lines. The higher *TIP1.1* expression of NR compared with R+ lines may be associated with a higher stomatal conductance and transpiration, together with a lower $\delta^{13}C$, and eventually to a higher water use and grain yield of the formers under environments other than very severe drought conditions. Our results are in the line of a favourable role of aquaporins favouring plant water transport and photosynthesis (Moshelion *et al.*, 2015). Those strong differences in *TIP1.1* expression between NR and R+ groups

could have greatly influence water status, especially facilitating the water transport through cytosolic and vacuole compartments in NR lines and, subsequently, influencing plant metabolism (Vera-Estrella *et al.*, 2004; Forrest and Bhave, 2007).

Regardless the yield environment, *TIP 1.1* was underexpressed in R+ compared to NR lines (Table 5), which supports a different mechanism of water transport between both groups of lines. Notably, the NR plants expressed higher expression of aquaporin gene which may be associated with a higher stomatal conductance and transpiration, together with a lower $\delta^{13}C$, in NR compared with R+ lines and eventually to a higher water use and grain yield of the formers in environments other than very severe drought conditions. Our results are in the line of a favourable role of aquaporins favouring plant water transport and photosynthesis (Moshelion *et al.*, 2015). Those strong differences in TIP 1.1 expression between NR and R+ groups could have greatly influence water status, especially facilitating the water transport through cytosolic and vacuole compartments in NR lines and, subsequently, influencing plant metabolism (Vera-Estrella *et al.*, 2004; Forrest and Bhave, 2007).

An overview of a general physiological and transcriptional switches between R+ and NR lines (regardless the yielding environment) (Fig. 4) showed that higher transpiration capacity in NR lines appears to be associated with higher aquaporin expression, suggesting better water transport. Moreover NR group showed better biomass (NDVI) and greenness aspect (a* and v*), which besides a better water status may be also associated to a more efficient N remobilization by *GS1* overexpression; this is in line with the importance of GS enzymes for yield production (Martin *et al.*, 2006; Yousfi *et al.*, 2016). Moreover and except for the most stressed environments the NR lines exhibited not only higher productivity but also higher biomass and nitrogen grain yield. Also the over expression of *DREB2* in R+ lines may play a positive role at the leaf level under water stress conditions, in terms of increasing the chlorophyll levels and nitrogen assimilation and the photosynthetic

carbon fixation by Rubisco. Moreover, under scenarios of severe water stress *DREB2* may have a positive role limiting the water loss by the plant. That fits with a recent study in wheat where a major QTL was reported to control the transpirative to VPD in wheat (Schoppach *et al.*, 2016). This QTL harboured several genes involved in ABA signalling and its interaction with *DREB2A* and root hydraulics.

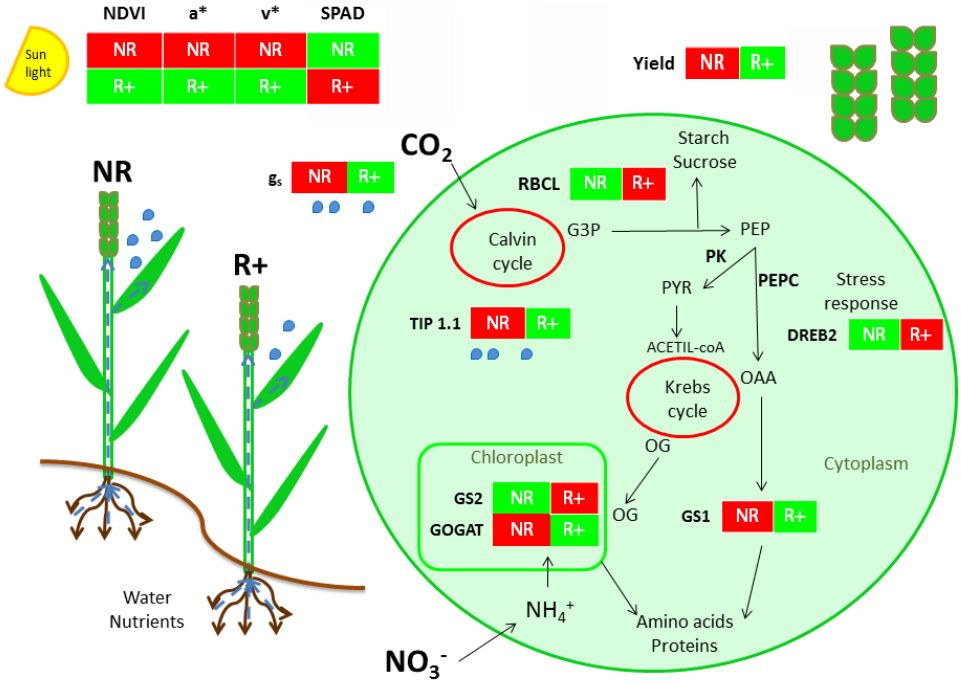


Figure 4. Overview of the changes in physiological traits and gene expression between very restrictive (R+) and non-restrictive (NR) durum wheat lines. The scheme shows the significant mean expression of better or up-regulated values (green) and means lower or down-regulation values (green) of significant traits evaluated in across all field trials and the integrated pathway of N and C metabolism.

Acetil-coA, Acetil co-enzyme A; CO_2, carbon dioxide; OG, 2-oxoglutarate; OAA, oxaloacetate; G3P, glyceraldehyde 3-phosphate; PEP, phosphoenol pyruvate; PYR, pyruvate; NH4+, ammonium and NO_3^-, nitrate. For trait and transcript descriptions see Tables 3 and 4.

5 Conclusions

This study provides evidence on how ability of wheat lines to restrict or not the transpiration may affect agronomical performance under a wide range of environmental conditions in the Mediterranean. Moreover the study highlights the complexity of physiological and molecular mechanisms associated with this different transpirative response to high VPD. The restrictive transpiration capacity is a successful strategy when water source is limited, whereas the non-restrictive transpiration capacity is applicable to wetter environments where NR genotypes exhibit larger biomass and produce higher yield. At the gene expression level both groups of lines are regulated by DREB transcription factors and dehydrins. However, the results suggest that the higher grain yields of NR lines is in line with a better water status of NR lines, associated with more active aquaporin, together with specific adaptations in carbon and nitrogen metabolism driven by regulation of genes encoding key enzymes. Also strong and significant association of vegetation indices with transcript abundances for dehydrin and C metabolism enzymes in NR lines revealed that a higher transpiration suggest better water transport of nutrients through water flux, driven by gene regulation to enhance yield gains. The negative but marginal correlation between plant leaf area and leaf transpiration suggest trade-offs between these traits are minor and supports further studies to explore the feasibility of this trait to select wheat genotypes better adapted to Mediterranean conditions.

Acknowledgements

This study was supported by the AGL2016-76527-R project from MINECO, Spain. SM was the recipient of a fellowship “Presidente de la República PRONABEC-III” from Peruvian Government. The authors thank the Unitat de Genòmica of the CCiTUB for their technical assistance.

6 References

Acreche MM, Briceño-Félix G, Sánchez JAM, Slafer GA. 2008. Physiological bases of genetic gains in Mediterranean bread wheat yield in Spain. European Journal of Agronomy 28, 162–170.

Alva AK, Paramasivam S, Fares A, Delgado JA, Mattos D, Sajwan K. 2006. Nitrogen and irrigation management practices to improve nitrogen uptake efficiency and minimize leaching losses. Journal of Crop Improvement 15, 369–420.

Araus JL, Cabrera-Bosquet L, Serret MD, Bort J, Nieto-Taladriz MT. 2013. Comparative performance of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ for phenotyping durum wheat adaptation to a dryland environment. Functional Plant Biology 40, 595.

Araus JL, Slafer G a., Royo C, Serret MD. 2008. Breeding for yield potential and stress adaptation in cereals. Critical Reviews in Plant Sciences 27, 377–412.

Araus JL, Villegas D, Aparicio N, del Moral LFG, El Hani S, Rharrabti Y, Ferrio JP, Royo C. 2003. Environmental factors determining carbon isotope discrimination and yield in durum wheat under mediterranean conditions. Crop Science 43, 170.

Ashgari R, Ebrahimzadeh H. 2006. Drought stress increases the expression of wheat leaf Ribulose - 1, 5-bisphosphate carbolylase/oxygenase protein. Iranian Journal of Science & Technology 30, 1–7.

Belko N, Zaman-Allah M, Diop NN, Cisse N, Zombre G, Ehlers JD, Vadez V. 2013. Restriction of transpiration rate under high vapour pressure deficit and non-limiting water conditions is important for terminal drought tolerance in cowpea. Plant Biology 15, 304–316.

Blum A. 2009. Effective use of water (EUW) and not water-use efficiency (WUE) is the target of crop yield improvement under drought stress. Field Crops Research 112, 119–123.

Budak H, Kantar M, Yucebilgili Kurtoglu K. 2013. Drought tolerance in modern and wild wheat. The Scientific World Journal 16, 1–16.

Cabrera-Bosquet L, Albrizio R, Nogués S, Araus J Luis. 2011. Dual $\Delta^{13}C/\delta^{18}O$ response to water and nitrogen availability and its relationship with yield in field-grown durum wheat. Plant, Cell and Environment 34, 418–433.

Casadessús J, Kaya Y, Bort J, et al. 2007. Using vegetation indices derived from conventional digital cameras as selection criteria for wheat breeding in water-limited environments. Annals of Applied Biology 150, 227–236.

Ceccarelli S, Grando S, Maatougui M. 2010. Plant breeding and climate changes. The Journal of Agricultural Science 148, 627–637.

Cheng L, Wang Y, He Q, Li H, Zhang X, Zhang F. 2016. Comparative proteomics illustrates the complexity of drought resistance mechanisms in two wheat (*Triticum aestivum* L.) cultivars under dehydration and rehydration. BMC Plant Biology 16, 1-23

Choudhary S, Mutava RN, Shekoofa A, Sinclair TR, Prasad PVV. 2013. Is the stay-green trait in sorghum a result of transpiration sensitivity to either soil drying or vapor pressure deficit? Crop Science 53, 2129–2134.

Choudhary S, Sinclair TR. 2014. Hydraulic conductance differences among sorghum genotypes to explain variation in restricted transpiration rates. Functional Plant Biology 41, 270–275.

Danyluk J, Carpentier E, Sarhan F. 1996. Identification and characterization of a low temperature regulated gene encoding an actin-binding protein from wheat. FEBS Lett 389, 324–327.

Devi MJ, Sinclair TR, Jain M, Gallo M. 2016. Leaf aquaporin transcript abundance in peanut genotypes diverging in expression of the limited-transpiration trait when subjected to differing vapor pressure deficits and aquaporin inhibitors. Physiologia Plantarum 156, 387–396.

Elazab A, Bort J, Zhou B, Serret MD, Nieto-Taladriz MT, Araus JL. 2015. The combined use of vegetation indices and stable isotopes to predict durum wheat grain yield under contrasting water conditions. Agricultural Water Management 158, 196–208.

Engels C, Fuganti-Pagliarini R, Marin SRR, Marcelino-Guimarães FC, Oliveira MCN, Kanamori N, Mizoi J, Nakashima K, Yamaguchi-Shinozaki K, Nepomuceno AL. 2013. Introduction of the *rd29A:AtDREB2A* ca gene into soybean (*Glycine max* L. Merril) and its molecular characterization in leaves and roots during dehydration. Genetics and Molecular Biology 36, 556–565.

Farquhar G, Richards RA. 1984. Isotopic composition of plant carbon correlates with Water-Use Efficiency of wheat genotypes. Australian Journal of Plant Physiology 11, 539–532.

Ficklin SP, Feltus FA. 2013. A systems-genetics approach and data mining tool to assist in the discovery of genes underlying complex traits in *Oryza sativa*. PLoS ONE 8. 1-13

Forrest KL, Bhave M. 2007. Major intrinsic proteins (MIPs) in plants: A complex gene family with major impacts on plant phenotype. Functional and Integrative Genomics 7, 263–289.

Gahlaut V, Jaiswal V, Kumar A, Gupta PK. 2016. Transcription factors involved in drought tolerance and their possible role in developing drought tolerant cultivars with emphasis on wheat (*Triticum aestivum* L.). Theoretical and Applied Genetics 129, 2019–2042.

Gholipoor M, Choudhary S, Sinclair TR, Messina CD, Cooper M. 2013. Transpiration response of maize hybrids to atmospheric vapour pressure deficit. Journal of Agronomy and Crop Science 199, 155–160.

Gholipoor M, Prasad PVV, Mutava RN, Sinclair TR. 2010. Genetic variability of transpiration response to vapor pressure deficit among sorghum genotypes. Field Crops Research 119, 85–90.

Habash DZ, Baudo M, Hindle M, et al. 2014. Systems responses to progressive water stress in durum wheat. PLoS ONE 9, 1–21.

Hirel B, Le Gouis J, Ney B, Gallais A. 2007. The challenge of improving nitrogen use efficiency in crop plants: Towards a more central role for genetic variability and

quantitative genetics within integrated approaches. Journal of Experimental Botany 58, 2369–2387.

Hoagland DR, Arnon DI. 1950. The water-culture method for growing plants without soil. California Agricultural Experiment Station Circular 347, 1–32.

Hove RM, Ziemann M, Bhave M. 2015. Identification and expression analysis of the barley (*Hordeum vulgare* L.) aquaporin gene family. PLoS ONE 10, 1-21

Hu H, Xiong L. 2014. Genetic engineering and breeding of drought-resistant crops. Annual Review of Plant Biology 65, 715–741.

Huseynova IM, Aliyeva DR, Mammadov AC, Aliyev JA. 2015. Hydrogen peroxide generation and antioxidant enzyme activities in the leaves and roots of wheat cultivars subjected to long-term soil drought stress. Photosynthesis Research 125, 279–289.

IGC. 2017. Cerelas Market report 2016-2017.

Kanno K, Suzuki Y, Makino A. 2017. A small decrease in rubisco content by individual suppression of *RBCS* genes leads to improvement of photosynthesis and greater biomass production in rice under conditions of elevated CO_2. Plant and Cell Physiology 58, 635–642.

Kholová J, Hash CT, Kakkera A, Koová M, Vadez V. 2010a. Constitutive water-conserving mechanisms are correlated with the terminal drought tolerance of pearl millet [*Pennisetum glaucum* (L.) R. Br.]. Journal of Experimental Botany 61, 369–377.

Kholová J, Hash CT, Kumar PL, Yadav RS, Koová M, Vadez V. 2010b. Terminal drought-tolerant pearl millet [*Pennisetum glaucum* (L.) R. Br.] have high leaf ABA and limit transpiration at high vapour pressure deficit. Journal of Experimental Botany 61, 1431–1440.

Kholová J, Nepolean T, Tom Hash C, Supriya A, Rajaram V, Senthilvel S, Kakkera A, Yadav R, Vadez V. 2012. Water saving traits co-map with a major terminal drought tolerance quantitative trait locus in pearl millet [*Pennisetum glaucum* (L.) R. Br.].

Molecular Breeding 30, 1337–1353.

Kholová J, Vadez V. 2013. Water extraction under terminal drought explains the genotypic differences in yield, not the anti-oxidant changes in leaves of pearl millet (*Pennisetum glaucum*). Functional Plant Biology 40, 44–53.

Komatsu S, Kamal AHM, Hossain Z. 2014. Wheat proteomics: proteome modulation and abiotic stress acclimation. Frontiers in Plant Science 5, 684.

Kosová K, Vitámvás P, Prášil IT. 2014a. Wheat and barley dehydrins under cold, drought, and salinity - what can *LEA-II* proteins tell us about plant stress response? Frontiers in Plant Science 5, 1–6.

Kosová K, Vítámvás P, Prášil IT. 2014b. Proteomics of stress responses in wheat and barley-search for potential protein markers of stress tolerance. Frontiers in plant science 5, 1–14.

Langridge P, Reynolds MP. 2015. Genomic tools to assist breeding for drought tolerance. Current Opinion in Biotechnology 32, 130–135.

Li Y, Ye W, Wang M, Yan X. 2009. Climate change and drought: a risk assessment of crop-yield impacts. Climate Research 39, 31–46.

Liu H, Able AJ, Able JA. 2017. Genotypic water-deficit stress responses in durum wheat: association between physiological traits, microRNA regulatory modules and yield components. Functional Plant Biology 44, 538–551.

Lobell DB, Roberts MJ, Schlenker W, Braun N, Little BB, Rejesus RM, Hammer GL. 2014. Greater Sensitivity to Drought Accompanies Maize Yield Increase in the U.S. Midwest. Science 344, 516–519.

Lopes MS, Araus JL, Van Heerden PDR, Foyer CH. 2011. Enhancing drought tolerance in C 4 crops. Journal of Experimental Botany 62, 3135–3153.

Martin A, Lee J, Kichey T, et al. 2006. Two cytosolic glutamine synthetase isoforms of maize are specifically involved in the control of grain production. The Plant Cell 18, 3252–3274.

Medina S, Vicente R, Amador A, Araus JL. 2016. Interactive effects of elevated [CO_2] and water stress on physiological traits and gene expression during vegetative growth in four durum wheat genotypes. Frontiers in Plant Science 7, 1–17.

Morran S, Eini O, Pyvovarenko T, Parent B, Singh R, Ismagul A, Eliby S, Shirley N, Langridge P, Lopato S. 2011. Improvement of stress tolerance of wheat and barley by modulation of expression of *DREB/CBF* factors. Plant Biotechnology Journal 9, 230–249.

Moshelion M, Halperin O, Wallach R, Oren R, Way DA. 2015. Role of aquaporins in determining transpiration and photosynthesis in water-stressed plants: Crop water-use efficiency, growth and yield. Plant, Cell and Environment 38, 1785–1793.

Mwadzingeni L, Shimelis H, Dube E, Laing MD, Tsilo TJ. 2016. Breeding wheat for drought tolerance: Progress and technologies. Journal of Integrative Agriculture 15, 935–943.

Nagy Z, Németh E, Guóth A, Bona L, Wodala B, Pécsváradi A. 2013. Metabolic indicators of drought stress tolerance in wheat: Glutamine synthetase isoenzymes and Rubisco. Plant Physiology and Biochemistry 67, 48–54.

Rampino P, Mita G, Fasano P, Borrelli GM, Aprile A, Dalessandro G, De Bellis L, Perrotta C. 2012. Novel durum wheat genes up-regulated in response to a combination of heat and drought stress. Plant Physiology and Biochemistry 56, 72–78.

Robertson M, Kirkegaard J, Rebetzke G, Llewellyn R, Wark T. 2016. Prospects for yield improvement in the Australian wheat industry: a perspective. Food and Energy Security 5, 1–16.

Roche D. 2015. Stomatal conductance is essential for higher yield potential of C 3 Crops. Critical Reviews in Plant Sciences 34, 429–453.

Salekdeh GH, Reynolds M, Bennett J, Boyer J. 2009. Conceptual framework for drought phenotyping during molecular breeding. Trends in Plant Science 14, 488–496.

Sanchez-Bragado R, Elazab A, Zhou B, Serret MD, Bort J, Nieto-Taladriz MT, Araus JL. 2014. Contribution of the ear and the flag leaf to grain filling in durum wheat inferred from the carbon isotope signature: Genotypic and growing conditions effects. Journal of Integrative Plant Biology 56, 444–454.

Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative CT method. Nature Protocols 3, 1101–1108.

Schoppach R, Fleury D, Sinclair TR, Sadok W. 2017. Transpiration sensitivity to evaporative demand across 120 years of breeding of australian wheat cultivars. Journal of Agronomy and Crop Science 203, 219–226.

Schoppach R, Sadok W. 2012. Differential sensitivities of transpiration to evaporative demand and soil water deficit among wheat elite cultivars indicate different strategies for drought tolerance. Environmental and Experimental Botany 84, 1–10.

Schoppach R, Sadok W. 2013. Transpiration sensitivities to evaporative demand and leaf areas vary with night and day warming regimes among wheat genotypes. Functional Plant Biology 40, 708–718.

Schoppach R, Taylor JD, Majerus E, Claverie E, Baumann U, Suchecki R, Fleury D, Sadok W. 2016. High resolution mapping of traits related to whole-plant transpiration under increasing evaporative demand in wheat. Journal of Experimental Botany 67, 2847–2860.

Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. 2003. Cytoscape: A software Environment for integrated models of biomolecular interaction networks. Genome Research 13, 2498–2504.

Sheshadri SA, Nishanth MJ, Simon B. 2016. Stress-mediated cis-lement transcription factor interactions interconnecting primary and specialized metabolism in planta. Frontiers in Plant Science 7, 1–23.

Streck N. 2003. Stomatal response to water vapor pressure deficit: an unsolved issue. Revista Brasileira de Agrociência 9, 317–322.

Talamè V, Ozturk NZ, Bohnert HJ, Tuberosa R. 2007. Barley transcript profiles under dehydration shock and drought stress treatments: A comparative analysis. Journal of Experimental Botany 58, 229–240.

Tardieu F, Granier C, Muller B. 2011. Water deficit and growth. Co-ordinating processes without an orchestrator? Current Opinion in Plant Biology 14, 283–289.

Tardieu F, Parent B, Caldeira CF, Welcker C. 2014. Genetic and physiological controls of growth under water deficit. Plant physiology 164, 1628–35.

Thomsen HC, Eriksson D, Møller IS, Schjoerring JK. 2014. Cytosolic glutamine synthetase: a target for improvement of crop nitrogen use efficiency? Trends in Plant Science 19, 656–663.

Tian H, Fu J, Drijber RA, Gao Y. 2015. Expression patterns of five genes involved in nitrogen metabolism in two winter wheat (*Triticum aestivum* L.) genotypes with high and low nitrogen utilization efficiencies. Journal of Cereal Science 61, 48–54.

Tsvetanov S, Atanassov A, Nakamura C. 2000. Gold responsive gene/protein families and cold/freezing tolerance in cereals. Biotechnology & Biotechnological Equipment 14, 3–11.

Vadez V, Kholova J, Medina S, Kakkera A, Anderberg H. 2014. Transpiration efficiency: New insights into an old story. Journal of Experimental Botany 65, 6141–6153.

Vera-Estrella R, Barkla BJ, Bohnert HJ, Pantoja O. 2004. Novel regulation of aquaporins during osmotic stress. Plant physiology 135, 2318–29.

Vergara-díaz O, Zaman-allah MA, Masuka B, Hornero A, Zarco-Tejada P, Prasanna BM, Cairns JE, Araus JL. 2016. A novel remote sensing approach for prediction of maize yield under different conditions of nitrogen fertilization. Frontiers in plant science 7, 1–13.

Vicente R, Pérez P, Martínez-Carrasco R, Usadel B, Kostadinova S, Morcuende R. 2015. Quantitative RT–PCR platform to measure transcript levels of C and N

metabolism-related genes in durum wheat: Transcript profiles in elevated [CO_2] and high temperature at different levels of N supply. Plant and Cell Physiology 56, 1556–1573.

Willigen C Vander, Pammenter NW, Mundree SG, Farrant JM. 2004. Mechanical stabilization of desiccated vegetative tissues of the resurrection grass Eragrostis nindensis: Does a *TIP 3;1* and/or compartmentalization of subcellular components and metabolites play a role? Journal of Experimental Botany 55, 651–661.

Yousfi S, Márquez AJ, Betti M, Araus JL, Serret MD. 2016. Gene expression and physiological responses to salinity and water stress of contrasting durum wheat genotypes. Journal of Integrative Plant Biology 58, 48–66.

Zadoks JC, Chang TT, Konzak CF. 1974. A decimal code for the growth stages of cereals. Weed Research 14, 415–421.

Zhang X, Liu S, Takano T. 2008. Overexpression of a mitochondrial ATP synthase small subunit gene (*AtMtATP6*) confers tolerance to several abiotic stresses in *Saccharomyces cerevisiae* and *Arabidopsis thaliana*. Biotechnology Letters 30, 1289–1294.

Zhang M, Ma D, Ma G, Wang C, Xie X, Kang G. 2017. Responses of glutamine synthetase activity and gene expression to nitrogen levels in winter wheat cultivars with different grain protein content. Journal of Cereal Science 74, 187–193.

Zhao P, Liu P, Yuan G, Jia J, Li X, Qi D, Chen S, Ma T, Liu G, Cheng L. 2016. New insights on drought stress response by global investigation of gene expression changes in sheepgrass (*Leymus chinensis*). Frontiers in plant science 7, 1-18.

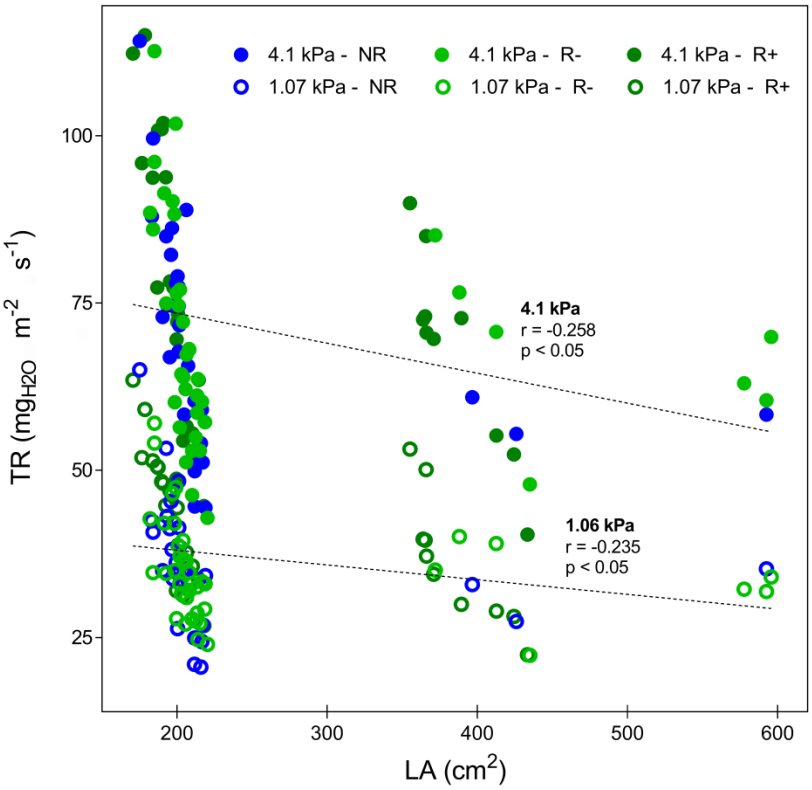
Supplementary Table S1. Primers for the housekeeping and target genes used for qRT-PCR analysis. Genes assayed and their names and sequence accession number are shown. The right-side column indicates the forward (F) and reverse (R) sequences of primers mentioned in Material and Methods section.

Gene	Name	Sequence 5´-3´
	Housekeeping genes	
18S	18S ribosomal subunit (M82356)	F: GGCCGCTCCTAGCCCTAATTG R: TGAGCACTCTAATTTCTTCAAAGTACG
UBI	Ubiquitin (Ta50503)	F: GCACCTTGGCGGACTACAACATTC R: GACACCGAAGACGAGACTTGTGAACC
	Target genes	
TIP 1.1	Aquaporin *TIP 1.1* (EU177566)	F: TGAGTTCCTTCTTCCTTCCTTCTTC R: TTTTTGCCCTGTCCTGTCGTAG
DREB1	Transcription factor *DREB1* (AF303376)	F: CACTCTCTTGGATGGTAGTGTCG R: GTGTATTCTCAGGTCCTCCTTTCC
DREB2	Transcription factor *DREB2B* (AB193608)	F: CTCTGAAACGATCAGGCGATGG R: GTGTATTCTCAGGTCCTCCTTTCC
SOD	Superoxide dismutase (KP696754)	F: GGGTGTGGCTAGCTTTGGAT R: TGCAGGTTTGACCCTTTGGT
WCOR	Actin-binding protein *WCOR719* (U58278)	F: TTCTTCATCCACTGGTCGCC R: GGAGCTGGCATACAGCATCT
GOGAT	Ferredoxin-dependent glutamate synthase (TC394038)	F: CGGCAATGGAGGCTGAGCAACA R: TGAGCCTGCTCGATGGTCACTGT
DHN16	Dehydrin *Td16* gen (X78429)	F:aCGAGGCCAAGCACAAGG R: TCTGCTTGGTCGTCTCCG
GS1	Cytosolic glutamine synthetase (DQ124209)	F:aAGGACGGCGGGTTCAA R: GCGATGTGCTCCTTGTGCTT
GS2	Chloroplastic glutamine synthetase (DQ124212)	F: GATGGAGGTTTCGACGTGAT R: CAAGTCAGGCGAAGTGAAA
PEPC	Phosphoenolpyruvate carboxylase (Y15897)	F: CAGACTGGCGAGCTCTTCTT R: GACGAAGCGTGGTTCTTGGA
PK	Pyruvate kinase (AK332778)	F: CCATGCTTGCCGATCCACGTCA R:aCGACAACGCGGTCATGCGA
ATPase	Chloroplastic ATP synthase β-subunit (M16843)	F: CCCTGCCCCTGCCACAACATTT R: GTTGCCAACGATCCGAGGCTGT
RBCL	Rubisco large subunit (KM668209.1)	F: CGTGCTCTACGTTTGGAGGA R: TTGGATACCATGAGGCGGG

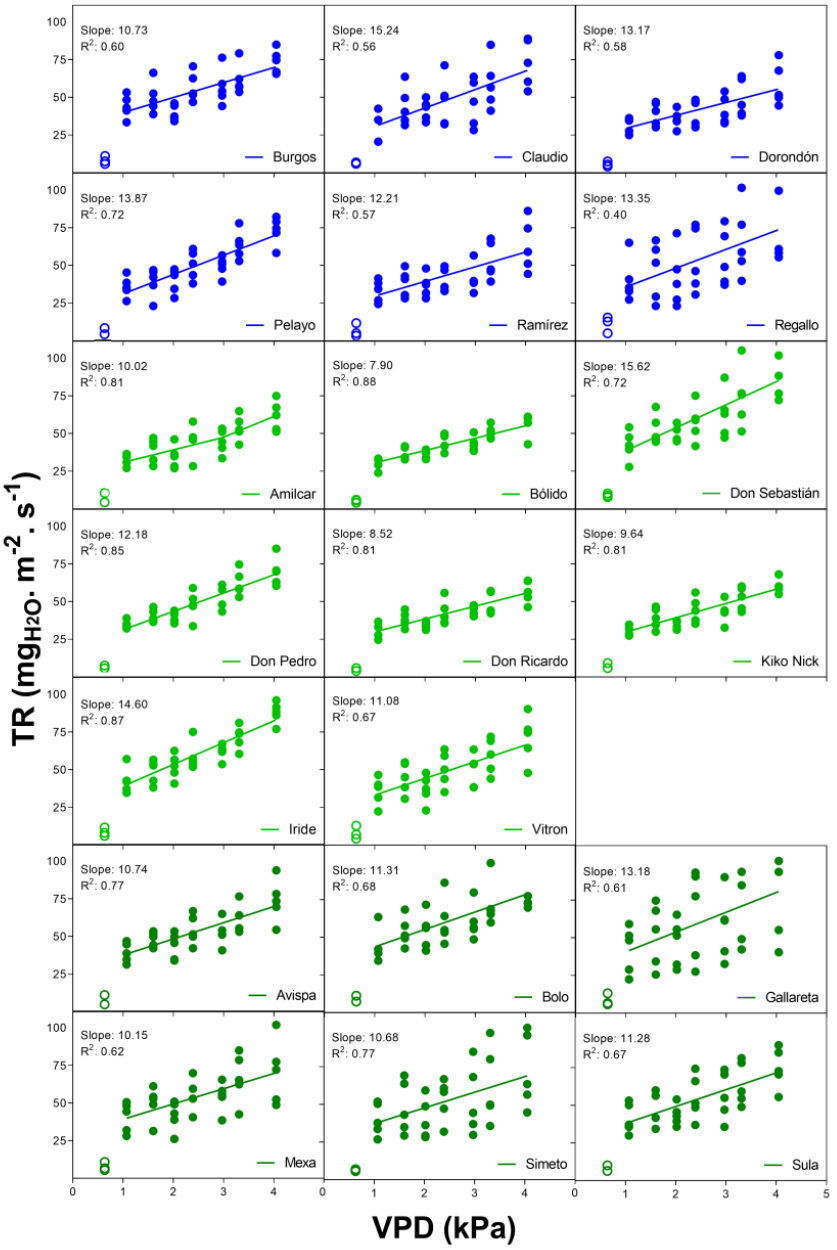
Supplementary Table S2. Linear adjustment of transpiration response (TR) to a variation in vapor pressure deficit (VPD) of 20 durum wheat lines. The lines were fitted to linear regressions (p<0.001) for the transpiration response to a changes in VPD between 1.07 kPa and 4.1 kPa. The values represent the mean of five biological replications. The parameters evaluated are the slope of TR vs. VPD with its R^2 of the fitting curve, and the TR at 1.07kPa, 2.02 kPa and 4.1 kPa. In the bottom is shown the average comparison between non-restrictive (NR) and restrictive (R- and R+) lines according LSD test (p<0.05). The TR is expressed as $mg_{H2O}\ m^{-2}\ s^{-1}$ and the slope in $mg_{H2O}\ m^{-2}\ s^{-1}\ kPa^{-1}$.

	Class	*Line*	*Slope*	*R^2*	*TR (1.07 kPa)*	*TR (2.02 kPa)*	*TR (4.1 kPa)*
Non-restrictive (NR)	NR	Burgos	10.73	0.604	43.90	39.66	73.93
	NR	Claudio	15.24	0.565	30.81	41.82	72.84
	NR	Dorondón	13.17	0.576	29.68	35.49	58.35
	NR	Pelayo	13.87	0.718	35.87	39.94	73.17
	NR	Ramírez	12.21	0.575	33.02	36.73	63.08
	NR	Regallo	13.35	0.403	40.27	41.42	77.70
Restrictive (R)	R -	Amilcar	10.02	0.814	31.21	34.54	61.71
	R -	Bólido	7.90	0.881	30.43	36.39	55.75
	R -	Don Ricardo	8.52	0.811	31.05	33.89	56.64
	R -	Don Pedro	12.18	0.855	34.45	38.98	69.84
	R -	Don Sebastián	15.62	0.718	42.17	49.08	90.30
	R -	Iride	14.60	0.872	42.74	52.12	87.82
	R -	Kiko Nick	9.64	0.814	31.57	34.99	61.97
	R -	Vitron	11.08	0.669	35.83	37.08	70.74
	R +	Avispa	10.74	0.771	39.30	43.54	73.88
	R +	Bolo	11.31	0.685	43.87	54.31	80.96
	R +	Gallareta	13.18	0.614	42.03	46.69	81.07
	R +	Mexa	10.15	0.62	40.64	41.64	70.50
	R +	Simeto	10.68	0.773	40.09	40.39	72.29
	R +	Sula	11.28	0.676	41.22	43.48	74.76
Non-restrictive	**NR**		**13.09 a**		**35.59 b**	**39.18 b**	**69.85 a**
Restrictive		**R-**	**11.20 ab**		**34.93 b**	**39.64 b**	**69.34 a**
		R+	**11.22 b**		**41.19 a**	**45.01 a**	**75.58 a**

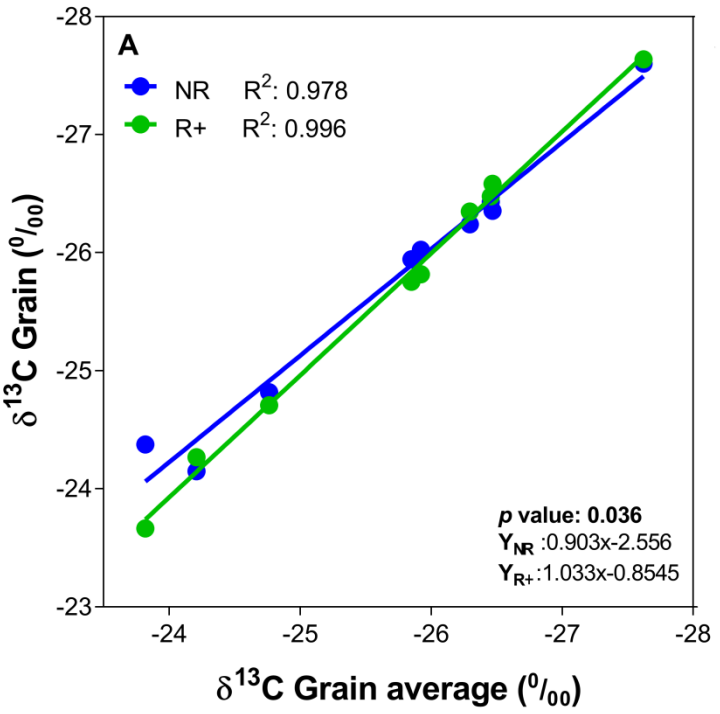
Supplementary Figure S1. Correlations between transpiration rate (TR) and the plant leaf area (LA) for the set of of 20 durum wheat lines. Pearson correlations were calculated at VPD values of 1.07 kPa (open circles) and 4.1 kPa (filled circles). Correlation coefficient and level of significance are included for each comparison.



Supplementary Figure S2. Transpiration rate (TR) of each of 20 durum wheat lines exposed to increasing VPD from 0.6 kPa to 4.1 kPa. Each curve expresses the linear regression between TR and VPD values in a range of 1.07 kPa to 4.1 kPa (full circles), and the TR values at 0.6 kPa (empty circles) for each durum wheat line. Plants were tested at the vegetative stage and values represent each biological replicate. All panels show the mean slope of the linear regression and the R^2 value.



Supplementary Figure S3. Relationship between grain carbon isotope composition ($\delta^{13}C$) and grain yield between very restrictive (R+) and the non-restrictive (NR) genotypes. The graph shows the linear regressions of the average grain $\delta^{13}C$ of NR and R+ groups versus the average $\delta^{13}C$ value for the whole set of 20 lines evaluated in each one of the nine field scenarios. The fitting curves on the figure were significant (p<0.001), Level of significance (p), between fitting lines as well as the determination coefficient (R^2) and the equation of each line are also indicated.



Chapter 4

Transpiration response and growth in pearl millet parental lines and hybrids bred for contrasting rainfall environments.

Respuesta de la transpiración y crecimiento en líneas parentales e híbridos de mijo mejorados para ambientes con precipitaciones contrastadas.

Susan Medina[1,2], S K Gupta[1] and Vincent Vadez[1*].

[1]International Crops Research Institute for semi-arid Tropics (ICRISAT), Crop Physiology Laboratory, Patancheru 502324, Greater Hyderabad, Andra Pradesh, India.

[2]Integrative Crop Ecophysiology Group, Plant Physiology Section, Faculty of Biology, University of Barcelona, Barcelona, Spain.

Article submitted to the journal / artículo enviado a la revista: Frontiers in Plant Science, May 2017

Transpiration response and growth in pearl millet parental lines and hybrids bred for contrasting rainfall environments.

Susan Medina[1,2], S K Gupta[1] and Vincent Vadez[1*].

[1]International Crops Research Institute for semi-arid Tropics (ICRISAT), Crop Physiology Laboratory, Patancheru 502324, Greater Hyderabad, Andra Pradesh, India.

[2]Integrative Crop Ecophysiology Group, Plant Physiology Section, Faculty of Biology, University of Barcelona, Barcelona, Spain.

*Corresponding author:

Vincent Vadez

International Crops Research Institute for semi-arid Tropics (ICRISAT), Crop Physiology Laboratory, Patancheru 502324, Greater Hyderabad, Telangana, india.

E-mail: V.VADEZ@cgiar.org

Type of article: Original Research Article

Number of figures: 5 (5 in color)
Figure 1 (1.5 columns)
Figure 2 (single column)
Figure 3 (1.5 columns)
Figure 4 (2 columns)
Figure 5 (1.5 columns)

Number of tables: 7 (0 in color)
Supplementary material: 1 figure and 3 tables
Figure 1S (2 column)

ABSTRACT

Under conditions of high vapour pressure deficit (VPD) and soil drying, restricting transpiration is an important avenue to gain efficiency in water use. The question we raise in this article is whether breeding for agro-ecological environments that differ for the rainfall have selected for traits that control plant water use. These are measured in pearl millet materials bred for zones varying in rainfall (8 combinations of parent and F_1hybrids, 18 F1-hybrids and then 40 F_1-hybrids). In all cases, we found an agro-ecological variation in the slope of the transpiration response to increasing VPD, and parental line variation in the transpiration response to soil drying within hybrids/parent combinations. The hybrids adapted to lower rainfall had higher transpiration response curves than those from the highest rainfall zones, but showed no variation in how transpiration responded to soil drying. The genotypes bred for lower rainfall zones showed lower leaf area, dry matter, thicker leaves, root development, and exudation, than the ones bred for high rainfall zone when grown in the low VPD environment of the greenhouse, but there was no difference in their root length neither on the root/shoot index in these genotypes. By contrast, when grown under high VPD conditions outdoors, the lower rainfall hybrids had the highest leaf, tiller and biomass development. Finally, under soil drying the genotypes from the lower rainfall accumulated less biomass than the ones from higher rainfall zone, and so did the parental lines compared to the hybrids. These differences in the transpiration response and growth clearly showed that breeding for different agro-ecological zones also bred for different genotype strategies in relation to traits related to plant water use.

Keywords: Adaptation, environment, rainfall, Pearl millet, VPD response, FTSW threshold, adaptation-environment variations, leaf development, growth.

HIGHLIGHTS

- Variation in transpiration response reflected breeding for agro-ecological zones
- Different growth strategies depended on the environmental conditions
- Different ideotypes reflected rainfall levels in specific agro-ecological zones

1 Introduction

Crops must enhance their productivity with less available water. The tolerance, or fitness, of a particular genotype to water limitations depends on its ability to match its water requirements to the water supply in specific environments (Vadez *et al.*, 2013*b*). Next to adapting the phenology and crop duration to fit water availability, genotypes with different canopy sizes are expected to have different water demands. Restricting water use by stomatal control is another avenue to fit water demand to water supply, although it may lead to either water being lost through evaporation or a lost opportunity for carbon fixation, suggesting that water saving is not a one-fit-all strategy. Therefore, understanding and analysing traits that contribute to crop fitness to specific stress environment, especially those that control plant water use, is a prerequisite to breed adapted cultivars to specific environments. The hypothesis of that paper is that some of these traits could have been influenced by the breeding history.

Pearl Millet (*Pennisetum glaucum L.)* is the second most important crop in India, this cereal is able to grow in the most arid zones, and its cultivation is being developed in the north arid and semi-arid regions of this country, these agro-ecological zones varying principally in the rainfall level. The *Lower rainfall zone* is located in Northern India, it is known as A1 zone (most arid zone or primary zone) and cover the territories of Western part of Rajasthan, and parts the states of Haryana and Gujarat, it has an annual rainfall level between 320-400 mm; its soil composition is sand and entysol (59%). On the other hand the *Higher rainfall zones* (A and B, being less arid than zone A1) are located in either the northern-central part of India or peninsular

India. The A zone (secondary zone) comprises the northern and north western part of India including the eastern Rajasthan and parts of Haryana, Gujarat and Uttar Pradesh; It has an annual rainfall level near to 400 mm with fine sand and entysol (31%) soil composition accounting low organic matter content. The B zone (tertiary zone) comprises the Peninsular Indian states of Maharashtra, Tamil Nadu and Karnataka; it annual rainfall level is among 400-520 mm and has heavy soil composition as entysol (28%) and alfisol (26%) (Manga and Kumar, 2011; Rai *et al.*, 2015). In effect those differences between soil profile and rainfall intensity and distribution in both zones may cause an effect on the crop adaptation and its breeding history.

Restricting transpiration under conditions of high evaporative demand is an important avenue to gain in efficiency of water use. During the last decade or so large genotypic variation in the restriction of water loss under high VPD has been found in different crop species (Reviewed in Vadez et al., 2014). How much the VPD-response depends on the environment where genotype/cultivars have evolved or for which they have been bred, is unknown. This trait is important because it leads to improved transpiration efficiency (TE). A restricted transpiration (lower TR) under high VPD in drought environments resulted in the increment of yield (Gholipoor *et al.*, 2010); Aparna et al., under review). This trait is also hypothesized to be explained by differences in the hydraulic characteristics of the plant.

Another option for controlling water use is for plants exposed to progressive water stress to reduce transpiration at high soil moisture levels, expressed as the fraction of transpirable soil water (FTSW) remaining in the soil. The genotypes that are more sensitive to soil drying initiate the stomatal closure at higher soil water content, which contributes to conserving soil water (Sinclair and Rufty, 2012; Vadez *et al.*, 2014) . This genetic variability in this response has been observed in cereals like pearl millet (Kholová *et al.*, 2010*b*), sorghum (Gholipoor *et al.*, 2012; Choudhary *et al.*, 2013) and also in legumes like chickpea (Zaman-Allah *et al.*, 2011) or groundnut (Devi et al., 2010). Henceforth, those two aspects mentioned above, the sensitivity of the

stomata under high VPD and soil drying, both contribute to a better conservation of soil water and may contribute to enhanced yields in scenarios with limited water (Sinclair, 2012). They are also supposed to enhance TE (Vadez *et al.*, 2014).

Therefore, the aim of this investigation was to assess different traits controlling plant water use in hybrids that were bred specifically for agro-ecological zones with different rainfalls. Specifically, the transpiration response to increasing VPD and possible mechanisms explaining it, transpiration response to soil drying, and the leaf canopy development, were assessed. A comparison was also made of these traits between the hybrids and their parental lines.

2 Materials and Method

2.1 Genetic material and location.

The genotypes collection of pearl millet (*Pennisetum glaucum L.)* had been bred in two agro-ecological scenarios: lower and higher rainfall zones of India. We assessed in total 22 genotypes developed in Zone A1 (higher rainfall), 19 genotypes in Zone A, and 18 in Zone B (lower rainfall) among three experiments (see table 1). In addition, 8 of these hybrids (4 from A1, 2 from A, and 2 from B zones) along with parental R- and B-lines were compared.

The first glasshouse experiment (Exp.1) was an assessment of the Transpiration rate response (TR) to increasing evaporative demand (vapour pressure deficit, VPD) and to soil drying response. This experiment was conducted in 2014 with (24 genotypes), i.e. 8 combinations of F_1 hybrids with their parental B line (male sterile) and R line (restorer); 4 combinations were bred for the lower rainfall zone (A1), and other 4 combinations were bred for the higher rainfall zones with half of them for zone A and the other half for zone B (Table 1). In the same way during 2015, a second experiment in glasshouse (Exp.2) of transpiration rate response to evaporative demand was conducted with 18 F_1 hybrids: 6 were bred for the lower rainfall zone

(A1) and 12 were bred for the zones A and B (see table 1). Furthermore, two additional experiment (Exp.3 and Exp.4) were conducted outdoors in 2015 and 2016 respectively, at the Leasyscan facility (Vadez et al., 2015) at ICRISAT with a larger number of F_1 hybrids (40 genotypes): 14 of them were bred for the lower rainfall zone (A1) and 26 were bred for the higher rainfall zones: 13 belonged to zone A and other 13 to zone B (Table 1). In Exp.3 the transpiration response to VPD was assessed in the LeasyScan platform under natural VPD increases. The purpose of Exp.3 and Exp.4 were to compare the canopy development of these hybrids, along with an assessment of the transpiration rate to natural increase in VPD (Exp.3). In Exp.3, the daily average temperature and relative humidity (RH) range was 22-28°C and 34-84 % respectively, while in Exp.4 the temperature and RH range was 26-31°C and 30-69%, respectively. All the experiments were conducted during February-April season of 2014 and 2015 at the ICRISAT campus in Patancheru (India): latitude 17°30´N; longitude 78°16´E; altitude 549m.

Table 1. Lists of pearl Millet parental (B line and R line) and F_1 hybrids tested in the transpiration response to VPD (Vapour Pressure Deficit) and Soil drying experiments. **Higher (A1) and lower (A and B) rainfall zones.**

Response to high VPD (Exp.1) and progressive soil drying (F_1 hybrids, B line and R line)			**Response to high VPD (Exp.2) (F_1 hybrids)**		**Response to high VPD and growth outdoors (Exp.3 and Exp.4) (F_1 hybrids)**			
Genotype	Class	Zone	Genotype	Zone	Genotype	Zone	Genotype	Zone
HOPE 2013-AHT-R-8	F_1	A1	HOPE-2014 AHT-R-15	A1	HOPE-2014 AHT-R-1	A1	AHT A/K14-2	A
96666 B	B	A1	HOPE-2014 AHT-R-7	A1	HOPE-2014 AHT-R-9	A1	AHT A/K14-3	A
RIB 3135/18	R	A1	HOPE-2014 AHT-R-11	A1	HOPE-2014 AHT-R-14	A1	IHT A2 /K14-24	A
HOPE-2013 AHT-R-14	F_1	A1	HOPE 2013-AHT-R-8	A1	HOPE-2014 AHT-R-8	A1	AHT B/K14-22	A
843-22 B	B	A1	HOPE-2013 AHT-R-14	A1	HOPE-2014 AHT-R-16	A1	EMTT /K14-10	A
MRC S1-97-3-4-B-B-1-B-1-B	R	A1	HHB 67 imp	A1	HOPE-2014 AHT-R-17	A1	IHT A1 /K14-4	A
HOPE-2013 AHT-R-18	F_1	A1	AHT II/K14-7	A	HOPE-2014 AHT-R-4	A1	IHT A2 /K14-13	A
(EERC-HS-29)-B-13-4-5-2	B	A1	AHT A/K14-5	A	HOPE-2014 AHT-R-15	A1	IHT B1 /K14-5	B
88004 B	R	A1	AHT II/K14-9	A	HOPE-2014 AHT-R-7	A1	IHT B1 /K14-20	B
HHB 67 imp	F_1	A1	IHT A2 /K14-24	A	HOPE-2014 AHT-R-11	A1	AHT II/K14-14	B
843-22 B	B	A1	AHT A/K13-4	A	HOPE 2013-AHT-R-8	A1	AHT B/K14-20	B
H77/833-2-202	R	A1	AHT A/K13-5	A	HOPE-2013 AHT-R-14	A1	AHT II/K14-11	B
AHT A/K13-4	F_1	A	IHT B1 /K14-20	B	HOPE-2013 AHT-R-18	A1	AHT II/K14-20	B
ICMB 97222	B	A	AHT II/K14-20	B	HHB 67 imp	A1	IHT B1 /K14-10	B
MRC HS-130-2-2-1-B-B-3-B-B-B-1-3-1	R	A	IHT B1 /K14-10	B	AHT II/K14-7	A	IHT B1 /K14-26	B
AHT A/K13-5	F_1	A	AHT-II/K13-5	B	AEHT /K14-2	A	AHT II/K13-18	B
ICMB 04222	B	A	ICMH 1201	B	AHT II/K14-8	A	AHT II/K14-5	B
JBV 3 S1 -237-1-3-3-1-B	R	A	AHT-II/K13-24	B	AEHT /K14-18	A	AHT II/K13-5	B
AHT-II/K13-5	F_1	B			AHT A/K14-5	A	AHT II/K13-6	B
ICMB 99222	B	B			AHT II/K14-9	A	ICMH 1201	B
ICMV 96490-S1-15-1-2-1-1	R	B						
AHT-II/K13-24	F_1	B						
ICMB 98222	B	B						
(MC 94 C2-S1-3-2-2-2-1-3-B-B xAIMP 92901 S1-488-2-1-1-4-B-B)-B-2-2-2	R	B						

2.2 Transpiration response to Vapour pressure deficit (VPD) in controlled conditions

Exp.1 and Exp.2 were carried out in controlled conditions, with 5 biological replicates per genotype (n_1=120 and n_2=90). All plants were sown in 8 Kg pots filled with red soil and grown in glasshouse (17-35°C/ 65-35 %RH). Ten to fifteen days after sowing, each pot was thinned to a single plant. The pots were watered every 1-3 days with soft water and plants were grown for 30 days before the experiment started (Vegetative stage: Zadocks scale 24-26, depending of each genotype). One day before the TR experiment, all pots were watered and allowed to drain overnight to reach soil capacity in the pot; the following morning each pot was covered with a plastic sheet and a layer of plastic beads to minimize soil evapotranspiration. After that, the pots were transferred to a Conviron E-15 (Controlled Environments, Winnipeg, MB, Canada) growth chamber for acclimatization. The next day, the TR response to high VPD was performed in the chamber by exposing the plants organized in a complete randomized design to a controlled ladder of increasing VPD, applied by changing both temperature and humidity every hour from 7 am (23 °C/ 80 %RH) up to 4 pm (40 °C/ 45 %RH), at a constant light flux of ~450 µmoles. m^{-2}. s^{-1}. Plant transpiration was measured by weighing pots every hour in a bench electronic 10 Kg balance with a resolution of 0.1g (FBK, Kern & Sohn GmbH, Balingen, Germany), giving one transpiration value per plant at each VPD point. To avoid the plant size variation, in each plant the transpiration was normalized by its leaf area. After the last recorded weight, the plants were harvested by cutting the stem above 2 cm of the soil level, and the xylem exudate was collected immediately in 11-mL pre-weighted tubes containing cotton inside during 20 minutes, after that the tubes were closed and their weight was recorded. Subsequent, the leaf area was measured with a leaf area meter (LA meter LI3000 model, Li-Cor, Licoln, Nebraska, US), and finally the stem and leaves were dried at 60°C in an oven during 72 hours. The following day, the roots were carefully washed and the measurement of root length

was conducted using the scanning equipment and imaging software WinRizho (WinRizho ^{TM} Pro, Regent Instruments Inc., Quebec City, Canada).

2.3 Transpiration response to evaporative demand outdoors

A transpiration rate response to naturally increasing VPD conditions was performed outdoors during February-March 2015 (Exp.3). This period of the year is known to enjoy high temperature and low RH%, giving a high VPD condition. Six biological replicates per genotypes (n_3=240) and additional 6 pots without plant to estimate the evapotranspiration of bare soil; In brief, the platform is a laser scanner-based technique (PlantEye F300, Phenospex, Heerlen, The Netherlands) providing 3D point clouds from which plant parameters, including leaf area, are measured every two hours (Vadez et al., 2015). The temperature and RH (20-39° / 20-70 RH% range) were recorded each 30 minutes (Campbell Scientific, Logan, Utah, USA). The seeds were sown in 15 Kg pots filled with red soil; twelve days after, each pot was thinned leaving two plants per pot. One experimental unit consisted of two such pots, i.e. 4 plants per experimental unit. The pots were automatically watered every 1-3 day with soft water, the plants were grown for 34 days before the experiment started (vegetative stage: Zadocks scale 28-30, depending of each genotype). The day before the transpiration assessment, each pot was over-watered with 1L of soft water by the afternoon and let for drainage overnight. The transpiration assay was carried out over two consecutive days, by weighing each pot in an electrical 20Kg balance with a resolution of 0.1g (FBK, Kern & Sohn GmbH, Balingen, Germany) at three time points during the day: 6:30 am, 10:00 am and 3:00 pm. After the last weighing of the afternoon of the first day, all the plants were watered with 1L of soft water again, drained overnight and the next day the same weighing procedure was repeated. At the end of the second day all plants were harvested and dried during 72 hours at 60°C in an oven similarly to the experiments described above. The environmental temperature range was 21.8-39.4 °C and the relative humidity range was 21-67 RH%, leading to a range of VPD values of 0.8-5.9 KPa during the time frame of the

experiment. Based on this, the transpiration recorded between the first two time points (6:30 am (0.8 KPa)-10:00 am (3.4 KPa) was considered to correspond to a low to mild VPD period, whereas the transpiration in the second period (until 3:00 pm (5.3 KPa) was considered to take place during high VPD conditions. The leaf area 3d data was extracted from the platform data base to calculate the transpiration rate (unit) for each day of experiment. The relationship between the measured and scanned leaf area was validated with the reported transformation LA3d= 0.22LA+ 241 (Vadez *et al.*, 2015), where y is the 3D leaf area (the area measured by the scanner) and LA was the observed leaf area measured with Li 3000 leaf area meter. Later the transpiration rate was calculated after estimating the soil evaporation from the non-sown pots. To do so, it was considered that soil evaporation was maximum at a leaf area index (LAI) of zero, and nil at a LAI of 2. In between these boundary LAI values, soil evaporation was considered to be proportional to the LAI. Transpiration rate was then calculated by dividing transpiration values by the leaf area. To fit the data of TR and VPD levels, we applied a linear regression, and then the slopes were compared among the genotypes. The growth outdoors (Exp.3 and Exp.4) was evaluated with LA3d and plant height data generated by the phenospex platform. Temperature data were used to convert days after sowing data into equivalent days at 20°C to compare growth curves between both seasons, following earlier work (Parent and Tardieu, 2012).

2.4 Transpiration response to soil drying

The dry-down experiment was conducted in the glasshouse with semi-regulated temperature and humidity (17-35°C/ 65-35 %RH) during February-March 2014 with the same plant material used in Exp.1 with 10 biological replicates for each genotype (n_4=240). The seeds were sown in 8 Kg pots filled with Alfisol, after 10-12 days all pots were thinned to one single plant per pot. The plants grew under fully irrigated conditions during 30 days (Zadock stage: 26-32, depending on the genotype).

The afternoon before the dry-down started all pots were irrigated with soft water to soil capacity, let to drain overnight and covered with a plastic sheet and a layer of plastic beads to avoid water loss by evaporation. The next morning all pots were weighed and this measure was recorded as the initial weight at field capacity. Then 5 replicates of each genotype were assigned to a well-watered treatment (WW), in which transpiration was replenished every day; the other 5 replicates were assigned to water-deficit treatment (WS) with an irrigation regime that allowed a maximal transpiration water loss on each day, by replenishing water in excess of this allowed maximum. This procedure allowed similar kinetics of stress imposition to plants varying in size. All pots were weighed every morning (10:00 am), their daily transpiration was calculated, and each pot was irrigated according to its water regime. This procedure was maintained until the transpiration of the WS plants fell below 10% of that in their WW controls. Then, plants were harvested, the leaf area was measured in the WW plants, and dry weight measured after drying samples in an oven at 60°C during 72 hours.

The Fraction of Transpirable Soil Water (FTSW), as a soil water stress indicator, and the Normalized Transpiration Ratio (NTR) were calculated. First, the transpiration ratio (TR) of all plants was calculated by dividing each transpiration value by the mean of the transpiration of the WW plants, within each genotype. Then, to avoid variations on individual plant size a second normalization consisted of dividing TR values by an average of the TR obtained during the first 5 days, i.e. before any water stress occurred. Therefore, NTR values were centred on 1.0 during the well-watered period before the stress started in the soil, and then started decreasing from 1.0 when stress started. The drydown was over for a given genotype when the NTR value fell below 0.1, i.e. when transpiration of the WS plants fell below 10% of that in their WW controls. The change of NTR was plotted against the FTSW, the FTSW was expressed as the volumetric water content of the soil, it was calculated using the following equation: (daily weight-final weight)/(initial weight-daily weight). To fit the data plotted as NTR against FTSW, we applied a two-segment linear regression, and then the slope and the FTSW threshold were compared among the genotypes.

2.5 Data analysis

The multivariate analysis of the data was performed with all data of the experiments performed in this study. The Principal component analysis (PCA) was performed in R by reducing the dimensions of the trait variables to differ both transpiration and physiological response between higher and lower rainfall, separate PCA were performed for each rainfall zone, two analyses for traits of exp.1 to TR response to high VPD and other two analyses for the TR response to soil drying.

The statistical analysis of data for the TR response to increasing VPD in Exp.1 and Exp.2, and of data plotted as NTR against FTSW in the dry down experiment, was done by Segmental non-linear regression and Linear regression ((Y_1=slope1.X + intercept 1 and Y_2= slope2.X + intercept2) or Linear regression (Y1=slope1X + intercept 1)). Both regressions with best fitting curve model with 1000 iterations and parameter comparisons, and One-way ANOVA followed by Dunnett's multiple comparisons. The growth comparison in Exp.3 and Exp.4 were performed with Sigmodial and linear regression fit comparisons ($p<005$) for the LA and plant height curves, all tests were performed with the provider considerations using GraphPad Prism (version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). The analysis of the TR response outdoors plotting normalized TR against VPD in Exp.3, and the physiological parameters of all experiments were done by Analysis of Variance (ANOVA) test, Student-t test, LSD (Least significant differences) test, Linear regression, Pearson Correlation and Principal component analysis (PCA), all tests were performed using the provider indications with the Linear model tool in Stats R package (Core Team 2015). In all analyses the data was considered as significant are $p<0.05$ and all data shown in the tables are means and SEM.

3 Results

3.1 F1 hybrid response to high VPD and to progressive soil drying

The F_1 hybrid response to high VPD in glasshouse showed seasonal slopes variation. In Exp.1 all groups (A1, A and B) had similar average slopes under low VPD (*slope1:* $0.0053_{Exp.1}$ and $0.0055_{Exp.2}$*)* but different average slopes under high VPD (*slope2:* $0.0078_{Exp.1}$ and $0.0033_{Exp.2}$). There was a variation in the slope of the TR response to increasing VPD levels, under low VPD across Exp.1 (Fig. 1: A-H) and Exp.2 (Fig. 1: I-K and D). Across years, the TR response under low VPD (slope 1) of low and high rainfall hybrids was similar (0.0057, A1 zone - 0.0048, A zone – 0.0059, B zone). By contrast, across both years in those glasshouse experiments, the TR response under high VPD (*slope 2*) was higher in lower rainfall hybrids (0.0054) than in the hybrids from the B zone (0.0036 – p<0.05) (Fig. 1 and Table 2). Nevertheless, when the transpiration response to increasing VPD was measured in plants grown under high VPD outdoors condition, F_1 hybrid bred for higher rainfall and hybrids bred for lower rainfall had similar slopes (Table 3), suggesting an effect of the plant growth environment on the VPD response.

With regards to the transpiration response to progressive soil drying, the F_1 hybrids from high rainfall and low rainfall zones grown in glasshouse had a similar behaviour (Fig. 2: blue lines). All showed a water conservative behaviour with FTSW threshold that were relatively high, i.e. around 0.44-0.47 (see Table 4), and declining slopes not showing any significant difference.

Table 2. Slopes of the response to high VPD of pearl millet F_1 Hybrids from lower rainfall zone (A1) and higher rainfall zones (A and B) of India. The table shows the TR slope variation of the genotypes under low VPD (Slope 1) and high VPD (Slope 2) assayed in greenhouse during the Exp.1 and Exp.2 fitting a Segmented linear model (all are significant at P<0.001) and T-test(*p<0.05*). Means of five replicates and SE are shown.

	Hybrids (F_1)	Zone	Slope 1			Slope 2		
Lower rainfall	*HOPE-2014 AHT-R-15*	A1	0.0022	±	0.0002	0.0056	±	0.0000
	HOPE-2014 AHT-R-7	A1	0.0063	±	0.0001	0.0029	±	0.0000
	HOPE-2014 AHT-R-11	A1	0.0077	±	0.0000	0.0028	±	0.0000
	HOPE 2013-AHT-R-8	A1	0.0058	±	0.0001	0.0035	±	0.0000
	HOPE-2013 AHT-R-14	A1	0.0057	±	0.0000	0.0033	±	0.0000
	HHB 67 imp	A1	0.0060	±	0.0000	0.0056	±	0.0000
	**HOPE 2013-AHT-R-8*	A1	0.0070	±	0.0019	0.0068	±	0.0012
	**HHB 67 imp*	A1	0.0061	±	0.0031	0.0050	±	0.0019
	**HOPE-2013 AHT-R-14*	A1	0.0044	±	0.0022	0.0103	±	0.0014
	**HOPE-2013 AHT-R-18*	A1	0.0060	±	0.0016	0.0082	±	0.0010
Higher rainfall	*AHT II/K14-7*	A	0.0054	±	0.0001	0.0037	±	0.0000
	AHT A/K14-5	A	0.0017	±	0.0002	0.0051	±	0.0000
	AHT II/K14-9	A	0.0055	±	0.0003	0.0027	±	0.0001
	IHT A2 /K14-24	A	0.0053	±	0.0001	0.0015	±	0.0000
	AHT A/K13-4	A	0.0051	±	0.0000	0.0032	±	0.0000
	AHT A/K13-5	A	0.0059	±	0.0002	0.0033	±	0.0000
	**AHT A/K13-4*	A	0.0064	±	0.0040	0.0108	±	0.0025
	**AHT A/K13-5*	A	0.0036	±	0.0013	0.0086	±	0.0008
	IHT B1 /K14-20	B	0.0061	±	0.0000	0.0031	±	0.0000
	AHT II/K14-20	B	0.0069	±	0.0002	0.0017	±	0.0000
	IHT B1 /K14-10	B	0.0058	±	0.0001	0.0026	±	0.0000
	AHT-II/K13-5	B	0.0065	±	0.0001	0.0029	±	0.0000
	ICMH 1201	B	0.0061	±	0.0001	0.0036	±	0.0000
	AHT-II/K13-24	B	0.0059	±	0.0001	0.0030	±	0.0000
	**AHT-II/K13-5*	B	0.0046	±	0.0021	0.0069	±	0.0013
	**AHT-II/K13-24*	B	0.0054	±	0.0025	0.0056	±	0.0017
	A1 zone $_{average}$		***0.0057^{a}***	***±***	***0.009***	***0.0054^{a}***	***±***	***0.0005***
	A zone $_{average}$		***0.0048^{a}***	***±***	***0.0007***	***00048^{ab}***	***±***	***0.0004***
	B zone $_{average}$		***0.0059^{a}***	***±***	***0.0006***	***0.0036^{b}***	***±***	***0.0003***

Table 3. Transpiration response of F1 hybrid assayed outdoors (Exp.3). The table shows the slopes variation under high VPD of hybrids bred for lower rainfall zone (A1) and higher rainfall zones (A and B) fitted in a linear regression (all are significant at P<0.001) analysed with LSD test (p<0.05). Means of six replicates and SE are shown.

	Genotype	Zone	Slope			Intercept		
Lower rainfall	*HOPE-2014 AHT-R-17*	A1	0.0164	±	0.0014	-0.0427	±	0.0064
	HOPE-2014 AHT-R-11	A1	0.0161	±	0.0013	-0.0426	±	0.0057
	HOPE-2014 AHT-R-4	A1	0.0161	±	0.0015	-0.0409	±	0.0066
	HOPE-2013 AHT-R-14	A1	0.0157	±	0.0018	-0.0401	±	0.0079
	HOPE 2013-AHT-R-8	A1	0.0154	±	0.0014	-0.0434	±	0.0064
	HOPE-2014 AHT-R-1	A1	0.0146	±	0.0013	-0.0383	±	0.0056
	HOPE-2014 AHT-R-16	A1	0.0145	±	0.0022	-0.0385	±	0.0101
	HOPE-2014 AHT-R-9	A1	0.0140	±	0.0015	-0.0370	±	0.0065
	HOPE-2014 AHT-R-7	A1	0.0132	±	0.0014	-0.0328	±	0.0061
	HOPE-2014 AHT-R-8	A1	0.0130	±	0.0004	-0.0324	±	0.0019
	HOPE-2014 AHT-R-14	A1	0.0125	±	0.0015	-0.0323	±	0.0064
	HOPE-2013 AHT-R-18	A1	0.0117	±	0.0009	-0.0278	±	0.0040
	HOPE-2014 AHT-R-15	A1	0.0112	±	0.0013	-0.0231	±	0.0058
		$A1_{average}$	0.0140^{a}	±	0.0004	-0.0356^{a}	±	0.0020
Higher rainfall	*AEHT /K14-2*	A	0.0170	±	0.0023	-0.0450	±	0.0099
	AHT A/K14-2	A	0.0154	±	0.0015	-0.0444	±	0.0065
	AHT II/K14-9	A	0.0150	±	0.0014	-0.0411	±	0.0061
	AHT II/K14-8	A	0.0139	±	0.0011	-0.0361	±	0.0050
	AHT A/K14-3	A	0.0137	±	0.0013	-0.0322	±	0.0056
	AHT B/K14-22	A	0.0135	±	0.0014	-0.0331	±	0.0061
	EMTT /K14-10	A	0.0133	±	0.0013	-0.0346	±	0.0057
	IHT A2 /K14-24	A	0.0130	±	0.0006	-0.0332	±	0.0027
	AHT A/K14-5	A	0.0123	±	0.0025	-0.0311	±	0.0112
	AEHT /K14-18	A	0.0121	±	0.0012	-0.0312	±	0.0053
	IHT A1 /K14-4	A	0.0117	±	0.0013	-0.0272	±	0.0057
	AHT II/K14-7	A	0.0116	±	0.0013	-0.0269	±	0.0062
		$A_{average}$	0.0128^{a}	±	0.0004	-0.0317^{ab}	±	0.0018
	IHT B1 /K14-5	B	0.0171	±	0.0012	-0.0460	±	0.0052
	AHT II/K14-14	B	0.0171	±	0.0012	-0.0460	±	0.0052
	AHT II/K14-20	B	0.0158	±	0.0011	-0.0430	±	0.0049
	AHT II/K13-5	B	0.0152	±	0.0017	-0.0396	±	0.0073
	AHT II/K13-18	B	0.0146	±	0.0006	-0.0389	±	0.0028
	ICMH 1201	B	0.0141	±	0.0014	-0.0348	±	0.0062
	AHT II/K14-5	B	0.0141	±	0.0020	-0.0403	±	0.0092
	AHT B/K14-20	B	0.0138	±	0.0007	-0.0340	±	0.0030
	IHT B1 /K14-20	B	0.0135	±	0.0014	-0.0336	±	0.0061
	AHT II/K13-6	B	0.0128	±	0.0011	-0.0285	±	0.0051
	IHT B1 /K14-26	B	0.0126	±	0.0025	-0.0288	±	0.0106
	AHT II/K14-11	B	0.0115	±	0.0017	-0.0211	±	0.0077
	IHT B1 /K14-10	B	0.0106	±	0.0015	-0.0188	±	0.0070
		$B_{average}$	0.0130^{a}	±	0.0004	-0.0309^{b}	±	0.0019
	$Lower\ rainfall_{average}$		0.0140^{a}	±	0.0004	-0.0356^{a}	±	0.0020
	$Higher\ rainfall_{average}$		0.0129^{a}	±	0.0004	-0.0313^{a}	±	0.0018

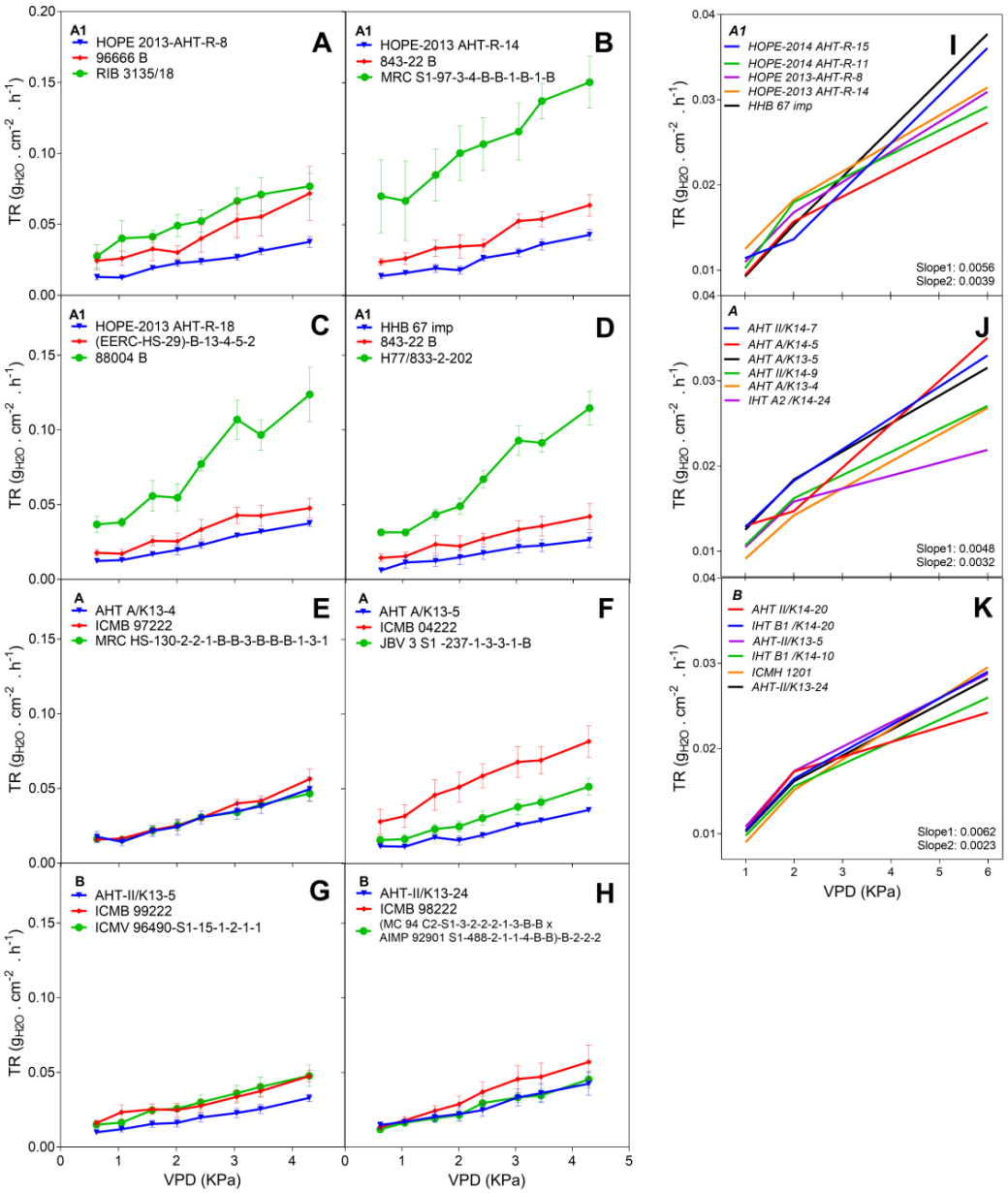


Figure 1. Transpiration response to high VPD of the combinations F1-Hybrids and parental (Exp.1 and Exp.2) genotypes bred for lower (A1) and higher (A and B) rainfall zones of India. Panels A-D show the response of the combinations [F_1 hybrids (blue), B line or male-sterile (red) and the R line or restorer (green)] bred in lower rainfall zone (A1); panels E-F and G-H show the response of higher rainfall genotypes bred in A and B zones respectively. Each curve shows a set of points with standard error. Panels I-J show the linear regression of the response to high VPD from hybrids bred in zones A1, A and B respectively. VPD: vapour pressure deficit, TR: Transpiration rate.

Table 4. Transpiration response to the progressive soil drying of F_1 Hybrid evolved in lower rainfall zone (A1) and higher rainfall zones (A and B). Differences analysed by LSD test (*p<0.05*). FTSW represents the fraction of transpirable soil water and the FTSW threshold is the FTSW at which the transpiration of plants exposed to water stress began to decline in comparison to fully irrigated controls. The last column provides a qualitative assessment of the response to soil drying.

	Hybrid	Zone	Slope	FTSW Threshold	Response
Higher rainfall	*HOPE-2013 AHT-R-18*	*A1*	2.67 ± 0.24	0.32 ± 0.02	Less conservative
	HOPE 2013-AHT-R-8	*A1*	2.29 ± 0.13	**0.44** ± 0.02	Conservative
	HHB 67 imp	*A1*	2.15 ± 0.14	**0.50** ± 0.03	Conservative
	HOPE-2013 AHT-R-14	*A1*	1.72 ± 0.13	**0.52** ± 0.03	Conservative
		$A1_{average}$	2.21^{a} ± 0.16	0.44^{a} ± 0.03	
Lower rainfall	*AHT A/K13-5*	*A*	2.20 ± 0.14	**0.45** ± 0.03	Conservative
	AHT A/K13-4	*A*	2.15 ± 0.09	**0.51** ± 0.02	Conservative
		$A_{average}$	2.17^{a} ± 0.11	0.48^{a} ± 0.02	
	AHT-II/K13-5	*B*	2.20 ± 0.11	**0.46** ± 0.02	Conservative
	AHT-II/K13-24	*B*	2.21 ± 0.12	**0.46** ± 0.02	Conservative
		$B_{average}$	2.20^{a} ± 0.11	0.46^{a} ± 0.02	
	Lower rainfall $_{average}$		**2.21^{a} ± 0.16**	**0.44^{a} ± 0.03**	**Conservative**
	Higher rainfall $_{average}$		**2.18^{a} ± 0.11**	**0.47^{a} ± 0.02**	**Conservative**

3.2 Responses to high VPD and progressive soil drying of the combinations of F1 hybrid and parental lines B and R

The profile of the combinations (F_1 hybrids and parental) shown in Table 5 indicated that F_1 hybrids adapted in both rainfall zones had lower declining NTR slopes and lower FTSW threshold than their parents (Fig. 2 and Suppl. Fig.1S). This relation was confirmed with the PCA analysis (see below), and this may have reflected their heterotic vigour. The difference in the slope of the transpiration response to increasing VPD in the R lines of the A1 zone was about two fold compared to the B lines and three fold compared to the hybrids.

Table 5. Transpiration response to high VPD and soil drying (Exp.1) among combinations of F_1 hybrids and parental lines (R and B) bred for lower (A1) and higher rainfall zones (A and B) of India. The table shows the slopes variation under high (Slope 2) and low (Slope 1) VPD, the FTSW threshold where the normalized transpiration rate (NTR) started to decline and the slope of the NTR decline upon soil drying beyond the FTSW threshold. Differences were analysed by LSD test *(p<0.05)*, values shown as mean and SE. FTSW: fraction of transpirable soil water and NTR: normalized transpiration.

	Genotype	Zone	Class	Response to High VPD						Response to soil drying					
				Slope 1			Slope 2			NTR Slope			FTSW threshold		
Lower rainfall	*HOPE 2013-AHT-R-8*	A1	F_1	**0.0070**	**±**	**0.0019**	**0.0068**	**±**	**0.0012**	**2.29**	**±**	**0.13**	**0.44**	**±**	**0.02**
	96666 B	A1	*B*	0.0062	±	0.0060	0.0092	±	0.0039	2.31	±	0.30	0.38	±	0.04
	RIB 3135/18	A1	*R*	0.0143	±	0.0071	0.0131	±	0.0044	4.77	±	0.54	0.20	±	0.02
	HOPE-2013 AHT-R-14	A1	F_1	**0.0044**	**±**	**0.0022**	**0.0103**	**±**	**0.0014**	**1.72**	**±**	**0.13**	**0.52**	**±**	**0.03**
	843-22 B	A1	*B*	0.0078	±	0.0045	0.0133	±	0.0028	3.03	±	0.19	0.30	±	0.01
	MRC S1-97-3-4-B-B-1-B-1-B	A1	*R*	0.0207	±	0.0189	0.0312	±	0.0118	-		-	-		-
	HOPE-2013 AHT-R-18	A1	F_1	**0.0060**	**±**	**0.0016**	**0.0082**	**±**	**0.0010**	**2.67**	**±**	**0.24**	**0.32**	**±**	**0.02**
	(EERC-HS-29)-B-13-4-5-2	A1	*B*	0.0090	±	0.0042	0.0094	±	0.0026	2.68	±	0.15	0.35	±	0.02
	88004 B	A1	*R*	0.0199	±	0.0085	0.0282	±	0.0053	3.40	±	0.29	0.25	±	0.02
	HHB 67 imp	A1	F_1	**0.0061**	**±**	**0.0031**	**0.0050**	**±**	**0.0019**	**2.15**	**±**	**0.14**	**0.50**	**±**	**0.03**
	843-22 B	A1	*B*	0.0081	±	0.0036	0.0042	±	0.0024	2.71	±	0.28	0.35	±	0.03
	H77/833-2-202	A1	*R*	0.0180	±	0.0055	0.0285	±	0.0034	4.51	±	0.44	0.19	±	0.01
Higher rainfall	*AHT A/K13-4*	A	F_1	**0.0064**	**±**	**0.0040**	**0.0108**	**±**	**0.0025**	**2.15**	**±**	**0.09**	**0.51**	**±**	**0.02**
	ICMB 97222	A	*B*	0.0071	±	0.0027	0.0134	±	0.0017	2.07	±	0.11	0.46	±	0.02
	MRC HS-130-2-2-1-B-B-3-B-B-B-1-3-1	A	*R*	0.0077	±	0.0022	0.0093	±	0.0014	2.90	±	0.24	0.30	±	0.02
	AHT A/K13-5	A	F_1	**0.0036**	**±**	**0.0013**	**0.0086**	**±**	**0.0008**	**2.20**	**±**	**0.14**	**0.45**	**±**	**0.03**
	ICMB 04222	A	*B*	0.0189	±	0.0073	0.0128	±	0.0046	1.99	±	0.20	0.42	±	0.03
	JBV 3 S1 -237-1-3-3-1-B	A	*R*	0.0076	±	0.0032	0.0114	±	0.0020	3.60	±	0.25	0.26	±	0.01
	AHT-II/K13-5	B	F_1	**0.0046**	**±**	**0.0021**	**0.0069**	**±**	**0.0013**	**2.20**	**±**	**0.11**	**0.46**	**±**	**0.02**
	ICMB 99222	B	*B*	0.0047	±	0.0032	0.0092	±	0.0020	3.78	±	0.46	0.22	±	0.02
	ICMV 96490-S1-15-1-2-1-1	B	*R*	0.0094	±	0.0027	0.0062	±	0.0018	1.58	±	0.13	0.50	±	0.03
	AHT-II/K13-24	B	F_1	**0.0054**	**±**	**0.0025**	**0.0056**	**±**	**0.0017**	**2.21**	**±**	**0.12**	**0.46**	**±**	**0.02**
	ICMB 98222	B	*B*	0.0126	±	0.0055	0.0120	±	0.0035	6.00	±	0.58	0.16	±	0.01
	(MC 94 C2-S1-3-2-2-2-1-3-B-Bx AIMP 920	B	*R*	0.0077	±	0.0029	0.0095	±	0.0018	5.10	±	0.68	0.16	±	0.02
Lower rainfall average		***F1***		**0.0059^{b}**	**±**	**0.0022**	**0.0076^{b}**	**±**	**0.0014**	**2.21^{c}**	**±**	**0.16**	**0.44^{a}**	**±**	**0.02**
		B line		0.0078^{ab}	±	0.0046	0.0090^{a}	±	0.0029	2.68^{b}	±	0.23	0.34^{b}	±	0.02
		R line		0.0182^{a}	±	0.0100	0.0252^{a}	±	0.0062	4.23^{a}	±	0.42	0.21^{b}	±	0.02
Higher rainfall average		***F1***		**0.0050^{a}**	**±**	**0.0025**	**0.0080^{a}**	**±**	**0.0016**	**2.19^{b}**	**±**	**0.12**	**0.47^{a}**	**±**	**0.02**
		B line		0.0108^{a}	±	0.0047	0.0118^{a}	±	0.0029	3.46^{a}	±	0.34	0.31^{b}	±	0.02
		R line		0.0081^{a}	±	0.0027	0.0091^{a}	±	0.0017	3.30^{a}	±	0.32	0.30^{b}	±	0.02

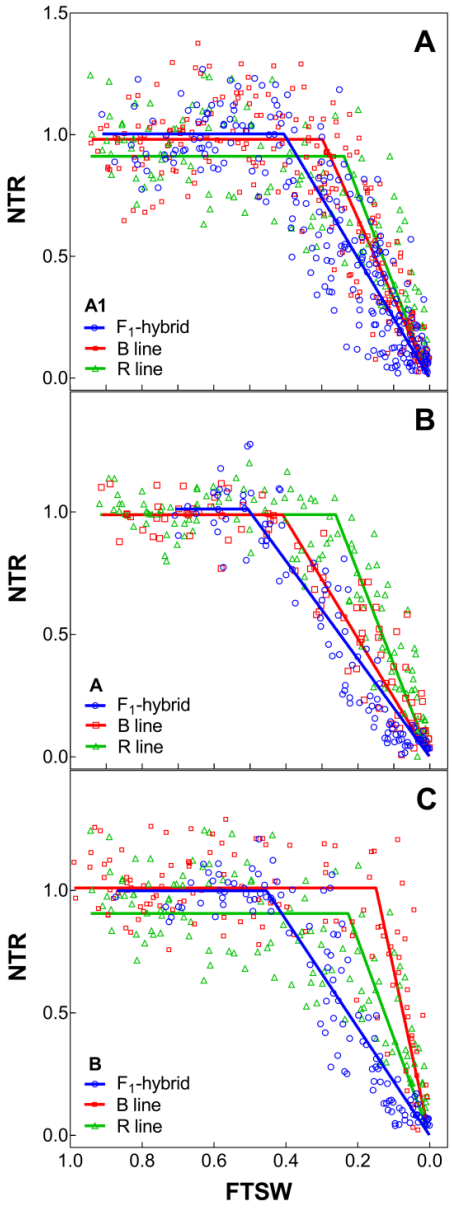


Figure 2. Transpiration response to soil drying of the combinations F_1 Hybrids and parental that were bred for lower (A1) and higher rainfall zones (B and C) of India. Panel A-C shows dry down response of combinations [F_1 hybrids (blue), B line or male-sterile (red) and the R line or restorer (green)] bred in zone A1, A and B respectively. Each biological replicate (circle) and its segmented regression line are represented. NTR: normalized transpiration rate, FTSW fraction of transpirable soil water

3.3 Physiological parameters in F1 hybrids of higher and lower rainfall zones

In the glasshouse experiments, having low VPD growth conditions (Table 5), F_1 hybrids from higher rainfall showed significantly higher leaf area (LA), exudation rate, root/shoot ratio, leaf thickness (SLA), and dry matter (TDM) than the ones from lower rainfall zones, but smaller root length. The exudation rate, or the exudation rate normalized by root length (RL) and root dry weight (RDW) were significantly larger for high rainfall zone hybrids than for the low rainfall zone hybrids. It should be noticed that for some of these parameters, there were also differences between the hybrids of the two higher rainfall zones. By contrast, in the outdoor experiment (Exp.3), only the total dry matter and tiller numbers were higher in the low rainfall than in the high rainfall hybrids (Table 6).

In the outdoors experiments, having high VPD conditions, the plants showed higher leaf area development in Exp.3 than in Exp.4 (Fig. 3A), while in both experiment they reached a similar plant height at the exponential growth phase (Fig 3B). Throughout the crop development phase that was measured in the different experiments, the VPD conditions were higher in Exp.4 than in Exp.3 (Fig. 3C). The daily increase in 3D leaf area was fitted to a linear regression as a function of days at 20ºC and the slope of that regression was higher in the A1 zone hybrids than in the B-zone hybrids (Fig. 3D). Similarly, the daily increase in plant height was fitted to a linear regression as a function of days at 20ºC and the slope of that regression was higher in the A1 zone hybrids than in the B-zone hybrids (Fig. 3D). As a consequence, hybrids bred in low rainfall zones had larger area and were taller than the high rainfall hybrids (Fig. 3: E, G) in this outdoor experiment under high VPD. According to the Pearson correlations we found a strong significant correlation (0.880; $p< 0.000$) between LA and RL (Fig. 4A), and more generally strong significant correlations between shoot and root traits (Fig 4B). By contrast, poor correlations were found between the net exudation rate with root length (0.3; $p< 0.001$) or with leaf area (0.35; $p<0.000$), and no correlation with the root dry weight (0.01; $p<0.855$) (Fig. 4B).

Table 5. Transpiration response to high VPD and soil drying (Exp.1) among combinations of F_1 hybrids and parental lines (R and B) bred for lower (A1) and higher rainfall zones (A and B) of India. The table shows the slopes variation under high (Slope 2) and low (Slope 1) VPD, the FTSW threshold where the normalized transpiration rate (NTR) started to decline and the slope of the NTR decline upon soil drying beyond the FTSW threshold. Differences were analysed by LSD test *(p<0.05)*, values shown as mean and SE. FTSW: fraction of transpirable soil water and NTR: normalized transpiration.

	Genotype	Zone	Class	Response to High VPD						Response to soil drying					
				Slope 1			Slope 2			NTR Slope			FTSW threshold		
Lower rainfall	*HOPE 2013-AHT-R-8*	A1	F_1	**0.0070**	**±**	**0.0019**	**0.0068**	**±**	**0.0012**	**2.29**	**±**	**0.13**	**0.44**	**±**	**0.02**
	96666 B	A1	*B*	0.0062	±	0.0060	0.0092	±	0.0039	2.31	±	0.30	0.38	±	0.04
	RIB 3135/18	A1	*R*	0.0143	±	0.0071	0.0131	±	0.0044	4.77	±	0.54	0.20	±	0.02
	HOPE-2013 AHT-R-14	A1	F_1	**0.0044**	**±**	**0.0022**	**0.0103**	**±**	**0.0014**	**1.72**	**±**	**0.13**	**0.52**	**±**	**0.03**
	843-22 B	A1	*B*	0.0078	±	0.0045	0.0133	±	0.0028	3.03	±	0.19	0.30	±	0.01
	MRC S1-97-3-4-B-B-1-B-1-B	A1	*R*	0.0207	±	0.0189	0.0312	±	0.0118	-		-	-		-
	HOPE-2013 AHT-R-18	A1	F_1	**0.0060**	**±**	**0.0016**	**0.0082**	**±**	**0.0010**	**2.67**	**±**	**0.24**	**0.32**	**±**	**0.02**
	(EERC-HS-29)-B-13-4-5-2	A1	*B*	0.0090	±	0.0042	0.0094	±	0.0026	2.68	±	0.15	0.35	±	0.02
	88004 B	A1	*R*	0.0199	±	0.0085	0.0282	±	0.0053	3.40	±	0.29	0.25	±	0.02
	HHB 67 imp	A1	F_1	**0.0061**	**±**	**0.0031**	**0.0050**	**±**	**0.0019**	**2.15**	**±**	**0.14**	**0.50**	**±**	**0.03**
	843-22 B	A1	*B*	0.0081	±	0.0036	0.0042	±	0.0024	2.71	±	0.28	0.35	±	0.03
	H77/833-2-202	A1	*R*	0.0180	±	0.0055	0.0285	±	0.0034	4.51	±	0.44	0.19	±	0.01
Higher rainfall	*AHT A/K13-4*	A	F_1	**0.0064**	**±**	**0.0040**	**0.0108**	**±**	**0.0025**	**2.15**	**±**	**0.09**	**0.51**	**±**	**0.02**
	ICMB 97222	A	*B*	0.0071	±	0.0027	0.0134	±	0.0017	2.07	±	0.11	0.46	±	0.02
	MRC HS-130-2-2-1-B-B-3-B-B-B-1-3-1	A	*R*	0.0077	±	0.0022	0.0093	±	0.0014	2.90	±	0.24	0.30	±	0.02
	AHT A/K13-5	A	F_1	**0.0036**	**±**	**0.0013**	**0.0086**	**±**	**0.0008**	**2.20**	**±**	**0.14**	**0.45**	**±**	**0.03**
	ICMB 04222	A	*B*	0.0189	±	0.0073	0.0128	±	0.0046	1.99	±	0.20	0.42	±	0.03
	JBV 3 S1 -237-1-3-3-1-B	A	*R*	0.0076	±	0.0032	0.0114	±	0.0020	3.60	±	0.25	0.26	±	0.01
	AHT-II/K13-5	B	F_1	**0.0046**	**±**	**0.0021**	**0.0069**	**±**	**0.0013**	**2.20**	**±**	**0.11**	**0.46**	**±**	**0.02**
	ICMB 99222	B	*B*	0.0047	±	0.0032	0.0092	±	0.0020	3.78	±	0.46	0.22	±	0.02
	ICMV 96490-S1-15-1-2-1-1	B	*R*	0.0094	±	0.0027	0.0062	±	0.0018	1.58	±	0.13	0.50	±	0.03
	AHT-II/K13-24	B	F_1	**0.0054**	**±**	**0.0025**	**0.0056**	**±**	**0.0017**	**2.21**	**±**	**0.12**	**0.46**	**±**	**0.02**
	ICMB 98222	B	*B*	0.0126	±	0.0055	0.0120	±	0.0035	6.00	±	0.58	0.16	±	0.01
	(MC 94 C2-S1-3-2-2-2-1-3-B-BxAIMP 92901	B	*R*	0.0077	±	0.0029	0.0095	±	0.0018	5.10	±	0.68	0.16	±	0.02
Lower rainfall $_{average}$			***F_1***	**0.0059^{b}**	**±**	**0.0022**	**0.0076^{b}**	**±**	**0.0014**	**2.21^{c}**	**±**	**0.16**	**0.44^{a}**	**±**	**0.02**
			B line	0.0078^{ab}	±	0.0046	0.0090^{a}	±	0.0029	2.68^{b}	±	0.23	0.34^{b}	±	0.02
			R line	0.0182^{a}	±	0.0100	0.0252^{a}	±	0.0062	4.23^{a}	±	0.42	0.21^{b}	±	0.02
Higher rainfall $_{average}$			***F_1***	**0.0050^{a}**	**±**	**0.0025**	**0.0080^{a}**	**±**	**0.0016**	**2.19^{b}**	**±**	**0.12**	**0.47^{a}**	**±**	**0.02**
			B line	0.0108^{a}	±	0.0047	0.0118^{a}	±	0.0029	3.46^{a}	±	0.34	0.31^{b}	±	0.02
			R line	0.0081^{a}	±	0.0027	0.0091^{a}	±	0.0017	3.30^{a}	±	0.32	0.30^{b}	±	0.02

Table 6. Physiological parameters comparisons between F1 hybrids bred for the lower (A1) and higher (A and B) rainfall zones, grown in in glasshouse (upper section) and outdoors (bottom section). Comparisons were performed with LSD test *(p<0.05)*.

	F_1 Hybrid Zone	LA (cm^2)	TDM (g)	SLA ($cm^2.g^{-1}$)	Root Shoot	Ex ($g.h^{-1}$)	RDW (g)	RL (cm)	Ex-RL ($g.h^{-1}.cm^{-1}$)	Ex-RDW ($g.h^{-1}.g^{-1}$)	DM control (g)	DM stress (g)
Glasshouse	A1	700.39^{c}	2.52^{a}	218.82^{b}	0.29^{b}	0.15^{b}	0.71^{b}	114.84^{a}	0.0018^{a}	0.28^{b}	35.20^{b}	25.67^{b}
	A	783.48^{b}	2.40^{ab}	215.77^{b}	0.28^{b}	0.18^{b}	0.70^{ab}	88.17^{b}	0.0031^{b}	0.40^{a}	35.78^{ab}	26.81^{ab}
	B	937.34^{a}	2.48^{b}	256.98^{a}	0.38^{a}	0.22^{a}	0.77^{a}	118.75^{a}	0.0025^{c}	0.41^{a}	37.68^{a}	28.35^{a}
	Low rainfall	**700.39^{b}**	**2.52^{a}**	**218.82^{b}**	**0.29^{b}**	**0.15^{b}**	**0.71^{a}**	**114.84^{a}**	**0.0018^{b}**	**0.28^{b}**	**35.20^{b}**	**25.67^{b}**
	High rainfall	**860.41^{a}**	**2.44^{b}**	**236.37^{a}**	**0.33^{a}**	**0.20^{a}**	**0.73^{a}**	**103.46^{b}**	**0.0028^{a}**	**0.40^{a}**	**36.73^{a}**	**27.58^{a}**

	Zone	$LA_{Exp.3}$ (cm^2)	TDM (g)	SLA ($cm^2.g^{-1}$)	Tillers	Height (mm)	Morning TR (10 am) ($g\ H_2O.mm^{-2}$)	Afternoon TR (3 pm) ($g\ H_2O.mm^{-2}$)	$LA_{Exp.4}$ (cm^2)
Outdoors	A1	6536^{a}	28.37^{a}	230^{ab}	7.80^{a}	288.86^{a}	0.19^{a}	0.68^{a}	3544^{a}
	A	5752^{a}	27.44^{a}	246^{a}	7.14^{a}	266.57^{b}	0.18^{a}	0.65^{a}	3044^{b}
	B	6313^{a}	27.80^{a}	226^{b}	5.45^{b}	288.11^{ab}	0.20^{a}	0.69^{a}	2572^{c}
	Low rainfall	**6536^{a}**	**28.37^{a}**	**230^{a}**	**7.80^{a}**	**288.86^{a}**	**0.19^{a}**	**0.68^{a}**	**3544^{a}**
	High rainfall	**6541^{a}**	**27.62^{b}**	**236^{a}**	**6.30^{b}**	**277.34^{a}**	**0.20^{a}**	**0.67^{a}**	**2808^{b}**

LA is the leaf area, TDM is the aerial dry matter, *SLA* is the specific leaf area which is the LA per gram of leaf dry matter and indicates how thin are the leaves, *Root shoot* is the ratio between root and aerial dry weight, *Ex* is the xylem exudate, *RDW* is the root dry weight, *RL* is the root length, *Ex-RL* is the xylem exudate normalized by root length, *Ex-RDW* is the xylem exudate normalized by root dry weight, *DM control* is the aerial dry matter under optimal conditions, *DM stress* is the aerial dry matter under water stress due to progressive soil drying, $LA_{Exp.3}$ is the LA in cm equivalent to LA_3d (recorded by the scanner) in Exp.3, *Tillers* is the number of tillers developed each the plant, *Height* is the plant height, *Morning TR* is the transpiration rate at 10 am accounting the LA-3d at low VPD conditions, *Afternoon TR* is the transpiration rate at 10 am accounting the LA-3d at high VPD conditions, and $LA_{Exp.4}$ is the LA in cm equivalent to LA_3d in Exp.4

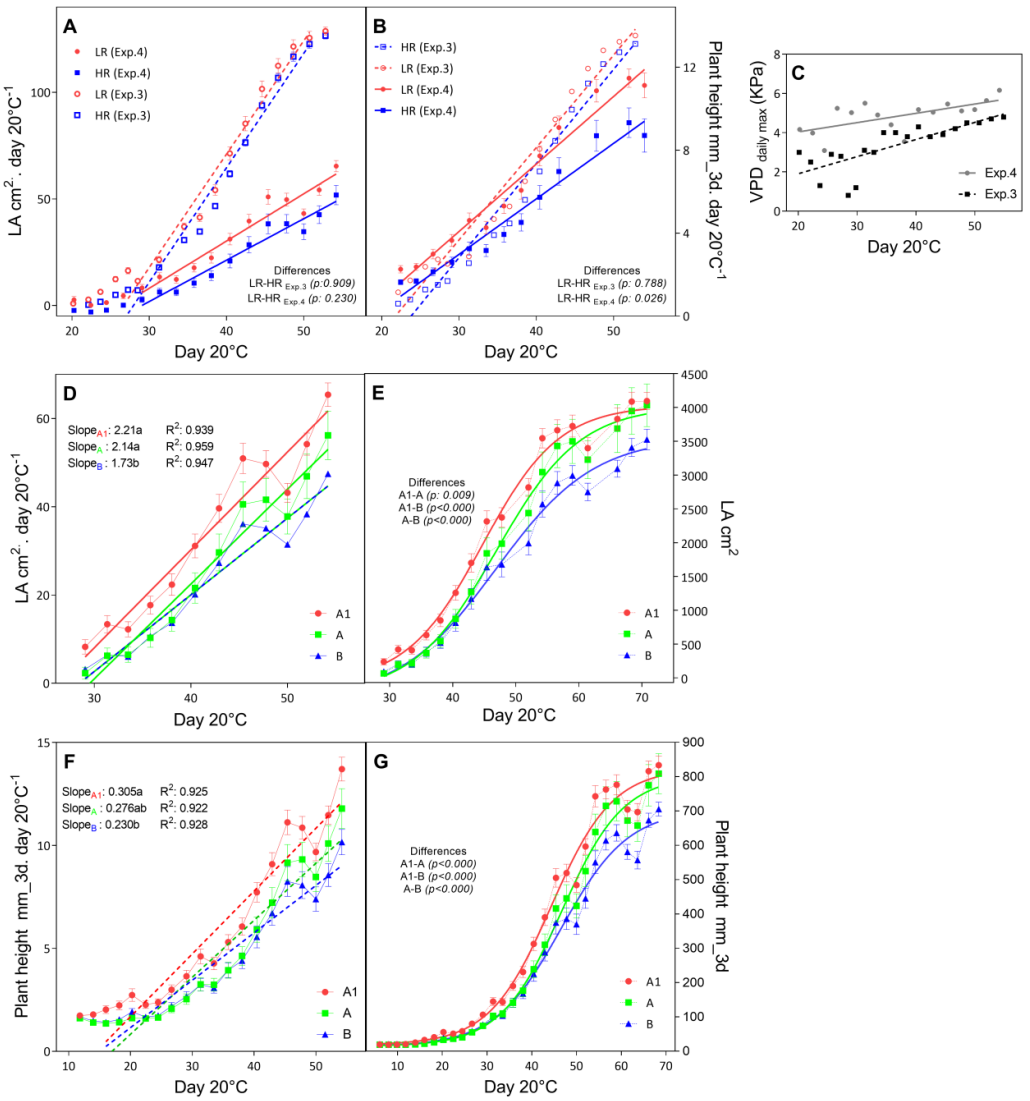


Figure 3. Growth development of F_1 hybrids bred for higher (HR) and lower rainfall (LR) zones. The upper panels show the comparison of increases in leaf area (A) and plant height (B) per unit of days at 20ºC within higher (HR, blue) and lower (LR, red) rainfall zones within two consecutive years (Exp.3 and Ex.4) where the maximum VPD levels (C) were different. The mid panel the development of leaves as the daily increase in LA (D) and the total LA per day at 20°C (E). Similarly the bottom panels show the daily increase (F) and total plant height (G) measured by the scanner (mm). Comparisons of slopes ($p<0.05$) were performed by linear regression of daily increase rates and are indicated with letters; and Sigmoidal regression were performed to compare total growth curves ($p<0.05$). Differences are indicated.

3.4 Physiological parameters of the combinations (F1 hybrid and parental) from higher and lower rainfall zones

The physiology of the combinations (F_1 hybrid and parental) showed significant differences between the F_1 hybrids, B and R lines in both rainfall zones (Table 7). The F_1 hybrids showed significant larger values for leaf area, TDM, root dry weight (RDW), exudate, RL and normalized exudation (Ex-RL, Ex-SDW, Ex-RDW), also while evaluated in response to soil drying under stress and optimal conditions their dry matter was higher than the B- and R-lines, which confirmed the hybrid heterotic effects. In both cases the hybrid showed lower root/shoot ratio than the parental. There was also a large difference between the R lines compared to B and F1 in both zones. Especially in A1 zone, the R-line were the smallest in most of the traits such as LA, TDW, RDW, SLA, exudate and normalized exudation, also under soil drying showed the lowest LA.

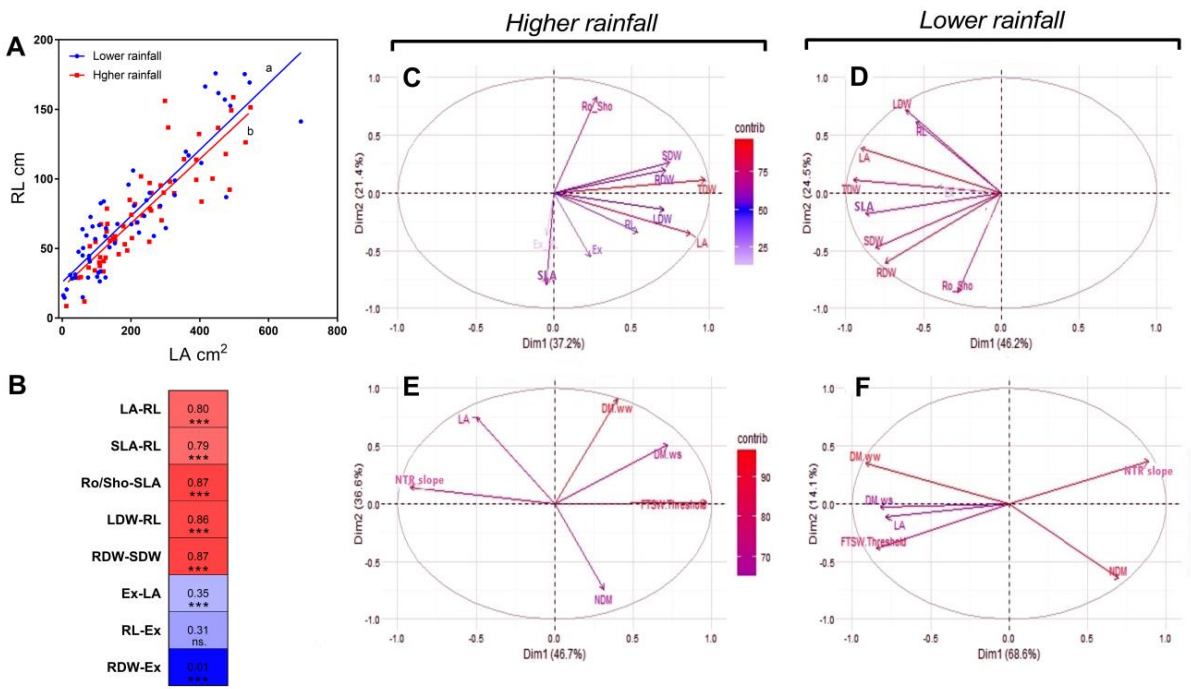
Table 7. Physiological parameters comparisons between combinations of F1 hybrids and parental (B and R lines) that evolved in lower rainfall zone (A1) and higher rainfall zones (A and B). Left Section shows the parameters of TR response to High VPD (left) and to progressive soil drying (right). Comparisons performed with LSD test (p<0.05). For abbreviation see legend of table 6. NDM is the DM stress normalized by DM control.

		Response to High VPD										Response to soil drying			
		LA	TDM	RDW	Root Shoot	Exudate	SLA	RL	Ex-RL	Ex-SDW	Ex-RDW	LA	DM stress	DM control	NDM
	Class	(cm^2)	(g)	(g)		($g.h^{-1}$)	($cm^2.g^{-1}$)	(cm)	($g.h^{-1}.cm^{-1}$)	($g.h^{-1}.g^{-1}$)	($g.h^{-1}.g^{-1}$)	(cm^2)	(g)	(g)	
Lower rainfall	F_1	366.85^{a}	2.44^{a}	0.45^{a}	0.19^{c}	0.18^{a}	187.40^{a}	114.84^{a}	0.0018^{a}	0.38^{a}	0.41^{a}	1453.64^{a}	25.67^{a}	35.20^{a}	0.74^{b}
	B line	168.19^{b}	1.56^{b}	0.31^{b}	0.20^{b}	0.08^{b}	127.90^{b}	61.10^{b}	0.0014^{b}	0.27^{b}	0.26^{b}	1365.73^{ab}	22.91^{b}	25.13^{b}	0.94^{a}
	R line	73.13^{c}	1.25^{c}	0.30^{c}	0.24^{a}	0.07^{c}	68.36^{c}	45.93^{c}	0.0015^{b}	0.25^{c}	0.23^{c}	834.50^{b}	19.03^{b}	20.94^{b}	0.92^{a}
Higher rainfall	F_1	341.87^{a}	2.22^{a}	0.41^{a}	0.19^{c}	0.26^{a}	184.36^{a}	103.46^{a}	0.0028^{a}	0.61^{a}	0.66^{a}	1925.87^{a}	27.58^{a}	36.73^{a}	0.75^{b}
	B line	155.38^{c}	1.44^{c}	0.31^{c}	0.23^{a}	0.10^{b}	126.38^{c}	53.92^{c}	0.0018^{c}	0.36^{c}	0.37^{c}	2040.01^{a}	21.18^{b}	27.34^{b}	0.80^{a}
	R line	211.12^{b}	1.75^{b}	0.33^{b}	0.20^{b}	0.14^{c}	147.25^{b}	71.84^{b}	0.0021^{b}	0.43^{b}	0.53^{b}	1635.37^{a}	19.56^{b}	25.93^{b}	0.77^{b}

3.5 Comparative trait analysis between higher and lower rainfall zones

A multivariate PCA analysis performed with data of Exp.1 showed the variation of the physiological parameters and their contribution in each rainfall zone. Under well-watered conditions all plant traits had positive loading on the main vector, both for the low and high rainfall hybrids (Fig. 4: C,D). On the second main vector, the different plant traits were distributed across the X-axis, with no major difference between low and high rainfall hybrids, except that SLA and the exudation rate had a strong negative loading for the high rainfall hybrids (Fig. 4C) whereas it had no weight in the low rainfall hybrids (Fig. 4D). The aerial dry matter (TDW) and LA were the most influent traits in both zones, and RDW was highly influent in low rainfall zone. Under soil progressive soil drying (Fig. 4: E,F), aerial dry matter (DM), NTR slope and FTSW threshold were the most influent traits in both zones. In low rainfall zone FTSW threshold and LA were located in the same quadrant respect to the main two main vectors (82%) showing their close relation on the main two vectors, while in high rainfall zone these traits were opposite on the main vector (46%) showing their independence.

Moreover, a set of highly significant correlations (see supplementary Table 1S; Fig. 4B) between growth traits from Exp.1 and Exp.2 showed coordinated relationships between aerial and root growth. This was also shown in the linear regression between LA and RL (r: 0.8 ***) represented in figure 4 (A) in both rainfall zones of evolution.



VPD: *vapor pressure deficit, NTR slope: slope of normalized transpiration in the response to soil drying., DM ww: dry matter in optimal conditions, DM ws: dry matter under water stress, NDM: normalized dry matter, FTSW fraction of transpirable soil water, FSTW threshold: FTSW at which the transpiration of plants exposed to water stress began to decline LA: Leaf area, LDW: leaf dry weight, SDW: stem dry weight, TDM: aerial dry matter, SLA: specific leaf area, Ex: xylem exudation, Ex-RL: exudation normalized by root length, RL: root length, RDW: root dry weight, Ro_Sho: root shoot index, Dim: dimension, contrib: contribution, ww: well-watered, ws: water stress.*

Figure 4. Pearson correlations and PCA analysis of physiological and transpiration related traits under optimal conditions as in the response soil drying between higher and lower rainfall genotypes. Panel A shows the coordinated growth leaf-root representing the linear regression between leaf area and root length within higher rainfall and lower rainfall genotypes, as panel B shows the main highest and lowest Pearson´s correlations between phenotypic traits in both zones in a correlation chart between physiological traits. Panels C and D explain the influence of physiological traits in Exp.1 for both rainfall zones under optimal conditions; as in water stress the relation of transpiration traits and biomass are shown in E and F.

4 Discussion

A schematized physical and function representation of high and low rainfall hybrids is shown in figure 5 as a mean of summarizing the main findings. In brief, the hybrids bred in high (HR) and low rainfall (LR) zones had different transpiration response to high VPD depending on the VPD level of their growth environment (Fig. 5A), the largest differences were found between the hybrids bred in A1 (LR) and B (HR) zones. When they grew in greenhouse (low VPD), the lower rainfall hybrids transpired more than higher rainfall ones under high VPD conditions, they had smaller leaf area and biomass, thinner leaves (higher SLA). Their canopy development under high VPD outdoors was opposite, where lower rainfall hybrids had larger and thicker leaves (LA, TDM, SLA) than the high rainfall zone hybrids. In addition, the roots (RDW) and xylem exudates (Exudate, Ex_RL and Ex_RDW) were higher in high rainfall hybrids. Regardless of their target breeding zones, genotypes showed a close relationship between root and canopy area, suggesting a coordinated growth between root and shoot. The parental lines were different from the hybrids in most of the traits evaluated, which reflected the heterotic effect, although in the A1 zone parent/hybrid combinations, the R-line was particularly contrasting with F_1 (Fig. 5B).

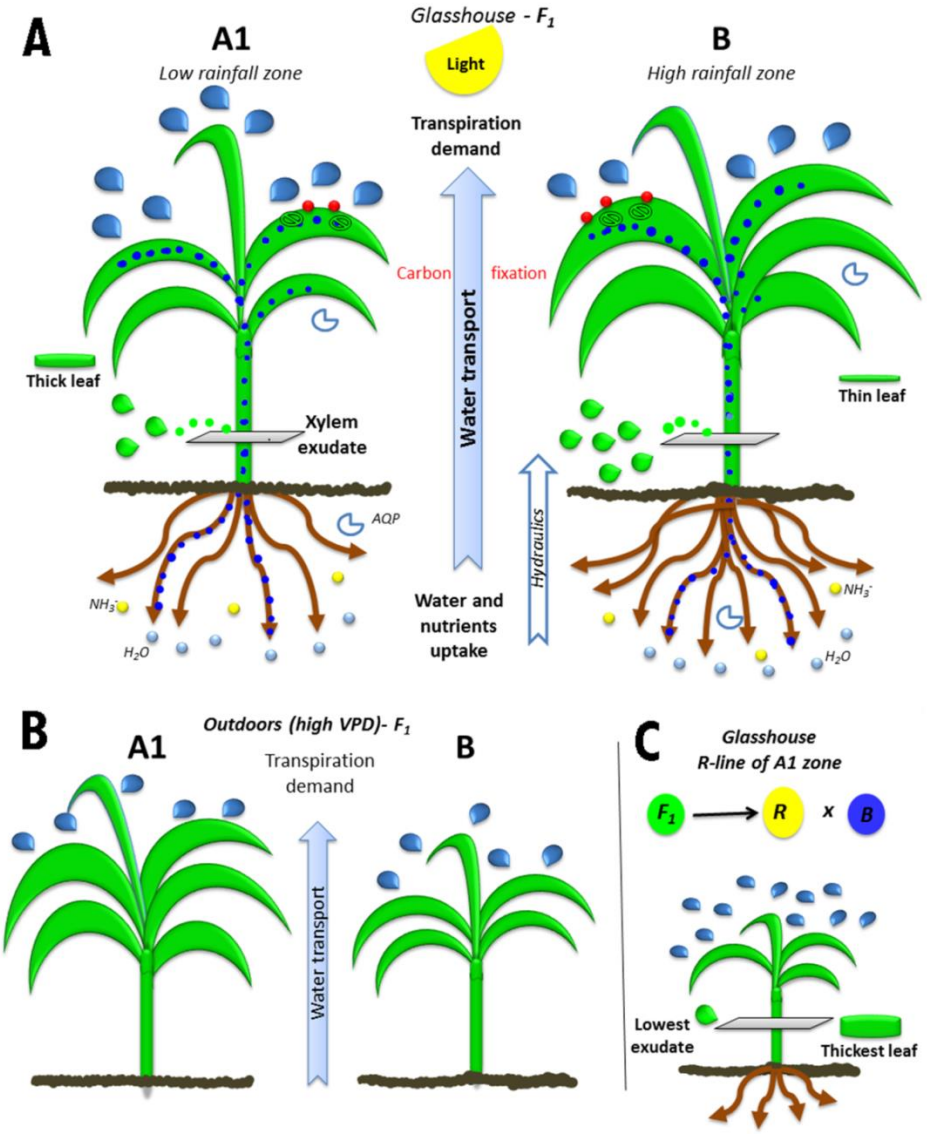


Figure 5. Ideotypes of A1 and B plants bred in high and low rainfall zone. The A panel illustrates the water transport (blue dots) from roots to leaves trough xylem (green drops) due to transpiration demand (blue drops: transpiration) and the integration of carbon fixation in the leaves (CO_2: red points) by the stomata and nutrient uptake by roots (blue circles: water and yellow circles: nitrate) in glasshouse conditions. Panel B shows the growth and transpiration ideotypes of A1 and B zone hybrids grown outdoors, Panel C shows the parental R-line of A1 zone which is the most contrasting line.

4.1 Transpiration response to increases in VPD

When grown in glasshouse conditions, the low rain fall zone hybrids did not restrict the transpiration under increasing VPD conditions whereas the high rainfall zone (B) hybrids did, although the two groups of genotypes did not display any transpiration rate differences under low VPD conditions. By contrast, when the plants were grown in outdoors conditions, the hybrids from the different zones did not display any difference in the transpiration response to increasing VPD. The former observation is consistent with earlier report on a pearl millet hybrid developed for the A1 zone, HHB67, and which did not display any transpiration restriction under increasing VPD conditions, compared to another line bred for better endowed environment and which displayed a transpiration restriction under high VPD conditions (Kholová *et al.*, 2010*a*). The interpretation could be made that genetic material having evolved, or being bred, for A1-types of environments where the rainfalls are erratic and in very sandy soil, would have likely developed adaptation strategies favouring a rapid water uptake before the water is lost to either infiltration or soil evaporation. On the contrary, genetic material bred for B-type environments with wetter conditions and deeper soil with higher clay content could have favoured a transpiration restriction under high VPD, i.e. when the water cost of fixing carbon is the highest (Vadez *et al.*, 2013*b*) . A similar observation could be done from recent report on phaseolus species, where drought adapted lima and tepary beans showed almost no sensitivity to increasing VPD conditions (Medina *et al.*, 2017).

The fact that in outdoors conditions, exposed to hotter/dryer conditions, there was no difference in the transpiration response to increasing VPD conditions between the hybrids developed for different rainfall zones, suggests an interaction between the transpiration response and the VPD conditions prevailing in the growing environment. Our interpretation is that the transpiration demand under high VPD conditions during growth would have prompted the plant development to cater for such a high water demand. Several earlier report can be interpreted in the same

way. In a work on turfgrass, Sermons and colleagues (Sermons *et al.*, 2012) showed that while plants restricted transpiration under high VPD conditions when grown under cool conditions, close to those of the adaptation zone of that particular specie, the transpiration restriction was much weaker when the plants were grown under higher temperature. Similar observation linking the degree of transpiration control under increasing VPD conditions to the temperature in the growing environment was made in soybean (Seversike *et al.*, 2013). In another study on pearl millet, it was also shown that a number of plant traits were altered by growing in a higher VPD environment, in particular there was less of a transpiration restriction in lines that usually restrict transpiration under increasing VPD, and there was also some effect on the root anatomy (endodermal cell size), which was hypothesised to relate to root hydraulic conductivity differences (Kholova *et al.*, 2016). This need of limiting the stomata closure to maintain higher photosynthetic rate and increase the leaf duration in drought-deciduous species was previously reported in nutrient deficit habitats (Rodriguez-Iturbe *et al.*, 2001). Therefore, more work would be needed to test side by side if there is indeed an effect of the VPD in the growth conditions on the transpiration response to transient step increases in VPD.

4.2 The absence of difference in the FTSW thresholds

Equally important under soil moisture-limited conditions, this water conservative behaviour with early stomata closure was the same in both rainfall hybrids; both declined at high soil moisture content and slowly, and a higher penalty on biomass production occurred in lower rainfall genotypes. Previous studies in superior genotypes of pearl millet reported that the lower daily transpiration which consequently drives NTR under drought conditions resulted in lower FTSW thresholds (Kholová *et al.*, 2010*a*). On the contrary, the fitness of our hybrids showed higher FTSW thresholds and subsequent lower NTR slope upon further decrease in

soil moisture. It is not clear why no difference in the FTSW threshold were found. The intuitive hypothesis that genetic material adapted to erratic rainfall pattern, or hybrids bred for the A1 zone here, would favour a behaviour of using soil water instead of losing it through soil evaporation, was here rejected. This decline on TR at high soil water content in hybrids was also reported as a phenomenon related to low hydraulic conductance (Choudhary and Sinclair, 2014).

4.3 Growth strategies

Leaf area and root area were closely related, suggesting that leaf and root growth worked in a closely coordinated manner to respond in both directions to the leaf demand of photosynthesis and transpiration, as the root demand for water and nutrient uptake (Fig. 5A). This coordinated metabolism was supported in other studies as linked to the leaf stomatal closure, which gathers a signal of abscisic acid (ABA) in the xylem while the roots is sensing the decrease in soil moisture (Laffray and Louguet, 1990). Also according to previous studies in several plant communities (Chenopodiaceae, Poaceae, Fabaceae and Asteraceae) and plant types (C3 and C4 grasses, and legumes) of Chinese arid and semi-arid zones, plants show a pattern of positive correlation between root and leaf traits like SLA-SRL and nitrogen content in both organs, they also affirmed that the correspondence aboveground–belowground leads to a strong whole-plant economic strategy of conserving or acquiring carbon and nutrient resources (Liu *et al.*, 2010). Recent work in pearl millet also showed that growth under high VPD conditions affected some traits of the root anatomy like the size of the endodermal cells, suggesting indeed a tight linkage in the development of root and shoot traits (Kholova *et al.*, 2016).

Outdoors, exposed to hotter conditions where VPD raised to ~5 KPa, the lower rainfall hybrids produced more tillers, accumulated more biomass, and had a higher leaf growth (Fig. 5: A). The tillering production is indeed a strategy for successful adaptation to unfavourable environment, where additional reproductive tillers can

compensate the loss of panicles to water stress. Earlier report mentions this as a strategy to minimize crop failure that also carries a yield penalty (Van Oosterom *et al.*, 2003; Kim *et al.*, 2010). This strategy is not only under genetic control but also under environmental control in cereals such as sorghum (Van Oosterom *et al.*, 2003; Kim *et al.*, 2010). Contrary results were found under the lower VPD growth conditions of the glasshouse, where high rainfall hybrids developed larger canopy than the low rainfall hybrids. This was consistent with earlier report of lower rainfall hybrids reporting a smaller canopy when grown outdoors during the rainy season in the LeasyScan platform (Vadez *et al.*, 2015). Our interpretation is similar to the one above to explain the absence of transpiration restriction under high VPD in the low rainfall hybrids: In A1-types of environments, with likely frequent events of high VPD conditions (between rain gaps), adapted genotypes are likely to be those that are able to sustain leaf expansion. It is known that high VPD conditions restrict leaf expansion in maize, although there is large genotypic variation (Reymond *et al.*, 2003; Caldeira *et al.*, 2014). Then under low VPD conditions, it is also understandable that high rainfall hybrid would be those developing a larger canopy to maximize light capture in an environment that does not have water limitation. This would also be supported by the higher exudate rate of these high rainfall hybrids in the low VPD growth conditions. This is where also the size could explain in part the differences in the transpiration response to increasing VPD between the low and high rainfall hybrids, where larger canopy B-hybrids would have a propensity to have restricted transpiration under high VPD because of canopy size. The difference in the transpiration response to increasing VPD between the hybrids and their parents, and the fact that R-lines showed much higher transpiration rates slope response to increasing VPD than their hybrids, would comfort this interpretation.

4.4 Differences in the combinations of hybrids and parental lines

Our experiments showed different behaviour between the parental and the hybrids in vegetative stage, the hybrids showing their heterotic superiority in biomass production. Interestingly, our study suggests that the ability to regulate the TR could have been conferred by any of the two parental; in our lower rainfall genotypes this capacity is given by the B line parent. Contrasting with the higher rainfall genotypes where the donor is the restorer parent. So the high slope of response in the A1 hybrid could be driven by the R line? During the last decades, some studies conducted in the development history of hybrids reported that the line x pollinator interaction was not significant for grain either biomass under favorable conditions, and there was low heritability in stress scenarios in the north part of India, which is our low rainfall zone (Yadav *et al.*, 2000). Later the single cross based on *CMS (cytoplasmic male sterility)* technique was improved to *single top crosses* with top restorer lines, because mostly the breeding programs looked for yield increment in non-extreme environments. However, the approach in the Indian breeding of these hybrids was the adaptation to arid zone via restorer line (R line) which are arid zone landraces and confer the adaptive characters (increased productivity) to the hybrid (Yadav *et al.*, 2009) this is the cross type of the population used in this experiment.

5 Conclusions

In this investigation we have shown that the breeding history had an impact on several traits playing a central role in plant water use. High rainfall hybrids did restrict transpiration under high evaporative demand while low rainfall hybrids did not, and the latter were also able to maintain a larger canopy under high evaporative demand. These traits in the low rainfall hybrids could also be interpreted as part of a strategy of adaptation to low and erratic rainfall consisting of maximizing water use when available. Such results open an opportunity to include such traits as part of the breeding selection process.

ACKNOWLEDGMENT

We thank the GEMS group from Crop physiology-ICRISAT for technical assistance in the experiments and S.K Gupta for providing the plant material. Operational expenses were funded by a grant from USAID (Feed the Future Innovation lab for Climate Resilient Pearl Millet). Also SM was the recipient of a fellowship "Presidente de la República PRONABEC-III" from Peruvian Government.

AUTHOR CONTRIBUTIONS

SM and VV conceived and designed the experiments. SM conducted the experimental work. SM and VV contributed to the data analysis and interpreted the results. SM wrote the paper under the supervision of VV, revised the manuscript, read and approved the final manuscript.

6 References

Caldeira, C. F., Bosio, M., Parent, B., and Jeanguenin, L. (2014). A Hydraulic Model Is Compatible with Rapid Changes in Leaf Elongation under Fluctuating Evaporative Demand and Soil Water Status. Plant Physiol. 164, 1718–1730. doi:10.1104/pp.113.228379.

Choudhary, S., Mutava, R. N., Shekoofa, A., Sinclair, T. R., and Prasad, P. V. V. (2013). Is the stay-green trait in sorghum a result of transpiration sensitivity to either soil drying or vapor pressure deficit? Crop Sci. 53, 2129–2134. doi:10.2135/cropsci2013.01.0043.

Choudhary, S., and Sinclair, T. R. (2014). Hydraulic conductance differences among sorghum genotypes to explain variation in restricted transpiration rates. Funct. Plant Biol. 41, 270–275. doi:10.1071/FP13246.

Gholipoor, M., Prasad, P. V. V., Mutava, R. N., and Sinclair, T. R. (2010). Genetic variability of transpiration response to vapor pressure deficit among sorghum genotypes. F. Crop. Res. 119, 85–90. doi:10.1016/j.fcr.2010.06.018.

Gholipoor, M., Sinclair, T. R., and Prasad, P. V. V. (2012). Genotypic variation within sorghum for transpiration response to drying soil. Plant Soil 357, 35–40. doi:10.1007/s11104-012-1140-8.

Kholová, J., Hash, C. T., Kakkera, A., Koová, M., and Vadez, V. (2010a). Constitutive water-conserving mechanisms are correlated with the terminal drought tolerance of pearl millet [Pennisetum glaucum (L.) R. Br.]. J. Exp. Bot. 61, 369–377. doi:10.1093/jxb/erp314.

Kholová, J., Hash, C. T., Kumar, P. L., Yadav, R. S., Koová, M., and Vadez, V. (2010b). Terminal drought-tolerant pearl millet [Pennisetum glaucum (L.) R. Br.] have high leaf ABA and limit transpiration at high vapour pressure deficit. J. Exp. Bot. 61, 1431–1440. doi:10.1093/jxb/erq013.

Kholova, J., Zindy, P., Mallayee, S., Baddam, R., Tharanya, M., Kaliamoorthy, S., et al. (2016). Component traits of plant water use are modulated by vapor pressure deficit in pearl millet [Pennisetum glaucum (L.) R. Br.]. Funct. Plant Biol., 423–437. doi:10.1071/FP15115.

Kim, H. K., Van Oosterom, E., Dingkuhn, M., Luquet, D., and Hammer, G. (2010). Regulation of tillering in sorghum: Environmental effects. Ann. Bot. 106, 57–67. doi:10.1093/aob/mcq079.

Laffray, D., and Louguet, P. (1990). Stomatal responses and drought resistance. Bull. la Soc. Bot. Fr. 137, 47–60. doi:10.1080/01811789.1990.10826986.

Liu, G., Freschet, G. T., Pan, X., Cornelissen, J. H. C., Li, Y., and Dong, M. (2010). Coordinated variation in leaf and root traits across multiple spatial scales in Chinese semi-arid and arid ecosystems. New Phytol. 188, 543–553. doi:10.1111/j.1469-8137.2010.03388.x.

Manga, V. K., and Kumar, A. (2011). Cultivar Options for Increasing Pearl Millet Productivity in Arid Regions. Indian J. Fundam. Appl. Life Sci. 1, 200–208.

Medina, V., Berny-Mier Teran, J. C., Gepts, P., and Gilbert, M. E. (2017). Low stomatal sensitivity to vapor pressure deficit in irrigated common, lima and tepary beans. F. Crop. Res. 206, 128–137. doi:10.1016/j.fcr.2017.02.010.

Parent, B., and Tardieu, F. (2012). Temperature Responses of Developmental Processes Havenot Affected By Breeding in Different Ecological Areas for 12 Crop Species. New Phytol. 194, 760–774.

Rai, K. N., Gupta, S. K., Govindaraj, M., and Yadav, H. P. (2015). Pearl Millet Improvement for enhanced productivity-strategies and impact. Indian Farming 62. doi:10.1080/00461520.2012.749445.

Reymond, M., Muller, B., Leonardi, A., Charcosset, A., and Tardieu, F. (2003). Combining Quantitative Trait Loci Analysis and an Ecophysiological Model to Analyze the Genetic Variability of the Responses of Maize Leaf Growth to Temperature and Water Deficit. Plant Physiol. 131, 664–675. doi:10.1104/pp.013839.

Rodriguez-Iturbe, I., Porporato, A., Laio, F., and Ridolfi, L. (2001). Plants in water-controlled ecosystems: active role in hydrologic\r processes and response to water stress I. Scope and general\r outline. Adv. Water Resour. 24, 697–705.

Sermons, S. M., Seversike, T. M., Sinclair, T. R., Fiscus, E. L., and Rufty, T. W. (2012). Temperature influences the ability of tall fescue to control transpiration in response to atmospheric vapour pressure deficit. Funct. Plant Biol. 39, 979–986. doi:10.1071/FP12172.

Seversike, T. M., Sermons, S. M., Sinclair, T. R., Carter, T. E., and Rufty, T. W. (2013). Temperature interactions with transpiration response to vapor pressure deficit among cultivated and wild soybean genotypes. Physiol. Plant. 148, 62–73. doi:10.1111/j.1399-3054.2012.01693.x.

Sinclair, T. R. (2012). Is transpiration efficiency a viable plant trait in breeding for crop improvement? Funct. Plant Biol. 39, 359–365. doi:10.1071/FP11198.

Sinclair, T. R., and Rufty, T. W. (2012). Nitrogen and water resources commonly limit crop yield increases, not necessarily plant genetics. Glob. Food Sec. 1, 94–98. doi:10.1016/j.gfs.2012.07.001.

Vadez, V., Kholová, J., Hummel, G., Zhokhavets, U., Gupta, S. K., and Hash, C. T. (2015). LeasyScan: A novel concept combining 3D imaging and lysimetry for high-throughput phenotyping of traits controlling plant water budget. J. Exp. Bot. 66, 5581–5593. doi:10.1093/jxb/erv251.

Vadez, V., Kholova, J., Medina, S., Kakkera, A., and Anderberg, H. (2014). Transpiration efficiency: New insights into an old story. J. Exp. Bot. 65, 6141–6153. doi:10.1093/jxb/eru040.

Vadez, V., Kholova, J., Zaman-Allah, M., and Belko, N. (2013). Water: The most important “molecular” component of water stress tolerance research. Funct. Plant Biol. 40, 1310–1322. doi:10.1071/FP13149.

Van Oosterom, E. J., Bidinger, F. R., and Weltzien, E. R. (2003). A yield architecture framework to explain adaptation of pearl millet to environmental stress. F. Crop. Res. 80, 33–56. doi:10.1016/S0378-4290(02)00153-3.

Yadav, O. P., Bidinger, F. R., and Singh, D. V. (2009). Utility of pearl millet landraces in breeding dual-purpose hybrids for arid zone environments of India. Euphytica 166, 239–247. doi:10.1007/s10681-008-9834-y.

Yadav, O. P., Weltzien-Rattunde, E., Bidinger, F. R., and Mahalakshmi, V. (2000). Heterosis in landrace-based topcross hybrids of pearl millet across arid environments. Euphytica 112, 285–295. doi:10.1023/A:1003965025727.

Zaman-Allah, M., Jenkinson, D. M., and Vadez, V. (2011). A conservative pattern of water use, rather than deep or profuse rooting, is critical for the terminal drought tolerance of chickpea. J. Exp. Bot. 62, 4239–4252. doi:10.1093/jxb/err139.

SUPPLEMENTAL INFORMATION

Supplementary Table 1 Correlation Analysis by Pearson Method – Two sided of genotypes (parental and F1 hybrid) evolved in higher rainfall zone (A1) and lower rainfall zones (A and B) during 2014 and 2015.

Correlation matrix		LA	SLA	LDW	RDW	RL	Ro_Sho	SDW	TDW	Ex	Ex-LA	Ex-LDW	Ex-RDW	Ex-RL	Ex-SDW	Ex-TDW
Cor	LA	1	0.310	0.800	0.460	**0.880**	-0.030	0.510	0.850	0.350	-0.440	0.090	0.050	0.040	-0.020	-0.030
p value		1	0.000	0.000	0.000	**0.000**	0.631	0.000	0.000	0.000	0.000	0.188	0.461	0.693	0.800	0.663
Cor	SLA	0.310	1	-0.090	0.230	0.790	**-0.870**	0.180	0.080	0.150	-0.220	**0.850**	-0.010	0.130	-0.360	**-0.900**
p value		0.000	1	0.207	0.001	0.000	**0.000**	0.010	0.269	0.025	0.002	**0.000**	0.913	0.154	0.000	**0.000**
Cor	LDW	0.800	-0.090	1	0.110	0.860	0.110	0.110	0.690	0.290	-0.250	-0.180	0.180	0.010	0.210	0.190
p value		0.000	0.207	1	0.104	0.000	0.116	0.097	0.000	0.000	0.000	0.009	0.011	0.910	0.002	0.006
Cor	RDW	0.460	0.230	0.110	1	0.760	0.180	**0.870**	0.710	**0.010**	-0.450	-0.010	-0.350	-0.070	-0.350	-0.020
p value		0.000	0.001	0.104	1	0.000	0.008	**0.000**	0.000	**0.855**	0.000	0.898	0.000	0.464	0.000	0.737
Cor	RL	0.880	0.790	0.860	0.760	1	-0.310	0.750	0.870	**0.310**	-0.370	0.090	0.130	-0.070	0.100	0.100
p value		0.000	0.000	0.000	0.000	1	0.001	0.000	0.000	**0.001**	0.000	0.306	0.173	0.457	0.258	0.266
Cor	Ro_Sho	-0.030	-0.870	0.110	0.180	-0.310	1	0.240	0.240	-0.090	-0.040	-0.840	-0.110	-0.090	0.240	0.950
p value		0.631	0.000	0.116	0.008	0.001	1	0.000	0.000	0.200	0.556	0.000	0.105	0.306	0.000	0.000
Cor	SDW	0.510	0.180	0.110	0.870	0.750	0.240	1	0.800	0.040	-0.430	-0.060	-0.310	-0.060	-0.360	0.080
p value		0.000	0.010	0.097	0.000	0.000	0.000	1	0.000	0.559	0.000	0.389	0.000	0.501	0.000	0.268
Cor	TDW	0.850	0.080	0.690	0.710	0.870	0.240	0.800	1	0.210	-0.470	-0.150	-0.120	-0.010	-0.140	0.170
p value		0.000	0.269	0.000	0.000	0.000	0.000	0.000	1	0.003	0.000	0.028	0.081	0.927	0.050	0.014
Cor	Ex	0.350	0.150	0.290	0.010	0.310	-0.090	0.040	0.210	1	0.210	0.510	0.800	0.860	0.720	0.120
p value		0.000	0.025	0.000	0.855	0.001	0.200	0.559	0.003	1	0.002	0.000	0.000	0.000	0.000	0.080
Cor	Ex_LA	-0.440	-0.220	-0.250	-0.450	-0.370	-0.040	-0.430	-0.470	0.210	1	0.140	0.500	0.440	0.440	0.110
p value		0.000	0.002	0.000	0.000	0.000	0.556	0.000	0.000	0.002	1	0.041	0.000	0.000	0.000	0.129
Cor	Ex_LDW	0.090	0.850	-0.180	-0.010	0.090	-0.840	-0.060	-0.150	0.510	0.140	1	0.400	0.950	0.060	-0.740
p value		0.188	0.000	0.009	0.898	0.306	0.000	0.389	0.028	0.000	0.041	1	0.000	0.000	0.364	0.000
Cor	Ex_RDW	0.050	-0.010	0.180	-0.350	0.130	-0.110	-0.310	-0.120	0.800	0.500	0.400	1	0.860	**0.860**	0.150
p value		0.461	0.913	0.011	0.000	0.173	0.105	0.000	0.081	0.000	0.000	0.000	1	0.000	0.000	0.032
Cor	Ex_RL	0.040	0.130	0.010	-0.070	-0.070	-0.090	-0.060	-0.010	0.860	0.440	0.950	0.860	1	0.890	0.950
p value		0.693	0.154	0.910	0.464	0.457	0.306	0.501	0.927	0.000	0.000	0.000	0.000	1	0.000	0.000
Cor	Ex_SDW	-0.020	-0.360	0.210	-0.350	0.100	0.240	-0.360	-0.140	0.720	0.440	0.060	0.860	0.890	1	0.490
p value		0.800	0.000	0.002	0.000	0.258	0.000	0.000	0.050	0.000	0.000	0.364	0.000	0.000	1	0.000
Cor	Ex_TDW	-0.030	-0.900	0.190	-0.020	0.100	0.950	0.080	0.170	0.120	0.110	-0.740	0.150	0.950	0.490	1
p value		0.663	0.000	0.006	0.737	0.266	0.000	0.268	0.014	0.080	0.129	0.000	0.032	0.000	0.000	1

Cor: Pearson correlation coefficient
p value: significance

Supplementary Table 2 Physiological parameters of genotype triplets (parental and F1hybrid) evolved in higher rainfall zone (A1) and lower rainfall zones (A and B) during 2014.

ID	LA (cm²)			TDW (g)			RDW (g)			Root Shoot			Ex (g. h⁻¹)			SLA (cm².g⁻¹)			RL (cm)			Ex-RL (g .h⁻¹.cm⁻¹)			Ex-SDW (g.h⁻¹.g⁻¹)			Ex-RDW (g .h⁻¹.g⁻¹)		
1	381.880	±	9.240	2.534	±	0.045	0.438	±	0.005	0.179	±	0.003	0.283	±	0.011	193.366	±	1.707	108.440	±	2.903	0.00296	±	0.00012	0.544	±	0.022	0.672	±	0.026
2	183.108	±	7.071	1.700	±	0.040	0.334	±	0.004	0.211	±	0.004	0.080	±	0.004	125.318	±	1.861	77.381	±	1.053	0.00103	±	0.00006	0.272	±	0.017	0.242	±	0.013
3	97.145	±	3.418	1.402	±	0.026	0.276	±	0.007	0.196	±	0.004	0.072	±	0.007	86.976	±	2.747	37.829	±	0.770	0.00165	±	0.00012	0.200	±	0.014	0.246	±	0.018
4	232.280	±	8.222	1.656	±	0.038	0.376	±	0.008	0.232	±	0.004	0.203	±	0.009	166.477	±	3.281	77.247	±	2.548	0.00255	±	0.00017	0.504	±	0.029	0.480	±	0.018
5	86.183	±	1.595	1.110	±	0.008	0.234	±	0.006	0.210	±	0.005	0.051	±	0.003	94.568	±	1.589	34.960	±	1.237	0.00145	±	0.00006	0.245	±	0.011	0.222	±	0.008
6	24.121	±	1.656	0.884	±	0.009	0.244	±	0.007	0.284	±	0.008	0.043	±	0.002	31.472	±	2.002	25.558	±	0.943	0.00149	±	0.00005	0.252	±	0.013	0.169	±	0.006
7	475.389	±	8.746	3.016	±	0.049	0.512	±	0.008	0.172	±	0.003	0.149	±	0.011	210.478	±	1.569	152.411	±	1.757	0.00094	±	0.00007	0.217	±	0.016	0.300	±	0.021
8	173.640	±	4.114	1.634	±	0.030	0.328	±	0.009	0.211	±	0.006	0.054	±	0.005	134.938	±	1.510	58.832	±	1.521	0.00079	±	0.00006	0.142	±	0.011	0.145	±	0.010
9	71.022	±	2.564	1.284	±	0.016	0.318	±	0.005	0.248	±	0.003	0.067	±	0.005	66.342	±	1.753	57.837	±	1.534	0.00125	±	0.00009	0.259	±	0.019	0.227	±	0.017
10	377.858	±	12.429	2.560	±	0.053	0.482	±	0.012	0.189	±	0.002	0.070	±	0.003	179.266	±	3.847	121.253	±	4.174	0.00078	±	0.00005	0.145	±	0.007	0.174	±	0.009
11	229.843	±	6.549	1.808	±	0.027	0.332	±	0.004	0.189	±	0.003	0.147	±	0.003	155.759	±	3.062	73.250	±	1.466	0.00225	±	0.00009	0.420	±	0.014	0.451	±	0.010
12	100.249	±	4.234	1.418	±	0.029	0.345	±	0.005	0.254	±	0.004	0.102	±	0.005	88.669	±	2.015	62.484	±	1.862	0.00149	±	0.00006	0.295	±	0.013	0.286	±	0.014
13	309.957	±	12.043	2.068	±	0.057	0.338	±	0.013	0.157	±	0.003	0.094	±	0.006	178.573	±	2.439	85.369	±	3.402	0.00147	±	0.00011	0.244	±	0.015	0.378	±	0.029
14	245.546	±	8.018	1.832	±	0.034	0.308	±	0.005	0.177	±	0.004	0.050	±	0.003	168.657	±	2.225	75.508	±	1.786	0.00069	±	0.00004	0.139	±	0.010	0.153	±	0.007
15	222.336	±	6.917	1.870	±	0.025	0.328	±	0.008	0.179	±	0.005	0.100	±	0.003	140.207	±	2.528	71.626	±	1.507	0.00150	±	0.00007	0.300	±	0.008	0.354	±	0.017
16	324.482	±	11.517	2.186	±	0.058	0.368	±	0.004	0.184	±	0.004	0.363	±	0.016	180.995	±	3.209	90.964	±	2.976	0.00468	±	0.00021	0.823	±	0.039	0.951	±	0.034
17	60.745	±	2.863	1.056	±	0.019	0.260	±	0.002	0.261	±	0.005	0.057	±	0.005	68.007	±	2.691	24.424	±	1.063	0.00190	±	0.00010	0.231	±	0.015	0.220	±	0.018
18	220.570	±	7.356	1.878	±	0.023	0.345	±	0.009	0.182	±	0.004	0.164	±	0.012	147.740	±	2.883	78.610	±	1.604	0.00236	±	0.00021	0.457	±	0.038	0.715	±	0.075
19	407.960	±	7.315	2.366	±	0.034	0.474	±	0.007	0.202	±	0.002	0.312	±	0.019	206.149	±	1.290	121.435	±	2.381	0.00292	±	0.00019	0.838	±	0.050	0.722	±	0.043
20	158.432	±	5.556	1.272	±	0.027	0.298	±	0.007	0.250	±	0.006	0.188	±	0.015	151.178	±	1.388	55.344	±	1.858	0.00303	±	0.00017	0.771	±	0.059	0.864	±	0.089
21	198.648	±	7.069	1.496	±	0.040	0.334	±	0.010	0.227	±	0.003	0.096	±	0.004	159.092	±	1.848	64.916	±	2.317	0.00149	±	0.00002	0.347	±	0.008	0.291	±	0.008
22	325.077	±	10.559	2.276	±	0.049	0.452	±	0.010	0.204	±	0.003	0.261	±	0.013	171.740	±	2.592	116.072	±	2.640	0.00217	±	0.00009	0.559	±	0.022	0.600	±	0.031
23	155.793	±	5.364	1.608	±	0.030	0.360	±	0.005	0.229	±	0.002	0.100	±	0.003	117.680	±	1.684	60.420	±	1.537	0.00170	±	0.00006	0.288	±	0.005	0.271	±	0.007
24	202.937	±	5.127	1.752	±	0.021	0.324	±	0.004	0.191	±	0.004	0.213	±	0.010	141.955	±	1.740	72.228	±	1.675	0.00290	±	0.00014	0.589	±	0.030	0.754	±	0.047

2014 Hybrids $F_{1\ (1,4,7,10,13,16,19,22)}$ – B line $_{(2,5,8,11,14,17,20,23)}$ R line $_{(3,6,9,12,15,18,21,24)}$

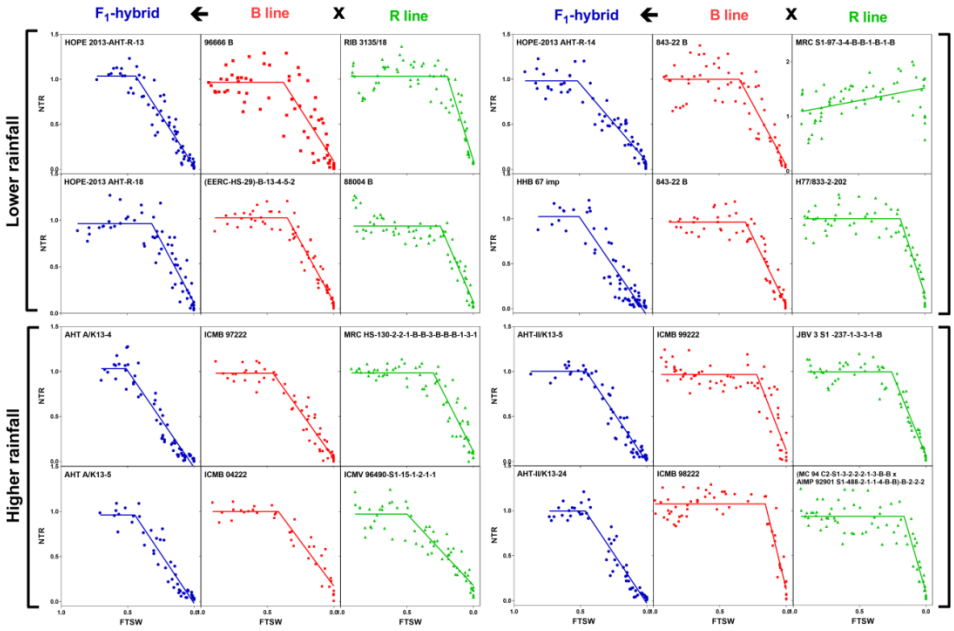
ID	Genotypes	ID	Genotype	ID	Genotype
Zone A1		*9*	*88004 B*	*17*	*ICMB 04222*
1	*HOPE 2013-AHT-R-8*	*10*	*HHB 67 imp*	*18*	*JBV 3 S1 -237-1-3-3-1-B*
2	*96666 B*	*11*	*843-22 B*	**Zone B**	
3	*RIB 3135/18*	*12*	*H77/833-2-202*	*19*	*AHT-II/K13-5*
4	*HOPE-2013 AHT-R-14*	**Zone A**		*20*	*ICMB 99222*
5	*843-22 B*	*13*	*AHT A/K13-4*	*21*	*ICMV 96490-S1-15-1-2-1-1*
6	*MRC S1-97-3-4-B-B-1-B-1-B*	*14*	*ICMB 97222*	*22*	*AHT-II/K13-24*
7	*HOPE-2013 AHT-R-18*	*15*	*MRC HS-130-2-2-1-B-B-3-B-B-B-1-3-1*	*23*	*ICMB 98222*
8	*(EERC-HS-29)-B-13-4-5-2*	*16*	*AHT A/K13-5*	*24*	*(MC 94 C2-S1-3-2-2-2-1-3-B-B x AIMP 92901 S1-488-2-1-1-4-B-B)-B-2-2-2*

Suplementary Table 3 Physiological parameters of F1- Hybrids evolved in higher rainfall zone (A1) and lower rainfall zones (A and B) during 2015.

ID	LA (cm^2)	TDW (g)	SDW (g)	RDW (g)	Root Shoot	Ex ($g.h^{-1}$)	SLA ($cm^2.g^{-1}$)	Ex-SDW ($g\text{-}h^{-1}.g^{-1}$)	Ex-RDW ($g.h^{-1}.g^{-1}$)
101	324.248 ± 6.750	2.766 ± 0.056	1.540 ± 0.028	0.989 ± 0.014	0.364 ± 0.002	0.141 ± 0.004	271.648 ± 3.419	0.098 ± 0.004	0.151 ± 0.006
102	298.372 ± 6.934	2.602 ± 0.050	1.433 ± 0.018	1.036 ± 0.020	0.426 ± 0.012	0.130 ± 0.004	260.001 ± 2.240	0.093 ± 0.003	0.135 ± 0.005
103	258.952 ± 4.600	2.299 ± 0.026	1.283 ± 0.009	0.751 ± 0.007	0.328 ± 0.001	0.149 ± 0.006	255.563 ± 1.109	0.112 ± 0.004	0.188 ± 0.007
104	257.448 ± 2.408	2.497 ± 0.027	1.378 ± 0.014	0.984 ± 0.012	0.395 ± 0.003	0.111 ± 0.003	233.252 ± 1.617	0.084 ± 0.003	0.117 ± 0.004
105	246.598 ± 4.042	2.552 ± 0.028	1.545 ± 0.030	0.921 ± 0.015	0.370 ± 0.007	0.153 ± 0.005	252.771 ± 5.907	0.105 ± 0.004	0.177 ± 0.006
106	298.520 ± 6.018	2.877 ± 0.024	1.552 ± 0.020	1.180 ± 0.019	0.409 ± 0.005	0.125 ± 0.006	228.198 ± 2.941	0.085 ± 0.004	0.117 ± 0.006
107	306.356 ± 10.047	2.669 ± 0.033	1.397 ± 0.006	1.042 ± 0.005	0.398 ± 0.004	0.094 ± 0.006	237.753 ± 3.402	0.068 ± 0.004	0.095 ± 0.006
108	308.218 ± 5.461	2.716 ± 0.017	1.376 ± 0.005	1.047 ± 0.009	0.386 ± 0.003	0.101 ± 0.004	230.292 ± 3.057	0.074 ± 0.003	0.099 ± 0.004
109	334.934 ± 7.030	2.702 ± 0.034	1.441 ± 0.012	1.089 ± 0.013	0.410 ± 0.005	0.123 ± 0.007	266.028 ± 3.067	0.084 ± 0.004	0.122 ± 0.008
110	290.312 ± 4.104	2.299 ± 0.017	1.099 ± 0.029	0.912 ± 0.016	0.408 ± 0.010	0.069 ± 0.002	245.145 ± 4.109	0.066 ± 0.002	0.080 ± 0.003
111	340.242 ± 5.704	2.856 ± 0.057	1.533 ± 0.030	1.137 ± 0.034	0.391 ± 0.006	0.166 ± 0.003	267.025 ± 3.582	0.115 ± 0.003	0.171 ± 0.006
112	340.824 ± 5.247	2.798 ± 0.050	1.492 ± 0.028	1.030 ± 0.019	0.367 ± 0.001	0.200 ± 0.005	264.301 ± 2.405	0.145 ± 0.006	0.212 ± 0.008
113	280.062 ± 0.759	2.454 ± 0.022	1.362 ± 0.005	1.165 ± 0.007	0.479 ± 0.004	0.079 ± 0.002	266.409 ± 3.750	0.059 ± 0.001	0.068 ± 0.002
114	340.850 ± 4.809	2.087 ± 0.087	0.988 ± 0.046	1.014 ± 0.027	1.339 ± 0.273	0.160 ± 0.006	747.338 ± 75.653	0.180 ± 0.044	0.198 ± 0.012
115	247.846 ± 2.658	2.643 ± 0.026	1.384 ± 0.009	0.951 ± 0.035	0.355 ± 0.011	0.127 ± 0.002	212.083 ± 5.730	0.093 ± 0.002	0.166 ± 0.007
116	341.684 ± 4.159	2.914 ± 0.031	1.511 ± 0.020	1.075 ± 0.016	0.371 ± 0.005	0.233 ± 0.009	245.137 ± 1.847	0.162 ± 0.007	0.237 ± 0.012
117	341.040 ± 9.892	2.959 ± 0.075	1.538 ± 0.038	1.021 ± 0.028	0.345 ± 0.004	0.179 ± 0.007	239.397 ± 2.543	0.116 ± 0.003	0.185 ± 0.006
118	289.562 ± 6.172	2.731 ± 0.051	1.459 ± 0.021	1.266 ± 0.044	0.460 ± 0.011	0.124 ± 0.001	239.718 ± 4.089	0.089 ± 0.002	0.114 ± 0.003

2015 Hybrids (F_1)

ID	Genotypes	ID	Genotype	ID	Genotype
Zone A1		**Zone A**		**Zone B**	
1	HOPE-2014 AHT-R-15	9	AHT II/K14-7	17	IHT B1 /K14-20
2	HOPE-2014 AHT-R-7	10	AHT A/K14-5	18	AHT II/K14-20
3	HOPE-2014 AHT-R-11	11	AHT II/K14-9	19	IHT B1 /K14-10
4	HOPE 2013-AHT-R-8	12	IHT A2 /K14-24	20	AHT-II/K13-5
5	HOPE-2013 AHT-R-14	13	AHT A/K13-4	21	ICMH 1201
6	HHB 67 imp	14	AHT A/K13-5	22	AHT-II/K13-24



Supplementary Figure 1. Individual profile of soil drying response of each genotype evolved in low and high rainfall zones of India. The upper panels show the dry down response of combinations [F_1 hybrids (blue), B-line or sterile male (red) and R-line or restorer (green)] bred in higher rainfall zone, and the bottom panels show the ones bred in lower rainfall zones. Each biological replicate is shown as a circle and it segmented regression is shown as a line. NTR: normalized transpiration rate, FTSW fraction of transpirable soil water.

TR transpiration rate, FTSW fraction of transpirable soil water

Chapter 5

Water flux patterns and aquaporin dynamics from transpiration demand to root hydraulics in Pearl millet hybrids.

Patrones de flujo de agua y dinámica de acuaporinas: desde la demanda transpirativa hasta la hidráulica de raices en híbridos de mijo.

Susan Medina[1, 2], Aparna Kakkera[1], P Sudhakar Reddy[1], and Vincent Vadez[1]

[1]International Crops Research Institute for semi-arid Tropics (ICRISAT), Crop Physiology Laboratory, Patancheru 502324, Greater Hyderabad, Andra Pradesh, India.

[2]Integrative Crop Ecophysiology Group, Plant Physiology Section, Faculty of Biology, University of Barcelona, Barcelona, Spain.

Article in preparation for further publication / Artículo en preparación para su publicación

Water flux patterns and aquaporin dynamics from transpiration demand to root hydraulics in Pearl millet hybrids.

Susan Medina[1, 2], Aparna Kakkera[1] , P Sudhakar Reddy[1], and Vincent Vadez[1]

[1]Crop Physiology Laboratory, International Crops Research Institute for Semi-Arid Tropics, Patancheru, India.

[2]Integrative Crop Ecophysiology Group, Plant Physiology Section, Faculty of Biology, University of Barcelona, Barcelona, Spain.

ABSTRACT

Water saving traits matter for adapting to water stress in pearl millet and previous studies showed that these traits may relate to water transport in the root cylinder, involving aquaporins via their putative role in influencing plant hydraulics. There is genetic variation for these traits, a variation that also depends on the breeding history. Here we confirm that water saving traits – i.e. the transpiration response to increasing VPD and plant growth under high VPD conditions – differed with the breeding history. Then we test the relationship between water saving traits and water transport pathways in the root cylinder, first by testing the transpiration response to aquaporin inhibition, then by assessing the effect of pressurizing the root system on plant transpiration, by measuring the root hydraulic conductivity of contrasting genotypes, and then by assessing the pattern of transcript abundances of three aquaporins under high VPD conditions. This work was done in 4 hybrids bred in higher and lower rainfall zones of India. The hybrids bred for lower rainfall increased their transpiration rate more than the higher rainfall hybrids when the root system was pressurized; this former group also showed lower root hydraulic conductivity. The root growth of lower rainfall hybrids was superior exhibiting higher size, tips, root hairs, metaxylem vessel number and thinner endodermis cells than higher

rainfall ones; similarly its aerial growth under high VPD was also higher. The lower rainfall hybrids showed higher up-regulation of PIP 2;3 in roots and down-regulation in leaves than lower rainfall hybrids; both groups exhibited similar transcript profiles of PIP 2;6 and TIP 2;2 and the transpiration decline following aquaporin inhibitor application was similar in both group, despite a non-significant trend for a higher inhibition in the lower rainfall hybrids. These features suggest the breeding history had indeed an influence on the physiology of water transport in plant, involving root anatomical development and dependence on aquaporin in the root water transport pathways.

Keywords: Aquaporin, transpiration, plant hydraulics, gene expression, aquaporin inhibition, xylem, growth, pearl millet, PIP 2;3.

1 Introduction

Pearl Millet (*Pennisetum glaucum L*.) is the second crop in India with nutritional and agro-economic importance; it is one of the most adapted crops in arid environments, especially to conditions like the north regions of this country where the rainfall level is variable. It is also a critical crop for Sahelian regions where virtually no other crop can stand. In India pearl millet is bred for contrasting rainfall zones. Among these is a low rainfall zone known as zone A1 with an annual level of 320-400 mm (situated in the North most arid part of India, covering the regions of Western Rajasthan, and parts the states of Haryana and Gujarat), and a high rainfall zone known as zone B with annual level of 400-520 mm (situated in the Peninsular Indian states of Maharashtra, Tamil Nadu and Karnataka) (Manga and Kumar, 2011; Rai *et al.*, 2015; Vadez *et al.*, 2015). Those variations in water regimes also come with differences in the soil composition and in the nutrient availability of these soils. An earlier report (Medina et al., in preparation) indicate that pearl millet hybrids bred for these different rainfall zones do vary for traits that alter plant water usage, and in particular for the capacity of plants to control stomata opening under conditions of

high evaporative demand. The transpiration response to increases in vapour pressure deficit (VPD) has been indeed shown to play an important role in the adaptation to water stress in several plant species (Vadez *et al.*, 2013*b*), and especially in pearl millet (Kholová and Vadez, 2013). This trait is hypothesized to be related to the hydraulics characteristics of the plants (Vadez, 2014), and in particular the root hydraulics in which aquaporins appear to play a critical role in its regulation (Gambetta *et al.*, 2012; Liu *et al.*, 2014, 2015). The root anatomy also appears to have a role in the regulation of the root hydraulic capacity (Lynch and Brown, 2012; Vadez, 2014) Therefore, understanding the interplay between aquaporins expression, root anatomy, their combined role on root hydraulics, and how they may influence the transpiration response under high evaporative demand, is important, especially if these traits are to be used in a breeding context.

Root hydraulics determines water uptake intensities and water potential gradients within the plant. Its dynamics contribute in many nutritional and growth functions which are integrated with water-saving responses (Maurel *et al.*, 2010). When transpiration rates are high, the apoplastic path will be used together with water transport through the symplastic pathway, allowing a coarse regulation of water uptake, and the root hydraulic resistance will be low allowing a rapid uptake of water. Opposite, when transpiration rates are low, the apoplastic path will be less used and the hydraulic resistance will be high. Recent work in sorghum shows that transpiration increased to a different extent in genotypes when the roots were pressurized to partially lift their hydraulic resistance (Choudhary and Sinclair, 2014). Then, the role of water channels (aquaporins) in the cell-to-cell path allows a fine adjustment of water flow or a regulation of water uptake (Steudle and Peterson, 1998; Steudle, 2000; Hose *et al.*, 2001; Javot and Maurel, 2002; Vadez, 2014).

Plant aquaporin are membrane channels proteins that facilitate the transport of water and small neutral molecules across biological membranes which may contribute to several plant growth and developmental processes (Li *et al.*, 2014). Aquaporins are major intrinsic proteins (MIPs) which have larger diversity of isoforms

and cellular localizations, they allow water or other small uncharged molecules to pass along the osmotic gradient, defining a single pore (Kruse *et al.*, 2006). They may be regulated by an electrostatic potential allowing rapid water flux, whereas a negative potential reduces the water permeability (Hub *et al.*, 2010). The Plasma membrane Intrinsic Proteins (PIPs) are located in the plasma membrane while the Tonoplast Intrinsic Proteins (TIPs) are in the vacuolar membrane. These two aquaporin families will be studied in this research, due to its abundances in the major pathways for transcellular and intracellular water transport (Hub *et al.*, 2010). Aquaporins play a major role in the fine regulation of root hydraulics and nutrient transport in roots and leaves during whole plant growth and development stages (Forrest and Bhave, 2007), they are also involved in the conductance of xylem and transport of dissolved gases such as carbon dioxide or boric or silicic acid (Kaldenhoff *et al.*, 2008; Li *et al.*, 2014). Aquaporin over-expression is a widely used strategy to understand plant water relations under stress. Previous studies of its transcript abundances described elsewhere (Li *et al.*, 2014) showed that high aquaporin expression on transgenic plants may confer either higher resistance or higher sensitivity to stress (Maurel, 2007; Li *et al.*, 2014). Aquaporins can be inhibited by metal compounds such as mercury or silver (Nardini and Salleo, 2005), and these pharmacological treatments are often used to alter water fluxes in plant, the hydraulic conductivity, root architecture, and water relationships of plants (Nardini and Salleo, 2005). Mercurial compounds as $HgCl_2$ inhibit aquaporin in the cell-cell path by a steric mechanism which binds the aquaporin structure leading to channel inhibition (Niemietz and Tyerman, 2002; Savage and Stroud, 2007). And silver as $AgNO_3$ is also a potent inhibitors of the water permeability in the plasma membrane of root cells (Niemietz and Tyerman, 2002).

Roots architecture traits, such as root depth and/or root length density (RLD), are considered to be important for drought adaptation, and may be consistent with water extraction in depth soils although they do not always explain the degrees of differences in yield under stress (Ho *et al.*, 2005; Vadez, 2014). Root hairs and small xylem diameters also may improve root acquisition of water (Segal *et al.*, 2008;

Comas *et al.*, 2013), and N uptake (Lynch, 2013). A study in wheat also showed that root metaxylem sizes could largely influence the root hydraulic conductance (Passioura, 1983), and this led to the development of wheat cultivars with better adaptation to water stress (Richards, 2006). Whether anatomical differences in the xylem vessels could also be related to the transpiration response to VPD has not been tested in pearl millet, although differences in the endodermal cells between lines contrasting for the capacity to restrict transpiration under high VPD have been shown (Kholová *et al.*, 2016), opening the possibility of root anatomical differences leading possibly to differences in plant adaptation to water stress.

The objective of this study was then to test linkages between earlier reported water saving traits and features of the root hydraulic characteristics, involving measurement of the degree of dependence on the aquaporin-mediated pathways in the root cylinder, transpiration response upon root pressurization, and pattern of aquaporin transcript abundance under high vapour pressure deficit. This work was done in pearl millet hybrids bred for different rainfall zones, earlier reported to vary for water saving traits.

2 Material and methods

Hybrids of Pearl Millet (*Pennisetum glaucum L.)* bred for two very contrasting agro-ecological scenarios of India: lower (LR) and higher (HR) rainfall zones were assessed. These were 4 genotypes, 2 developed for the high rainfall Zone B (HR: AHT-II/K13-24 and AHT-II/K13-5), and 2 bred for the low rainfall Zone A1 (LR: HOPE 2013-AHT-R-14 and HOPE 2013-AHT-R-8). These four hybrids were assessed among three experiments in controlled conditions.

The first glasshouse experiment to test effect of inhibitors over the aquaporins in the cell-cell water transport path was an assessment of the Transpiration response (TR) to aquaporin inhibition under high evaporative demand (Exp 1) where the plants were grown in hydroponic system and tested in growth chambers. A second

experiment (Exp.2) to assess possible root hydraulics limitations for transpiration under increasing evaporative demand of higher and lower rainfall hybrids was conducted by assessing the TR response to root pressurization. A third experiment (Exp.3) to elucidate the possible role of three aquaporins in the water flow was assessed by measuring the aquaporin gene expression pattern in root and shoot under high evaporative demand. Finally, a fourth experiment (Exp.4) to assess the canopy development was assessed in the LeasyScan platform to assess the leaf development. The three first experiments (Exp.1, Exp.3 and Exp.3) were assessed in vegetative stage with five biological replications per genotype and treatment, while Exp.4 was assessed with three biological replicates , and assayed in a complete randomized design during the period February-April 2016, at ICRISAT campus in Patancheru (India): latitude 17°30´N; longitude 78°16´E; altitude 549m.

2.1 Experiment 1: Transpiration response (TR) to Aquaporin inhibition under high evaporative demand conditions

The purpose of this experiment was to assess the extent of transpiration inhibition upon aquaporin inhibitor treatment in pearl millet hybrids bred for different rainfall zones and known to contrast in their response to increasing vapour pressure deficit (VPD) conditions. The plants (n_1=60) grew during February–March 2016 in controlled hydroponic glasshouse environment (17-35°C/ 70-35%RH), the seeds germinated during 7 days in moisturized sand with nutrient solution (Hoagland solution pH 6-6.3: ($MgSO_4$ (2.05mM), K_2SO_4 (1.25mM), Fe-EDTA (0.04mM), $CaCl_2$ (3.3mM), KH_2PO_4 (0.5 mM), H_3BO_3 (4uM), $MnSO_4$ (6.6µM), $ZnSO_4$ (1.55µM), $CuSO_4$ (1,55µM), $CoSO_4$ (0.12µM) and Na_2MoO_4 (0.12 µM) diluted in deionized water). Then plantlets were transferred to the hydroponic system (500 ml flask filled with nutrient solution and aeration (0.5 KPa)), the solution was refilled every morning and completely replaced every third day. At 25 days after sowing (DAS), the TR to aquaporin inhibitors was tested under high VPD conditions. First, the plants in flasks were transferred to a growth chamber Conviron E-15 (Controlled Environments, Winnipeg, MB, Canada)

for overnight acclimation at VPD conditions of 0.5KPa (23°C/ 80%RH) with the same aeration as in the growing stage. The next day, light started at 6 am reaching to full light at 8:30 am, while the VPD was progressively increased, reaching a value of 3 Kpa (36°C/ 55%RH) at 8:30 am, the assay had three 2h periods: baseline (8:30-10:30 h), inhibition (10:30 -13:30 h) and recovery (13:30 -15:30 h) and the transpiration was recorded each 20 min by weighing all flasks, while VPD was maintained in 3KPa during all the experiment. The aquaporin inhibitors $HgCl_2$ and $AgNo_3$ were added (500 µl to each flask; $HgCl_2$ stock (100mM) and $AgNo_3$ stock (10mM)) at the end of baseline period reaching a final concentration of 100µM and 10µM respectively and tested during two hours. Finally, for recovery all flasks contents were replaced with 500 ml of new nutrient solution. Each time, controls and treated plants were assessed. At the end of the last period, the stem was cut and the xylem exudate was collected during 20 min in pre-weighted tubes with absorbent paper inside. Subsequent leaves and roots were collected, leaf area was measured with a Leaf area meter (LA meter LI3000 model, Li-Cor, Licoln, Nebraska, US) and the roots were carefully washed with dionized water and scanned with the WinRhizo^{TM} (WinRizho TM Pro, Regent Instruments Inc., Quebec City, Canada); finally all organs were dried at 60°C in an oven during 72 hours.

2.2 Experiment 2: TR response to a root pressurization

The purpose of this experiment was to measure the extent of transpiration increase in plants whose root system would be pressurized to lift a putative hydraulic limitation. Plants (n_2=80) grew during March 2016 in controlled glasshouse environment (18-35°C/ 70-35% RH), seeds were sown in cylindrical polystyrene bags containing 1.5 Kg of substrate (vertisol-sand 1:1) protecting root parts from light, watered each third day with 50% nutrient solution (described in Exp 1.). At 30 DAS, one half of the plants were transferred to pressure pots similar to those used earlier (Choudhary and Sinclair, 2014), removing the plastic bag and filling the empty space with soil, caring not to disturb the root system. Then those plants were watered and

acclimatized without sealing the lid during two days, subsequently the lid was sealed. Likewise the other half of the plants (controls) were transferred to similar pots, although not allowing to be pressurized. The soil surface of these control plants was covered with a layer of polystyrene sheet and plastic beads. The following morning (33 DAS) the pots to be submitted to pressure were sealed. Both control and pressure pots were then weighted in a bench electronic balance (FBK, Kern & Sohn GmbH, Balingen, Germany) every two hours to measure transpiration. After these initial two hours, a positive pressure was applied to the pressure pots to increase the hydrostatic water potential in the leaf xylem (Sinclair *et al.*, 2008), first during a 2 h period under 0.15 MPA and a subsequently during a second 2h period at 0.25 MPa. At the end of each of these periods, the pot weight was recorded in both pressurized and non-pressurized plants. Differences in weight allowed calculation of TR before pressurisation and within each positive pressure period, which were to be compared to the transpiration under environmental conditions in non-pressurized pots. After the last weighing, all plants were harvested and the leaf area of each plant was measured using an area meter (Model LI-3100, Li-Cor, Lincoln, NE, USA) to calculate the transpiration rate (TR). To estimate the limitation of the whole plant conductance in TR response, in the TR of pressurized plants was normalized by the TR of control plants. At the end of the experiment, the root hydraulic conductivity was estimated by removing the shoot of the plant (which had rested during 30 min after the last pressure level), leaving a stem segment of ~2cm length above the top of the pot lid. This stem segment was attached to 12cm long tube filled with cotton (pre-weighted), which was used to collect exuded xylem sap when pressure was applied to the pot and stored; three consecutive levels pressure were applied: 0.05, 0.15 and 0.25 MPa during 10 min each one. Xylem flux was determined from the amount of exudate collected over 10 min while pressure was applied. While in control plants no pressure was applied and only the root exudate was collected at ambient conditions during 10 minutes. At the end of the measurements, all roots were carefully washed with distilled water, scanned and measured with WinRhizo software to calculate the root length (RL). Root hydraulic conductance was the

relation between the flow rate of xylem by RL and the driving force (0.05, 0.15 and 0.25 MPa).

Additionally, in all roots a 2 cm segment removed from mid part of the longest root and stored in saline solution (NaCl 0.85%) and cut in fine slides, then root and aerial matter were dried and tubes with xylem exudate were weighted. All measurement was performed as in Exp 1. Root slices were stained with Acidic Fuchsine dye (5%) and visualized in microscopy at 10 X; further observations were processed in Image J software (https://imagej.nih.gov/ij/).

2.3 Experiment 3: TR to evaporative demand and Aquaporin expression

The purpose of this experiment was to assess the degree of aquaporin gene expression in plants exposed to an increased evaporative demand, in genotypes contrasting in their transpiration response to increasing VPD. Plants (n_3=80) grew during February-March 2016 period in glasshouse as in Exp 2. Seeds were sown in 8 Kg pots with sand, watered each two days with nutrient solution (as in Exp 1.). At 30 DAS all the plants had fully developed 6 leaves; they were fully irrigated (soft water), then sealed with a layer of plastic sheet and beads and drained overnight. The following day the TR to evaporative demand was assayed, first pots were acclimatized overnight in two growth chambers Conviron E-15 (Controlled Environments, Winnipeg, MB, Canada) at 0.5 VPD conditions (23°C/ 80% RH); equal set of plants in each chamber. The next day, one chamber was set under constant low VPD (1KPa: 25°C/ 70% RH), while the other was set under a VPD ladder of 1-4 KPa by changing temperature and humidity from (25°C/ 70% RH) to (40°C/ 45% RH). The plant transpiration was recorded by weighing each pot in an electronic bench (FBK, Kern & Sohn GmbH, Balingen, Germany) from 7:30 am to 4 pm, considering as baseline the first period from 7:30 to 8:30 where VPD reached 1KPa. Then TR response of plants subjected to a ladder of VPD were normalized by plants assayed in constant VPD. The aquaporin expression was assessed considering three target times (fig 3): (i) the initial low VPD point (M_L and M_H) of 1KPa at 8:30 h in both chambers at

morning, then in the afternoon at (ii) the final point (A_L) of constant low VPD period at 15:45 h (low VPD chamber) and (iii) the final point (A_H) of high VPD period at 15:50 h (chamber of VPD ladder). At these moments (A_L, A_H, M_L and M_H) leaf and root tissues samples were collected and immediately frozen in liquid nitrogen at -80°C. In leaves, a central part was cut from the last fully developed leaf which was previously measured the day before, then the plant was harvested and the root was immediately washed with deionized water and the central part of the root pool was sampled. After that, leaf area was measured and aerial matter was dried as in Exp 1 and 2.

For the gene expression assay, all frozen root and leaf tissues (n=160: 80 of C, 40 of T1 and 40 of T2) were ground in liquid nitrogen and subsequently RNA was isolated from 100 mg of tissue using the NucleoSpin® RNA Plant Kit (Macherey-Nagel, GmbH & Co. KG, Duren, Germany) following the manufacturer´s instructions, this kit included DNAase to eliminate residual genomic DNA. RNA was integrity was verified by electrophoresis in 1.2% Agarose gel, then quantified with Qubit® HS RNA Assay Kit (Thermo Fisher Scientific Inc, Massachusetts, USA) and stored at -70°C. Total RNA (500 ng) was used for cDNA synthesis using M-MuLV Reverse Transcriptase, Oligo d(T)23VN nucleotides and the RNase Inhibitor M0314S (New England Biolabs Inc., Massachusetts, US) following manufacturer´s instructions and stored in a ditulion of 10 ng/µl at -20°C. Three technical replicates were analysed per biological replicate while primer efficiency was validated in previous studies (Reddy *et al.*, 2015*a*,*b*); the primers encoding for housekeeping genes EIF4α, EF-1α and ACP, and target aquaporin genes PIP2;3, PIP2;6 and TIP 2;2 listed in Supplementary Table S1.

The qRT-PCR assay was performed using a Realplex Real- Time PCR system (Eppendorf, Germany) and SYBR Green mix (Bioline Reagents Limited, London, UK) in 96 optical-well-plates (Axygen, USA) sealed with ultra-clear sealing film (Platemax) in a reaction volume of 10µl; 5µl of 2x SensiMix SYBR No ROX mix (Bioline), 400 nM of each gene-specific primer, 1µl of diluted cDNA (1:10) and nuclease-free water. The thermal profile was as follows: initial denaturation of 2 min at 95°C, PCR cycling (40

cycles) of 15 s at 95°C and 30 s at 62◦C with fluorescent signal recording, and a final step of 15 s at 72 °C . After amplification, melt curves were generated for each reaction to ensure specific amplification. All qPCR reactions, including the non-template control, were performed technical triplicates. The final cycle threshold values (C_t) were recorded. The internal control genes encoding were used to normalize qRT-PCR results, which were widely used in previous reports (Reddy *et al.*, 2015*a*,*b*). The relative expression was analysed using the comparative Ct method (Schmittgen and Livak, 2008) as the changes between the expression of the target and reference genes (ΔC_t) using fold expression $2^{-\Delta Ct}$ and represented as a $\log_2$ transformation of the expression ratio. The comparison within treatments (C_1, C_2, T_1 and T_2) was expressed as fold change accounting $2^{-\Delta\Delta Ct}$ values (Schmittgen and Livak, 2008).

2.4 Experiment 4: Growth under high VPD conditions

The fourth experiment was meant to assay and confirm differences in aerial development found earlier, and putatively due to differences in the root capacity to uptake and transport water. The trial was performed outdoors during March-April 2016 (Exp.4) under naturally fluctuating high VPD conditions in the LeasyScan phenotyping platform facility (Vadez et al., 2015). This season usually have high temperatures and low RH%, giving a high VPD condition (~5 Kpa). Six biological replicates were assessed per each genotypes (n_4=24), this platform is a laser scanner-based technique providing 3D point clouds from which plant parameters, including leaf area and plant height . Those parameters together with temperature and RH (20-39° /20-70 RH%) were recorded each 120 minutes by the platform sensor set (PlantEye F300, Phenospex, Heerlen, The Netherlands). The seeds were sown in 15 Kg pots filled with red soil; twelve days after, each pot was thinned leaving two plants per pot. The pots were automatically watered every 1-3 day with soft water; the plants were grown during 48 DAS. The environmental temperature range was 16-41 °C and the relative humidity range was 12-87 RH%, with one rainfall day of 5mm leading a range of VPD values of 1.0-6.5 KPa during the time frame of the

experiment. The data of the parameters mentioned above was extracted from the platform data base to calculate the daily growth rate (unit) for each day at 20°C (Parent and Tardieu, 2012). The relationship between the measured and scanned leaf area was validated with the reported transformation LA3d= 0.22LA+ 241 (Vadez *et al.*, 2015), where y is the 3D leaf area (measured by the scanner) and LA was the observed leaf area measured with Li 3000 leaf area meter. To fit the data of leaf area and plant height in growth curves we applied a sigmoidal regression, and at the exponential growth phase a linear regression for leaf area, then the curves were compared among the rainfall zones.

2.5 Statistical analysis

To fit the data of TR response under aquaporin inhibitors In Exp.1, the TR of treated plants was normalized by control plants (non-treated) transpiration, the response to the inhibition was analysed by AUC (Area under the curve) test with 1000 iterations, where this model accounts the inhibition as the area under the transpiration of control plants in the 2D plot of NTR vs. time frame for each inhibitor. For the TR response to VPD and pressure in Exp.2, the transpiration of pressurized plants was normalized by control plants at both pressure periods. Similarly for the xylem exudate flow of pressurized roots was also normalized by control roots (non-pressurized) at each period of applied pressure, then the hydraulic conductivity was calculated as the slope of the linear regression of exudate flow and pressure applied. Both normalized transpiration and root hydraulic conductance slopes in Exp.2; as growth parameters and aquaporin expression rates in Exp.1 and Exp.3 were compared between the two groups of hybrids (low vs high rainfall) assessing LSD test ($p<0.05$).

To fit the data of TR and VPD levels in Exp.3, the baseline transpiration of plants at constant low VPD was used to normalize the transpiration when submitted to a VPD ladder. Then we applied a Two-segment linear regression (Y1=slope1.X + intercept 1 and Y2= slope2.X + intercept2) with 1000 iterations, and then the slopes and intercepts were compared among rainfall categories and treatments. While

aquaporin gene expression was calculated with comparative Ct method, and the expression relative to control genes was submitted to a $\log_2$ transformation in order have a linear scale for the values, and then all comparisons within treatments were performed with LSD test. To compare growth in Exp.4 we fitted the data of whole growth period within sigmoidal nonlinear regression, and linear regression for exponential growth phase, and then curves between rainfall categories were compared ($p<0.05$).

All AUC analysis, linear and nonlinear regressions and comparison in Exp.1, Exp.2, Exp.3 and Exp.4 were performed following provider considerations using GraphPad Prism (version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). The LSD (Least significant difference, $p<0.05$) and ANOVA analysis in all experiments to compare between higher and lower rainfall zones, accounting all data replications in each experiment were performed using R (R Development Core Team, 2008). In all analyses, a $p<0.05$ threshold was considered for significant differences and all bars show letters which indicate the mean comparison from LSD test.

3 Results

3.1 Inhibition of transpiration response

The transpiration response (TR) of plants significantly decreased due to the addition of inhibitors such as $HgCl_2$ and $AgNO_3$ (Exp.1) in the four hybrids tested (Supplemental fig 1S), without TR recovery after the inhibitor was replaced by nutrient solution. Clear differences were found between the TR restriction of Hg Cl_2 (25-50% over the NTR of control plants) (fig 1, A) and the transpiration restriction due to $AgNO_3$ (fig 1, B) which was only decreased by about 25% in all hybrids belonging to both rainfall zones. No differences were found between hybrids bred in low (LR) and high (HR) rainfall zones, in both cases the AUC rate was similar, although the AgNO3 effect seemed to be slightly lower in HR hybrids.

The transpiration rate in the control period was similar for hybrids bred in both zones (fig 1, A, B). The comparison of exudate flow normalized to root length (RL) (fig 1, C) showed a higher xylem flow of high rainfall hybrids (HR) in control conditions. Then the xylem flux in both groups was similarly limited by both inhibitors, being $HgCl_2$ the most aggressive and higher rainfall hybrids the most sensitive. The exudate flow was not significantly different after $AgNO_3$ treatment.

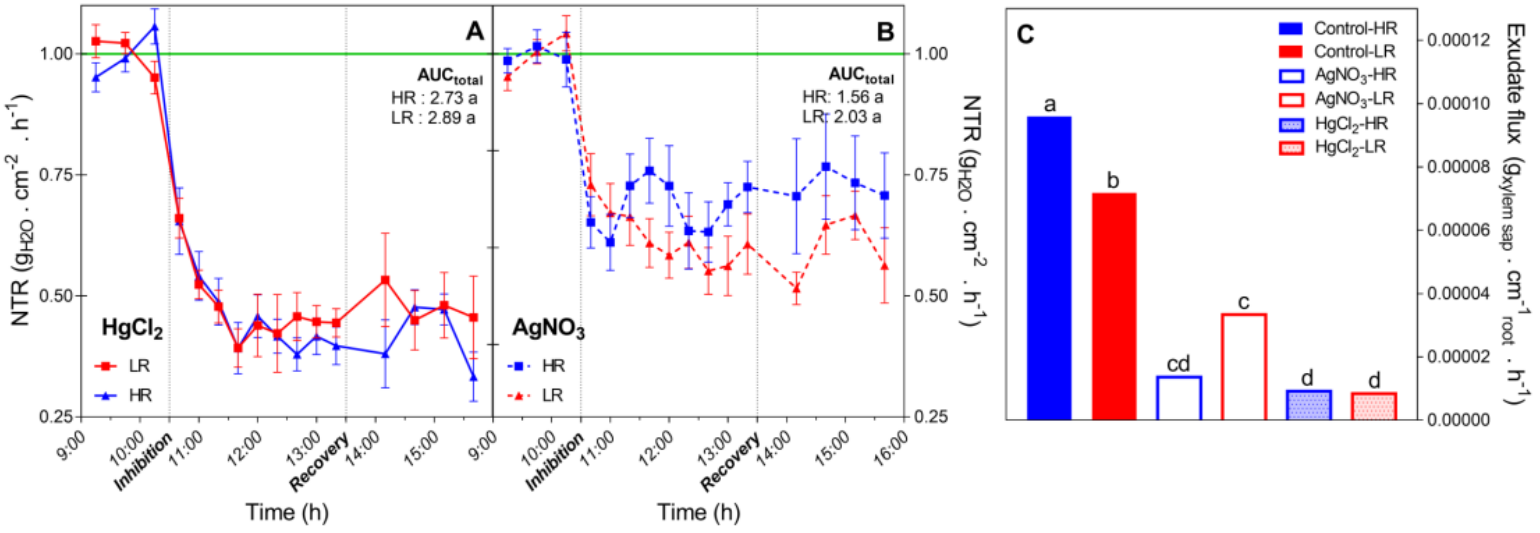


FIGURE 1. Aquaporin inhibition in hybrids bred in high (HR) and low (LR) rainfall zones. The transpiration response inhibition of HR and LR plants submitted to two inhibitors such as $HgCl_2$ (A) and $AgNO_3$ (B) with respect to non-inhibited plants (baseline-green) was analyzed by AUC test which accounts the area of the curve below the baseline. The transpiration rate of HR and LR (C) hybrids during the period before the inhibition, and the exudate flow relative to root length (D) are shown, both analyzed by LSD test (*p< 0.05)*. Letters indicate significant differences.

3.2 Hydraulic conductance limitations

The transpiration response with pot pressurization (Exp.2), showed a large increment of transpiration when the roots were put under pressure. The normalized transpiration (NTR) due to pressure (fig 2, A), i.e. the increase in transpiration compared to non-pressurized controls, increased by about 50 to 100% across hybrids and pressure treatments. Hybrids bred in low rainfall zone showed higher NTR than higher rainfall when the increment of root pressure reached 0.25 MPa, whereas in 1.5 MPa the same trend was visible although both groups' NTR were not significantly different. After the transpiration response to root pressure, the root hydraulic conductivity was calculated by assessing the exudate flow rate in response to root pressurization (fig 2, B). The slope of that response, normalized to root length provided the root hydraulic conductivity. This hydraulic conductivity was significantly lower in hybrids bred in low rainfall zone (slope: 105 g cm^{-1} h^{-1} MPa^{-1}) than in high rainfall hybrids (slope: 74).

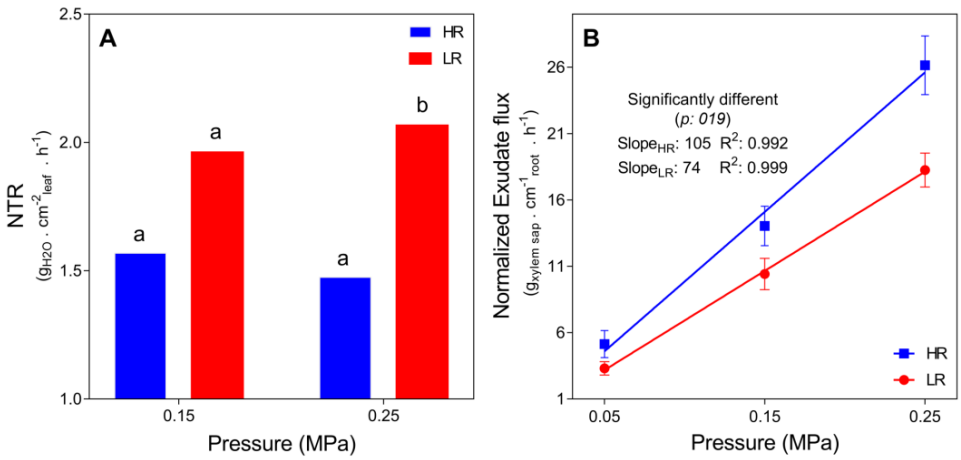


FIGURE 2. Hydraulic response in high (HR) and low (LR) rainfall hybrids to increments in root pressure. The limiting hydraulic conductance in leaves is presented as the normalized transpiration (NTR) response of HR and LR plants under two levels of increased root pressure (0.15 and 0.25 MPa) is shown in A; letters indicate significant differences analyzed by LSD test ($p<0.05$). The hydraulic conductance on both rainfall hybrids (B) is shown as the slope of increment of xylem flux normalized by root length, at each increased pressure level (0.05, 0.15 and 0.25 MPa). Curves were assessed by Linear Regression and compared with F test ($p<0.05$).

3.3 Transpiration dynamics under high and low VPD and aquaporin gene expression

The transpiration response (Exp.3) of high (blue) and low (red) rainfall hybrids under a ladder of VPD [(morning (M_L -2KPa) to afternoon (A_H - 4.5 KPa)] was normalized by their transpiration at constant low VPD [(morning (M_L -2KPa) to afternoon (A_L - 2 KPa)] showed in figure 3A. This normalized transpiration (NTR) showed a higher slope of increase upon increasing VPD levels (0.73) in low rainfall hybrids than in the high rainfall hybrids (slope: 0.44); while at low VPD their slopes were similar (0.09 and 0.08 respectively) which is shown in figure 3A.

The aquaporin gene expression assayed through TR-qPCR in leaves and roots at these morning and afternoon points (green circles), represented in figure 3A, showed that: (i) In general there was an up-regulation of PIP 2;6 and TIP 2;2 transcript abundances (Exp.3) with respect to control genes (Fig. 3B) of hybrids bred in high (HR) and low (LR) rainfall zones in leaves and roots; also there was a remarkable higher expression of TIP 2;2 in leaves and roots. By contrast, PIP 2;3 was only slightly up-regulated in the roots and down-regulated in the leaves. (ii) The comparison between expression under high (A_H) and low (A_L) VPD in the afternoon (Fig. 3B) showed a higher expression in the leaves under high VPD conditions for PIP 2;6 and TIP 2;2; while in roots it was opposite. In the case of PIP 2;3 under high VPD (A_H), its expression in LR hybrids was lower in the leaves and higher in the roots, whereas in HR genotypes it was the opposite (Fig. 3B). (iii) During the morning under low VPD (M_H and M_L), PIP 2;6 and PIP 2;3 transcript abundances were higher in the roots of HR plants, and also transcript abundances of TIP 2;2 were higher in both tissues of HR plants; by contrast the expression of PIP 2;3 in the leaves of HR hybrids was lower (Fig. 3B). Hence, comparing between afternoon and morning expression (supplemental table 2S - upper panel): PIP 2:3 transcript abundance was superior during the morning, for PIP 2:6 the timing expression was variable; and the morning expression of TIP 2;2 under constant low VPD was lower in LR hybrids in both tissues and higher in HR ones only in leaves.

Finally, the fold change in expression under high VPD conditions relative to low VPD in the afternoon (fig 3, C) exhibited that: (i) Both groups of hybrids expressed PIP 2;6 around two fold times more in the leaves, where expression in hybrids bred for low rainfall zone was slightly higher; and in roots both groups' expression was 1.2 folds less. (ii) Similarly, TIP 2;2 over-expression (around 1 fold) in leaves was higher in hybrids bred for high rainfall zones than for low rainfall. The under-expression of TIP 2;2 (around 1.5 folds) in the roots was slightly higher in lower rainfall hybrids. (iii) Interestingly for PIP 2;3 fold change in leaves, the hybrids bred in higher rainfall zone expressed 1.4 folds more, while lower rainfall hybrids expressed 1.1 folds less. It was the opposite in the roots, where high rainfall hybrids under-expressed this aquaporin by 6 folds while lower rainfall ones over-expressed it by 1.1 folds. The significance of these comparisons is shown in supplemental table 2S.

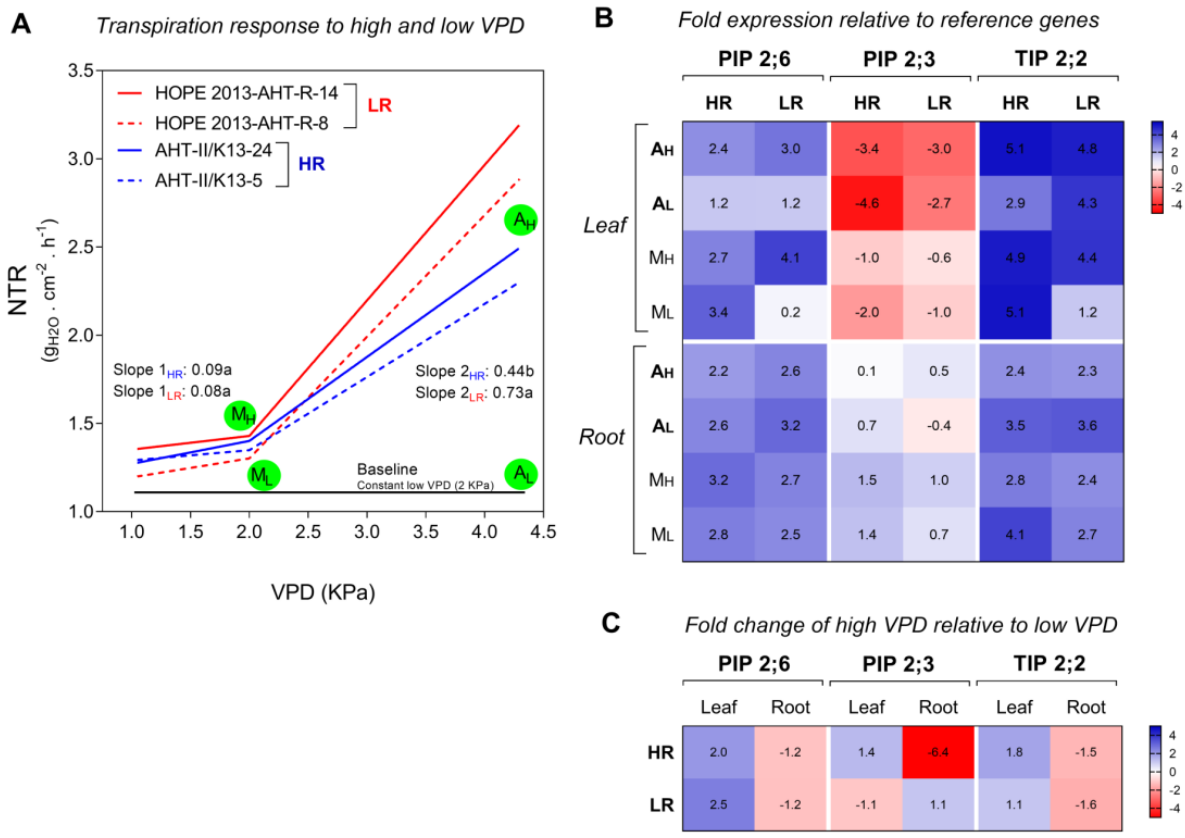


FIGURE 3. Aquaporin gene expression leaf and root tissues within transpiration response to high and low VPD of high (HR) and low (LR) rainfall hybrids. Panel A shows the normalized transpiration (NTR) responses of HR (AHT-II/K13-24 and AHT-II/K13-5) and LR (HOPE 2013-AHT-R-14 and HOPE 2013-AHT-R-8) plants assessed from morning (M) to afternoon (A) under a ladder of VPD [M_L (2KPa) and A_L (4KPa)], and normalized by its response at constant low VPD [M_C and A_C (2KPa)]; represented with red and blue curves differences ($p<0.05$) in slopes were analysed by Segmented regression and indicated with letters. Panel B shows these aquaporins expression related to reference gene which represented as $\log_2(2^{-\Delta Ct})$ during the morning and afternoon under a ladder of VPD (M_H and A_H) and constant low VPD (M_L and A_L) conditions. Panel C shows the fold change expression of aquaporins PIP 2;3. PIP2;6 and TIP 2;2 of high VPD respect to low VPD on the afternoon (A_H/A_L) of HR and LR hybrids; expressed as $\log_2 2^{-\Delta\Delta Ct}$ in leaf and root tissues. For detail in fold expression see Supplemental table 2S.

3.4 Plant growth: roots and aerial features

The root development in low rainfall hybrids, measured in Exp.1 in hydroponic system and Exp.2 grown in sand and soil (1:1), was superior to that in higher rainfall hybrids. Roots of low rainfall hybrids showed greater dry biomass (RDW), length (L), surface area (SA), volume (V), hair tips (T) and forks, as higher development of very small root hairs [small hairs (L, SA, V and T between 0 and 2) and high size (T, V, SA between 2 and 4) values of corresponding magnitudes] described in table 1 (Exp.1 and Exp.2). Moreover the root slides (fig 4, A-D) revealed wider endodermis cells (fig 4, E-I) and higher number of metaxylem vessels (MX, fig 4, J) in hybrids bred in low rainfall zones.

Assuming that a higher root development of lower rainfall genotypes, and its higher PIP 2;3 aquaporin abundances under high VPD can lead to a higher water and nutrient transport to the shoot, so this fact may produce higher development of the aerial part of the plant. So we tested both groups of hybrid under high VPD conditions outdoors. The aerial development (Exp.4) under high VPD conditions of hybrids bred for low rainfall showed significantly higher leaf area (fig 4, K) than higher rainfall plants, the slope of the former group at the exponential phase being higher (2.63) than the latter group (1.45). Also the plant height was significantly higher in low rainfall hybrids (data not shown). Moreover this low rainfall plants showed superiority in LA, stem dry weight (SDW) and total dry weight (TDW) reported in table 1 (Exp.1, Exp.2 and Exp.3).

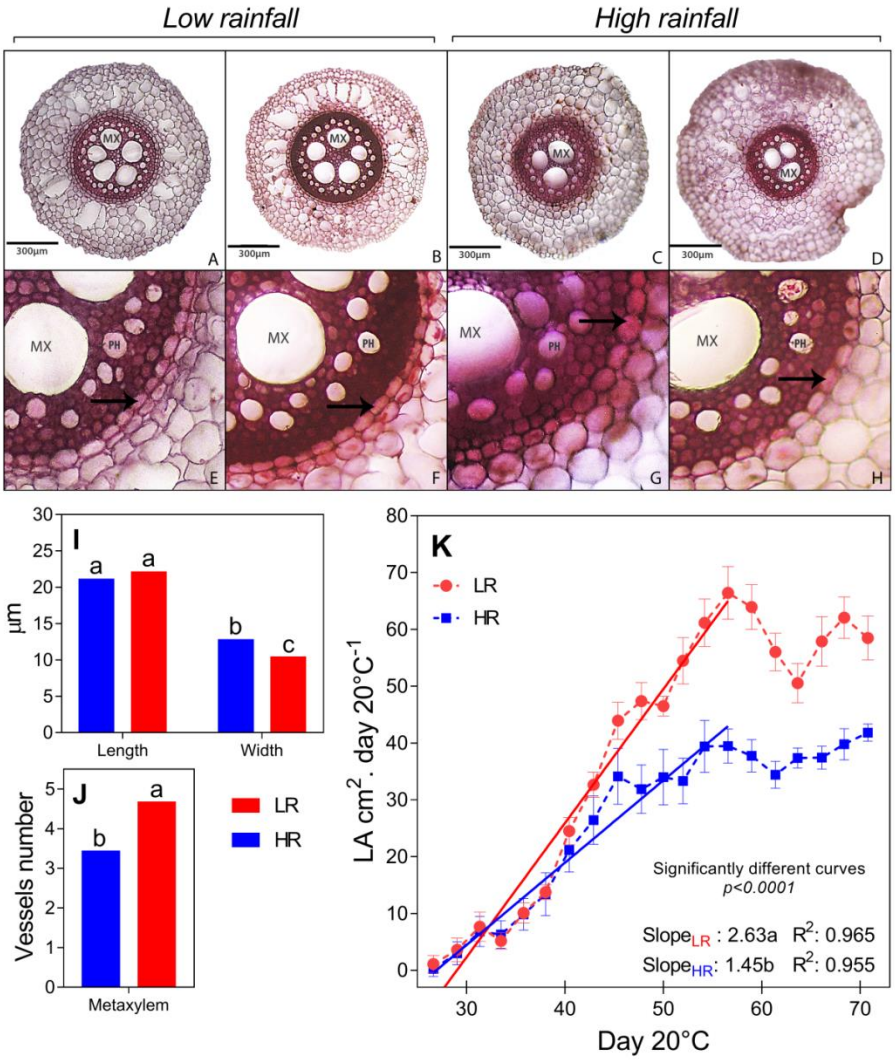


FIGURE 4. Anatomical differences of roots segments and aerial growth of hybrids bred in high (HR) and low (LR) rainfall zones. The upper panel shows transverse sections were cut freehand in the middle part from the root apex to the root basis of hybrids evolved in LR [HOPE 2013-AHT-R-14 (A,E) and HOPE 2013-AHT-R-8 (B,F)] and HR [AHT-II/K13-24 (C, G) and AHT-II/K13-5 (D,H)] zones. (A-D) General view of a root sections under 10 x 10 magnifications. (E-H) View of a quarter of stele, the endodermal cell layers are marked by an arrow, metaxylem (MX) and phloem (PH). Main differences in MX number (J) and endodermal cells size (I) are shown in the bottom panel. Differences were analysed by LSD test ($p<0.05$), letters indicate significant differences. The growth curves of leaf area (K) and its Linear regression for the exponential phase are show. Differences of slopes and curves are indicated.

TABLE 1. Root and aerial anatomical features of higher (HR) and lower (LR) rainfall hybrids. Mean of all traits are shown, differences were assessed by t-test are indicated (significant, *p< 0.05; ns, p>0.05)*.

Trait	HR	LR	*p* value	*Trait*	HR	LR	*p* value
Exp.2				*Exp.1*			
Aerial part				*Aerial part*			
LA (cm^2)	146	**217**	<0.000	*LA (cm^2)*	118	157	ns
SDW (g)	0.45	**0.64**	<0.000	*SDW (g)*	0.22	**0.38**	0.039
TDW (g)	1.44	**1.87**	<0.000	*TDW (g)*	0.72	**1.07**	0.049
Pressurized Roots				*Roots treated with $AgNO_3$*			
RDW (g)	0.64	0.40	0.007	*RDW (g)*	0.20	0.31	0.016
Length (cm)-L	4023	**5160**	0.03	*2<T<3*	0.5	**1.8**	0.013
Surf Area (cm^2)-SA	429	**578**	0.02	*V>4*	0.58	**1.19**	0.016
Avg Diam (mm)	0.34	0.36	ns	*SA>4*	10	**23**	0.028
LenPerVol ($cm.m^{-3}$)	4023	**5160**	0.03				
Volume (cm^3)-V	3.67	**5.18**	0.03				
Tips-T	14794	**19505**	0.03				
Forks	22290	**36654**	0.09				
0.00<L≤0.50	3230	**4096**	0.03	*Exp.3*			
0.50<L≤1.00	577	**736**	0.03	*Aerial part*			
1.00<L≤1.50	145	216	ns	*LA (cm^2)*	210	**317**	<0.000
1.50<L≤2.00	34	53	ns	*SDW (g)*	0.40	**0.86**	<0.000
2.00<L≤2.50	15	25	ns	*LDW (g)*	0.55	0.87	<0.000
2.50<L≤3.00	7	13	ns	*TDW (g)*	0.94	**1.72**	<0.000
0.00<SA≤0.50	185	**237**	0.007				
0.50<SA≤1.00	128	**163**	0.01				
1.00<SA≤1.50	57	85	0.03				
1.50<SA≤2.00	19	30	ns				
2.00<SA≤2.50	11	18	ns				
2.50<SA≤3.00	6	12	ns				
0.00<V≤0.50	0.41	**0.49**	<0.000				
0.50<V≤1.00	0.88	**1.03**	0.02				
1.00<V≤1.50	0.68	**0.92**	0.01				
1.50<V≤2.00	0.32	**0.46**	0.05				
2.00<V≤2.50	0.24	0.36	ns				
2.50<V≤3.00	0.17	**0.31**	0.06				
0.00<T≤0.50	14712	**19396**	0.03				
0.50<T≤1.00	67	85	ns				
1.00<T≤1.50	11	**15**	0.04				
1.50<T≤2.00	1.12	**3.57**	0.009				

4 Discussion

In summary remarkable differences were found between hybrids bred in low and high rainfall zones of India (Fig.5) and submitted to high VPD or hotter environments. The aquaporin inhibitors had a similar effect on the transpiration of both groups, despite a slight trend for a higher inhibition in the low rainfall hybrids. The hybrids bred in low rainfall zones had lower exudation rate, larger and more numerous metaxylem vessels, and thinner cells in the endodermis, than the hybrids bred for the higher rainfall. When roots were pressurized, transpiration dramatically increased in both hybrid types, and the transpiration increase was higher in the low rainfall hybrids while the roots hydraulic conductivity was lower than in the low rainfall hybrids. The high aquaporin expression of PIP 2;6 in the leaf seemed to contribute to the high transpiration response, while the much higher expression of aquaporin PIP 2;3 in the roots seemed to play a key role in the higher water transport of low rainfall hybrids upon increase in VPD. Those features may help in the superior growth in roots and canopy of the lower rainfall genotypes in hotter/dryer environments.

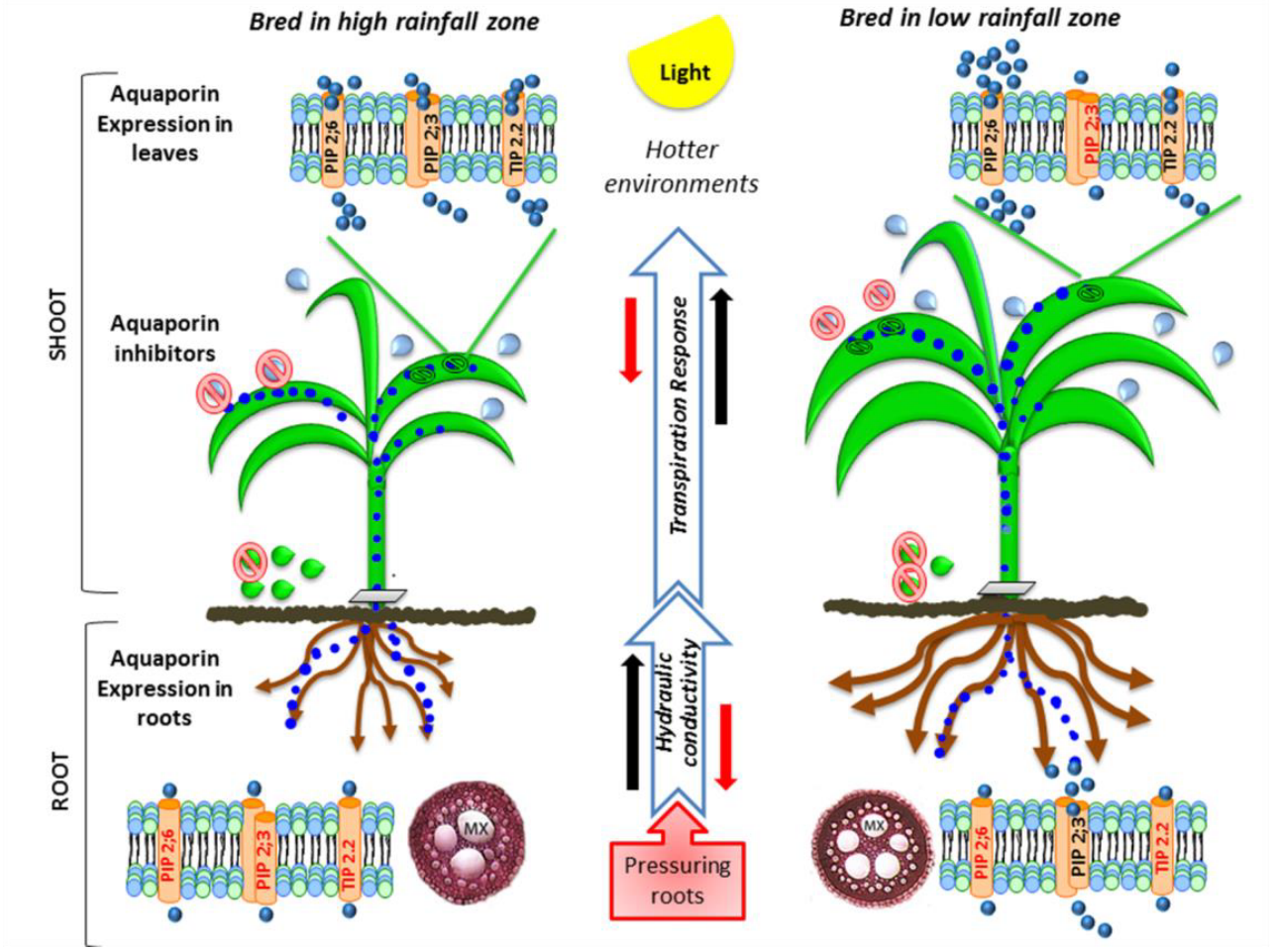


FIGURE 5. Ideotypes of hybrids bred in high and low rainfall zones in hotter conditions. The graph illustrates the water transport (blue dots) from roots to leaves trough xylem (green drops) due to transpiration demand (light blue drops: transpiration) under high VPD and its blockage by aquaporin inhibitors (red block circles), the effect on transpiration response and hydraulic conductivity of a pressurized root (red and black arrows), also the aquaporin expression in the membranes (upper and bottom orange pores in the membranes) under high VPD conditions as the differences in metaxylem vessels (steles).

4.1 Hydraulic conductance limitations

The root pressurization experiment revealed a hydraulic limitation in the roots of pearl millet, since transpiration increased dramatically upon pressure imposition. This hydraulic limitation was more important in the low rainfall hybrids, in which the transpiration increase following root pressurization was higher than in the high rainfall hybrids. These results were also supported by the measurement of a lower root hydraulic conductance of the low rainfall hybrids. The fact that the low rainfall hybrids had a larger number of metaxylem vessels than the high rainfall hybrids then suggests that the xylem was likely not where the hydraulic limitation took place. We interpret and hypothesize that the hydraulic limitation was likely to be in the root cylinder. It is unclear whether and how the lower sized endodermal cells in the low rainfall hybrids could contribute to this. The roots are known to be highly responsive to their environment, they alter their permeability due to changes of nutrient deficiency or stress, this flow plasticity may elucidate a non-universal rule for plants nutrient management (Javot and Maurel, 2002; Hodge, 2009). It is also unclear why the low rainfall hybrids had a higher slope in the transpiration response to increasing VPD conditions than the high rainfall hybrids. Our interpretation is that aquaporin-driven water flow through the root cylinder could be induced under high evaporative demand conditions in the low rainfall hybrids. Similar phenomenon was reported in barley, where the hydraulic conductance increased under a driving water uptake, so plant transpiration was more than doubled (Suku *et al.*, 2014) as in our study; those increments specially in low rainfall hybrids may respond to higher water flux through cell-to-cell radial path in roots. This could be interpreted from the higher PIP2;3 aquaporin gene up-regulation in the roots of low rainfall hybrids under high VPD conditions. This could also be inferred from the inhibition study where the transpiration inhibition by $AgNO_3$ was slightly higher in the low rainfall hybrids than in the high rainfall hybrids. Earlier reports point to a similar interpretation, possibly also with an additive effect of aquaporins in poplar where increments in hydraulic conductivity of xylem flux were related with higher expression of PIP 2;3 transcripts and other PIP families (Almeida-Rodriguez *et al.*, 2011); also in Arabidopsis where

AtPIP1;2 mediated portion of leaf water transport (Postaire *et al.*, 2010). Therefore, the overall interpretation – still a working hypothesis that needs further confirmation – is that hybrids from the low rainfall zone would depend more on aquaporin-mediated water transport in the root cylinder, and this pathway would be upregulated in situation of high water demand.

These low rainfall hybrids which were bred in the driest environments showed this increased canopy water demand and lower root hydraulic as drought adapted genotypes of grapevine described elsewhere (Gambetta *et al.*, 2012; Barrios-Masias *et al.*, 2015) or maize (Caldeira *et al.*, 2014) suggesting that hydraulic processes may respond to rapid changes in the evaporative demand as a possible adaptation to a specific environment in this group of genotypes. This kind of hydraulic efficiency selection or speciation of life history type was also reported in fire prone chaparral communities (Pratt *et al.*, 2010). This interaction of aquaporins with the hydraulic conductivity may be regulated by phosphorylation/dephosphorylation processes that activate aquaporins functions as in the case of leaf hydraulic regulations by PIP 2 aquaporins in Arabidopsis (Lee *et al.*, 2012; Prado *et al.*, 2013).

4.2 Inhibition of transpiration response due to aquaporin inhibitors

The transpiration in both rainfall hybrids in our study was highly inhibited by $HgCl_2$ and $AgNO_3$ (fig 1). The effect of these metallic inhibitor treatment is consistent with earlier report, for instance in sorghum where Hg caused decreased transpiration rates (Liu *et al.*, 2014), or in barley and *vicia faba* where Hg caused decreases in leaf stomatal conductance and carbon isotope discrimination, and led to down regulation of aquaporin transcripts (Terashima and Ono, 2002; Lopes *et al.*, 2013). The transpiration decreased in the $AgNO_3$ treated plants, although the transpiration decrease was less than in plants treated with mercury. Similar differences in the effects of inhibitors effect were found in previous studies in soybean (Sadok and Sinclair, 2010), and also in other reports of a low inhibition of transpiration by $AgNO_3$

(Devi *et al.*, 2016*b*). This could account for different population of aquaporin having different sensitivities to these inhibitors, as in poplar where PIP1 and PIP2 inhibition by mercurial compounds was related to a decrease of leaf hydraulic conductance (Lopez *et al.*, 2013). The fact that upon $AgNO_3$ treatment, the decline in transpiration was in the range of 25-40% indicates that both simplastic and apoplastic water transport pathways were both important for root water transport in pearl millet, possibly with a higher role for the apoplastic water transport. Aquaporins could also be less sensitive to $AgNO_3$ than to mercury compounds. Moreover, previous studies of genotypes which do not restrict transpiration under high VPD, reported no decrease on their transpiration when treated with AgNO3 (Devi *et al.*, 2012), which is contrary to our lower rainfall hybrids in which this inhibitor limited transpiration by about 40%.

The exudate inhibition due to Hgcl2 and AgNO3 (Fig. 1) was also strong, especially in high rainfall hybrids, suggesting here also a higher role of aquaporin for water transport in high rainfall hybrids, in situation of no transpiration (shoot removed). In peanut these two metallic inhibitors decreased TR by blocking AQPs and up-regulating the AQP transcripts, possibly to compensate blockage (Devi *et al.*, 2016*a*). Previous studies in wheat showed that $HgCl_2$ was responsible for blocking ~30% of aquaporin activity relative to water flux (Schoppach *et al.*, 2014), this negative effect may explain the higher reduction of xylem exudates in higher and lower rainfall hybrids under $HgCl_2$ exposure. It was intriguing to find that the bleeding rate was higher in the higher rainfall hybrids, whereas under high evaporative demand there seemed to be higher water transport in the low rainfall hybrids (see above section). This may suggest that while high rainfall hybrids secure a high "baseline" water transport, displayed by their high bleeding rates under a no-transpiration situation, under high evaporative demand low rainfall hybrids were able to secure a higher water transport to support transpiration, possibly with an enhanced role of aquaporin-mediated water transport. This can be also interpreted from the higher PIP2;3 upregulation in the roots of low rainfall hybrids under high VPD conditions, and also from their slightly higher transpiration inhibition by aquaporin inhibitors.

This interpretation is supported by previous studies showing that the ability to drive axial water flow was closely related with the root ability to increase its radial water flow mediated by the aquaporins while water uptaken (Schoppach *et al.*, 2014). Also a high radial water uptake across membranes was reported in wheat where the apoplastic pathways also contributed with the axial water flow (Fricke *et al.*, 2014). Therefore, our interpretation and hypothesis is that low rainfall hybrids likely have a better developed xylem vessel to support an abundant axial flow rate, and the tuning of water transport would then take place in the root axial flow path, mediated by aquaporins under conditions of high evaporative demand.

4.3 Transpiration dynamics under high and low VPD and aquaporin gene expression

The low rainfall hybrids transpired higher rates than high rainfall hybrids when exposed to high VPD, the latter group seemed then unable to channel enough water to support transpiration to the level of the low rainfall hybrids. All hybrids showed a circadian regulated expression pattern for tested aquaporins (Fig3. B), which was also demonstrated in PIP1s and PIP2s in previous studies (Lopez *et al.*, 2003, 2013). It is known that aquaporin over-expression had been linked with plant performances of higher water permeability (Maurel, 2007), related to retaining water and enhancing antioxidant activities (Zhou *et al.*, 2012). Our hybrids showed a dynamic pattern of expression of PIP 2 and TIP 2 families in roots and shoots. In poplar, when transpiration demand was increased, the water potential raised and the flow root-shoot was linked with the up-regulation of PIP2 aquaporins for genotypes that were used to grow under high relative humidity conditions as our high rainfall hybrids (Laur and Hacke, 2013). Moreover coinciding with PIP 2 family expression in the mesophyll cells of leaves and endodermis of rice roots, the PIP aquaporins that we tested also seemed to be involved in the water transport and in the mechanisms to maintain the osmotic balance (Li *et al.*, 2008).

Aquaporin PIP 2;6 was over-expressed in shoots and under-expressed in roots (Fig. 3C), this aquaporin was highly expressed in leaves of low rainfall hybrids as GhPIP 2.7 in cotton leaves under drought conditions (Zhang *et al.*, 2013). Also a similar isoform in rice showed that higher abundances in OsPIP2 were related to higher transpiration rates in rice (Sakurai-Ishikawa *et al.*, 2011). That suggests its role on adaptation to water limited environments as the case of our hybrids bred in very arid zones of India; and a role involved in water flux in the leaf due to transpiration demand. Aquaporin PIP 2;3 aquaporin expression pattern fits with the hydraulic conductance and transpiration profile of high and low rainfall hybrids (Fig. 3C and Fig. 2B). In high rainfall genotypes, its over-expression in leaves matched its high root hydraulic conductance, also in low rainfall hybrids the matches are also consistent with the increase water flow driven by higher transpiration demand, and with other studies where increased root radial hydraulic conductivity was associated with PIP 2 family abundances (Katsuhara *et al.*, 2003). This is probably an environmental effect over the breeding history in the response of PIP 2;3, each group expressed this aquaporin in a different way in both tissues. Previous studies also reported different ways of expression in PIP1 family (da Silva *et al.*, 2013). Coinciding with earlier report in groundnut (Devi *et al.*, 2016*a*), our high rainfall hybrids, which control more the water losses under high VPD, also down-regulate this PIP2;3 when VPD was increased. This down-regulation was also found in PIPs, which may explain a limitation in the water flow. On the contrary in low rainfall hybrids, the over-expression of this aquaporin matches their likely higher hydraulic conductivity under high VPD ability, as described in rice (Katsuhara *et al.*, 2003; Maurel, 2007). In fact, the positive root pressurization effect were similar to applying a negative pressure on the leaves, which is alike the high VPD conditions. TIP 2:2 seemed to be also expressed constitutively In both tissues, as TaTIP2;2 from Arabidopsis (Xu *et al.*, 2013), which is down-regulated by drought stress, similarly our low rainfall plants which were bred in the arid zones and showed higher abundances of this aquaporin. Moreover, high rainfall genotypes expressed higher amounts of TIP 2;2 in leaves, this can be related with its lower transpiration; previous studies had reported the

same decrease in transpiration in peanut (Devi *et al.*, 2016*a*,*b*). In low rainfall hybrids we found a decrease of TIP 2;2 when water was limited, and also a low root hydraulic conductance, which agrees with findings in grapevine (Zarrouk *et al.*, 2016). Contrary to reports in tomato, this constitutive aquaporin expression increased the water transport, whole-plant transpiration and fruit yield (Sade *et al.*, 2009), our high rainfall plants over-expressed it in roots and leaves but developed smaller leaf area (Fig. 4K) and canopy height.

4.4 Plant growth: roots and aerial features

Plants growth dynamic depends on the water and nutrients flow matching the evaporative demand and soil water content, that will balance the plant water status (Maurel *et al.*, 2010; Vadez, 2014; Caldeira *et al.*, 2014). The plant growth of our contrasting hybrids revealed a superiority in plant size accounting roots and shoots of lower rainfall genotypes (Fig. 4K and table 1), which also had higher transpiration response to hotter conditions and lower hydraulic conductance, opposite to higher rainfall hybrids. Moreover, this former group showed higher root hair, and root length of lateral roots which may suggest a better capacity of water uptake, also a higher number of metaxylem vessels, these features may suggest a highest axial flow of water which matches with its higher transpiration response under high VPD; this agrees previous reports of efficient xylem vessels and capillaries conduct the ascent of water driven by the transpiration demand in order to maintain the osmotic balance (Maurel, 1997). This agrees with a similar feature in wheat where water saving took place from lines having smaller xylem vessels (Richards and Passioura, 1989). The finding of thinner endodermis cells may suggest a smaller space across membranes in the cell-to-cell pathway, where the high abundances of PIP 2;3 aquaporin may be localized. The variation in endodermis cells size related to aquaporin expression was also reported previously in wheat where a genotype with high transpiration demand and Hg sensitive aquaporins also had thinner endodermis cells and smaller central metaxylem elements (Schoppach *et al.*, 2014) as our low

rainfall genotypes. In rice it was demonstrated that the presence of OsPIP2;5 in the proximal end of the endodermis and in the cells around the xylem vessels (Sakurai-Ishikawa *et al.*, 2011), may play a key role in the fine adjustment of the water flow. The lower number of metaxylem vessels of the root in higher rainfall hybrids contrasts with findings in legumes such as cowpea, soybean and common bean where it was reported that a higher number of xylem vessels as an indicator of adaptation to soils with higher water regimes (Purushothaman *et al.*, 2013). The higher aerial development (Fig. 4F and Table 1) of lower rainfall genotypes can be related to its higher capacity to drive water flux to the exchange of vapour in the stroma, as reported in Arabidopsis that a high hydraulic conductivity in the axial part of the plant was related with higher shoot development (Postaire *et al.*, 2010). This would also agree with result in maize showing a relationship between hydraulic conductivity and leaf expansion processes (Reymond *et al.*, 2003).

5 Conclusion

The influence of the breeding history for given adaptation environments, depending of the predictable or unpredictable rainfall periods, was reflected in features of the plant water flow patterns. The hybrids bred in lower and higher rainfall had indeed very contrasting performances in terms of transpiration response to pressure application (positive applied to the root, higher evaporative demand), and in its root hydraulic conductivity. Low rainfall hybrids had more of a root hydraulic conductivity limitation under regular conditions but were able to have larger increase in transpiration under higher evaporative demand. Our hypothesis is then of a facilitation of that extra water movement, through the axial root pathway, to be facilitated by aquaporins, in accordance with the higher upregulation of some aquaporins (PIP 2;3) in the roots of these hybrids under high VPD, while these low rainfall hybrids are set with a high axial water flow thanks to larger/more numerous metaxylem vessels. These different water flux performances may also reflect the different biomass development, reflecting a higher biomass development under high VPD of genotypes bred in lower rainfall zone.

AKNOWLEDGMENT

We thank the GEMS group from Crop physiology-ICRISAT for technical assistance in the experiments and PTTC molecular biology team for the facilities provided for gene expression experiments.

AUTHOR CONTRIBUTION

SM and VV conceived and designed the experiments. SM and AK conducted the experimental work. SM and VV contributed to the data analysis and interpreted the results. SM wrote the paper under the supervision of VV, revised the manuscript, all authors read and approved the final manuscript.

FUNDING

Operational expenses were funded by a grant from USAID (Feed the Future Innovation lab for Climate Resilient Pearl Millet). SM was also the recipient of a fellowship “Presidente de la República PRONABEC-III” from Peruvian Government.

6 References

Almeida-Rodriguez, A. M., Hacke, U. G., and Laur, J. (2011). Influence of evaporative demand on aquaporin expression and root hydraulics of hybrid poplar. Plant, Cell Environ. 34, 1318–1331. doi:10.1111/j.1365-3040.2011.02331.x.

Barrios-Masias, F. H., Knipfer, T., and McElrone, A. J. (2015). Differential responses of grapevine rootstocks to water stress are associated with adjustments in fine root hydraulic physiology and suberization. J. Exp. Bot. 66, 6069–6078. doi:10.1093/jxb/erv324.

Caldeira, C. F., Bosio, M., Parent, B., Jeanguenin, L., Chaumont, F. F., and Tardieu, F. F. (2014). A Hydraulic Model Is Compatible with Rapid Changes in Leaf Elongation

under Fluctuating Evaporative Demand and Soil Water Status. PLANT Physiol. 164, 1718–1730. doi:10.1104/pp.113.228379.

Choudhary, S., and Sinclair, T. R. (2014). Hydraulic conductance differences among sorghum genotypes to explain variation in restricted transpiration rates. Funct. Plant Biol. 41, 270–275. doi:10.1071/FP13246.

Comas, L. H., Becker, S. R., Cruz, V. M. V, Byrne, P. F., and Dierig, D. a (2013). Root traits contributing to plant productivity under drought. Front. Plant Sci. 4, 442. doi:10.3389/fpls.2013.00442.

da Silva, M. D., Silva, R. L. de O., Costa Ferreira Neto, J. R., Guimarães, A. C. R., Veiga, D. T., Chabregas, S. M., et al. (2013). Expression Analysis of Sugarcane Aquaporin Genes under Water Deficit. J. Nucleic Acids 2013, 1–14. doi:10.1155/2013/763945.

Devi, M. J., Sadok, W., and Sinclair, T. R. (2012). Transpiration response of de-rooted peanut plants to aquaporin inhibitors. Environ. Exp. Bot. 78, 167–172. doi:10.1016/j.envexpbot.2012.01.001.

Devi, M. J., Sinclair, T. R., Jain, M., and Gallo, M. (2016a). Leaf aquaporin transcript abundance in peanut genotypes diverging in expression of the limited-transpiration trait when subjected to differing vapor pressure deficits and aquaporin inhibitors. Physiol. Plant. 156, 387–396. doi:10.1111/ppl.12378.

Devi, M. J., Sinclair, T. R., and Taliercio, E. (2016b). Silver and zinc inhibitors influence transpiration rate and aquaporin transcript abundance in intact soybean plants. Environ. Exp. Bot. 122, 168–175. doi:10.1016/j.envexpbot.2015.10.006.

Forrest, K. L., and Bhave, M. (2007). Major intrinsic proteins (MIPs) in plants: A complex gene family with major impacts on plant phenotype. Funct. Integr. Genomics 7, 263–289. doi:10.1007/s10142-007-0049-4.

Fricke, W., Bijanzadeh, E., Emam, Y., and Knipfer, T. (2014). Root hydraulics in salt-stressed wheat. Funct. Plant Biol. 41, 366–378. doi:10.1071/FP13219.

Gambetta, G. A., Manuck, C. M., Drucker, S. T., Shaghasi, T., Fort, K., Matthews, M. A., et al. (2012). The relationship between root hydraulics and scion vigour across Vitis rootstocks: What role do root aquaporins play? J. Exp. Bot. 63, 6445–6455. doi:10.1093/jxb/ers312.

Ho, M. D., Rosas, J. C., Brown, K. M., and Lynch, J. P. (2005). Root architectural tradeoffs for water and phosphorus acquisition. Funct. Plant Biol. 32, 737–748. doi:10.1071/FP05043.

Hodge, A. (2009). Root decisions. Plant, Cell Environ. 32, 628–640. doi:10.1111/j.1365-3040.2008.01891.x.

Hose, E., Clarkson, D. T., Steudle, E., Schreiber, L., and Hartung, W. (2001). The exodermis: a variable apoplastic barrier. J. Exp. Bot. 52, 2245–2264. doi:10.1093/jexbot/52.365.2245.

Hub, J. S., Aponte-Santamaría, C., Grubmü, H., and De, B. L. (2010). Voltage-Regulated Water Flux through Aquaporin Channels In Silico. Biophysj 99, L97–L99. doi:10.1016/j.bpj.2010.11.003.

Javot, H., and Maurel, C. (2002). The role of aquaporins in root water uptake. Ann. Bot. 90, 301–313. doi:10.1093/aob/mcf199.

Kaldenhoff, R., Ribas-Carbo, M., Sans, J. F., Lovisolo, C., Heckwolf, M., and Uehlein, N. (2008). Aquaporins and plant water balance. Plant, Cell Environ. 31, 658–666. doi:10.1111/j.1365-3040.2008.01792.x.

Katsuhara, M., Koshio, K., Shibasaka, M., Hayashi, Y., Hayakawa, T., and Kasamo, K. (2003). Over-expression of a Barley Aquaporin Increased the Shoot/Root Ratio and Raised Salt Sensitivity in Transgenic Rice Plants. Plant Cell Physiol. 44, 1378–1383. doi:10.1093/pcp/pcg167.

Kholová, J., and Vadez, V. (2013). Water extraction under terminal drought explains the genotypic differences in yield, not the anti-oxidant changes in leaves of pearl millet (Pennisetum glaucum). Funct. Plant Biol. 40, 44–53. doi:10.1071/FP12181.

Kholová, J., Zindy, P., Malayee, S., Baddam, R., Murugesan, T., Kaliamoorthy, S., et al. (2016). Component traits of plant water use are modulated by vapour pressure deficit in pearl millet (Pennisetum glaucum (L.) R.Br.). Funct. Plant Biol. 43, 423–437. doi:10.1071/FP15115.

Kruse, E., Uehlein, N., and Kaldenhoff, R. (2006). The aquaporins. Genome Biol. 7, 206. doi:10.1186/gb-2006-7-2-206.

Laur, J., and Hacke, U. G. (2013). Transpirational demand affects aquaporin expression in poplar roots. J. Exp. Bot. 64, 2283–2293. doi:10.1093/jxb/ert096.

Lee, S. H., Chung, G. C., Jang, J. Y., Ahn, S. J., and Zwiazek, J. J. (2012). Overexpression of PIP2;5 Aquaporin Alleviates Effects of Low Root Temperature on Cell Hydraulic Conductivity and Growth in Arabidopsis. Plant Physiol. 159, 479–488. doi:10.1104/pp.112.194506.

Li, G., Santoni, V., and Maurel, C. (2014). Plant aquaporins: Roles in plant physiology. Biochim. Biophys. Acta - Gen. Subj. 1840, 1574–1582. doi:10.1016/j.bbagen.2013.11.004.

Li, G. W., Zhang, M. H., Cai, W. M., Sun, W. N., and Su, W. A. (2008). Characterization of OsPIP2;7, a water channel protein in rice. Plant Cell Physiol. 49, 1851–1858. doi:10.1093/pcp/pcn166.

Liu, P., Yin, L., Deng, X., Wang, S., Tanaka, K., and Zhang, S. (2014). Aquaporin-mediated increase in root hydraulic conductance is involved in silicon-induced improved root water uptake under osmotic stress in Sorghum bicolor L. J. Exp. Bot. 65, 4747–4756. doi:10.1093/jxb/eru220.

Liu, P., Yin, L., Wang, S., Zhang, M., Deng, X., Zhang, S., et al. (2015). Enhanced root hydraulic conductance by aquaporin regulation accounts for silicon alleviated salt-induced osmotic stress in sorghum bicolor L. Environ. Exp. Bot. 111, 42–51. doi:10.1016/j.envexpbot.2014.10.006.

Lopes, M. S., Iglesia-Turiño, S., Cabrera-Bosquet, L., Serret, M. D., Bort, J., Febrero, A., et al. (2013). Molecular and physiological mechanisms associated with root exposure to mercury in barley. Metallomics 5, 1305–1315. doi:10.1039/c3mt00084b.

Lopez, D., Venisse, J. S., Fumanal, B., Chaumont, F., Guillot, E., Daniels, M. J., et al. (2013). Aquaporins and leaf hydraulics: Poplar sheds new light. Plant Cell Physiol. 54, 1963–1975. doi:10.1093/pcp/pct135.

Lopez, F., Bousser, A., Sissoëff, I., Gaspar, M., Lachaise, B., Hoarau, J., et al. (2003). Diurnal Regulation of Water Transport and Aquaporin Gene Expression in Maize Roots: Contribution of PIP2 Proteins. Plant Cell Physiol. 44, 1384–1395. doi:10.1093/pcp/pcg168.

Lynch, J. P. (2013). Steep, cheap and deep: An ideotype to optimize water and N acquisition by maize root systems. Ann. Bot. 112, 347–357. doi:10.1093/aob/mcs293.

Lynch, J. P., and Brown, K. M. (2012). New roots for agriculture: exploiting the root phenome. Philos. Trans. R. Soc. B Biol. Sci. 367, 1598–1604. doi:10.1098/rstb.2011.0243.

Manga, V. K., and Kumar, A. (2011). Cultivar Options for Increasing Pearl Millet Productivity in Arid Regions. Indian J. Fundam. Appl. Life Sci. 1, 200–208.

Maurel, C. (1997). Aquaporins and Water Permeability of Plant Membranes. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 399–429. doi:10.1146/annurev.arplant.48.1.399.

Maurel, C. (2007). Plant aquaporins: Novel functions and regulation properties. FEBS Lett. 581, 2227–2236. doi:10.1016/j.febslet.2007.03.021.

Maurel, C., Simonneau, T., and Sutka, M. (2010). The significance of roots as hydraulic rheostats. J. Exp. Bot. 61, 3191–3198. doi:10.1093/jxb/erq150.

Nardini, A., and Salleo, S. (2005). Water stress-induced modifications of leaf hydraulic architecture in sunflower: Co-ordination with gas exchange. J. Exp. Bot. 56, 3093–3101. doi:10.1093/jxb/eri306.

Niemietz, C. M., and Tyerman, S. D. (2002). New potent inhibitors of aquaporins: Silver and gold compounds inhibit aquaporins of plant and human origin. FEBS Lett. 531, 443–447. doi:10.1016/S0014-5793(02)03581-0.

Parent, B., and Tardieu, F. (2012). Temperature Responses of Developmental Processes Havenot Affected By Breeding in Different Ecological Areas for 12 Crop Species. New Phytol. 194, 760–774.

Passioura, J. B. (1983). Roots and drought resistance. Agric. Water Manag. 7, 265–280. doi:10.1016/0378-3774(83)90089-6.

Postaire, O., Tournaire-Roux, C., Grondin, A., Boursiac, Y., Morillon, R., Schäffner, A. R., et al. (2010). A PIP1 aquaporin contributes to hydrostatic pressure-induced water transport in both the root and rosette of Arabidopsis. Plant Physiol. 152, 1418–30. doi:10.1104/pp.109.145326.

Prado, K., Boursiac, Y., Tournaire-Roux, C., Monneuse, J.-M., Postaire, O., Da Ines, O., et al. (2013). Regulation of Arabidopsis leaf hydraulics involves light-dependent phosphorylation of aquaporins in veins. Plant Cell 25, 1029–39. doi:10.1105/tpc.112.108456.

Pratt, R. B., North, G. B., Jacobsen, A. L., Ewers, F. W., and Davis, S. D. (2010). Xylem root and shoot hydraulics is linked to life history type in chaparral seedlings. Funct. Ecol. 24, 70–81. doi:10.1111/j.1365-2435.2009.01613.x.

Purushothaman, R., Zaman-allah, M., and Mallikarjuna, N. (2013). Root Anatomical Traits and Their Possible Contribution to Drought Tolerance in Grain Legumes. Plant Prod. Sci. 16, 1–8. doi:10.1626/pps.16.1.

Rai, K. N., Gupta, S. K., Govindaraj, M., and Yadav, H. P. (2015). Pearl Millet Improvement for enhanced productivity-strategies and impact. Indian Farming 62. doi:10.1080/00461520.2012.749445.

Reddy, P. S., Santosh, T., Rao, R. B., Sharma, K. K., and Vadez, V. (2015a). Genome-wide identification and characterization of the aquaporin gene family in Sorghum bicolor (L.). PLGENE 1, 18–28. doi:10.1016/j.plgene.2014.12.002.

Reddy, P. S., Srinivas Reddy, D., Sharma, K. K., Bhatnagar-Mathur, P., and Vadez, V. (2015b). Cloning and validation of reference genes for normalization of gene expression studies in pearl millet [Pennisetum glaucum (L.) R. Br.] by quantitative real-time PCR. PLGENE 1, 35–42. doi:10.1016/j.plgene.2015.02.001.

Richards, R. A. (2006). Physiological traits used in the breeding of new cultivars for water-scarce environments. Agric. Water Manag. 80, 197–211. doi:10.1016/j.agwat.2005.07.013.

Richards, R. A., and Passioura, J. B. (1989). A breeding program to reduce the diameter of the major xylem vessel in the seminal roots of wheat and its effect on grain yield in rain-fed environments. Aust. J. Agric. Res. 40, 943–950. doi:10.1071/AR9890943.

Reymond, M., Muller, B., Leonardi, A., Charcosset, A., and Tardieu, F. (2003). Combining Quantitative Trait Loci Analysis and an Ecophysiological Model to Analyze the Genetic Variability of the Responses of Maize Leaf Growth to Temperature and Water Deficit. Plant Physiol. 131, 664–675. doi:10.1104/pp.013839.

Sade, N., Vinocur, B. J., Diber, A., Shatil, A., Ronen, G., Nissan, H., et al. (2009). Improving plant stress tolerance and yield production: Is the tonoplast aquaporin SlTIP2;2 a key to isohydric to anisohydric conversion? New Phytol. 181, 651–661. doi:10.1111/j.1469-8137.2008.02689.x.

Sadok, W., and Sinclair, T. R. (2010). Transpiration response of “slow-wilting” and commercial soybean (Glycine max (L.) Merr.) genotypes to three aquaporin inhibitors. J. Exp. Bot. 61, 821–829. doi:10.1093/jxb/erp350.

Sakurai-Ishikawa, J., Murai-Hatano, M., Hayashi, H., Ahamed, A., Fukushi, K., Matsumoto, T., et al. (2011). Transpiration from shoots triggers diurnal changes in

root aquaporin expression. Plant, Cell Environ. 34, 1150–1163. doi:10.1111/j.1365-3040.2011.02313.x.

Savage, D. F., and Stroud, R. M. (2007). Structural Basis of Aquaporin Inhibition by Mercury. J. Mol. Biol. 368, 607–617. doi:10.1016/j.jmb.2007.02.070.

Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3, 1101–1108. doi:10.1038/nprot.2008.73.

Schoppach, R., Wauthelet, D., Jeanguenin, L., and Sadok, W. (2014). Conservative water use under high evaporative demand associated with smaller root metaxylem and limited trans-membrane water transport in wheat. Funct. Plant Biol. 41, 257. doi:10.1071/FP13211.

Segal, E., Kushnir, T., Mualem, Y., and Shani, U. (2008). Water uptake and hydraulics of the root hair rhizosphere. Vadose Zo. J. 7, 1027–1034. doi:10.2136/vzj2007.0122.

Sinclair, T. R., Zwieniecki, M. A., and Holbrook, N. M. (2008). Low leaf hydraulic conductance associated with drought tolerance in soybean. Physiol. Plant. 132, 446–451. doi:10.1111/j.1399-3054.2007.01028.x.

Steudle, E. (2000). Water uptake by plant roots: an integration of views. Plant Soil 226, 45–56. doi:10.1023/A:1026439226716.

Steudle, E., and Peterson, C. a (1998). How does water get through roots ? J. Exp. Bot. 49, 775–788. doi:10.1093/jxb/49.322.775.

Suku, S., Knipfer, T., and Fricke, W. (2014). Do root hydraulic properties change during the early vegetative stage of plant development in barley (Hordeum vulgare)? Ann. Bot. 113, 385–402. doi:10.1093/aob/mct270.

Terashima, I., and Ono, K. (2002). Effects of HgCl(2) on CO(2) dependence of leaf photosynthesis: evidence indicating involvement of aquaporins in CO(2) diffusion across the plasma membrane. Plant Cell Physiol. 43, 70–78. doi:10.1093/pcp/pcf001.

Vadez, V. (2014). Root hydraulics: The forgotten side of roots in drought adaptation. F. Crop. Res. 165, 15–24. doi:10.1016/j.fcr.2014.03.017.

Vadez, V., Kholová, J., Hummel, G., Zhokhavets, U., Gupta, S. K., and Hash, C. T. (2015). LeasyScan: A novel concept combining 3D imaging and lysimetry for high-throughput phenotyping of traits controlling plant water budget. J. Exp. Bot. 66, 5581–5593. doi:10.1093/jxb/erv251.

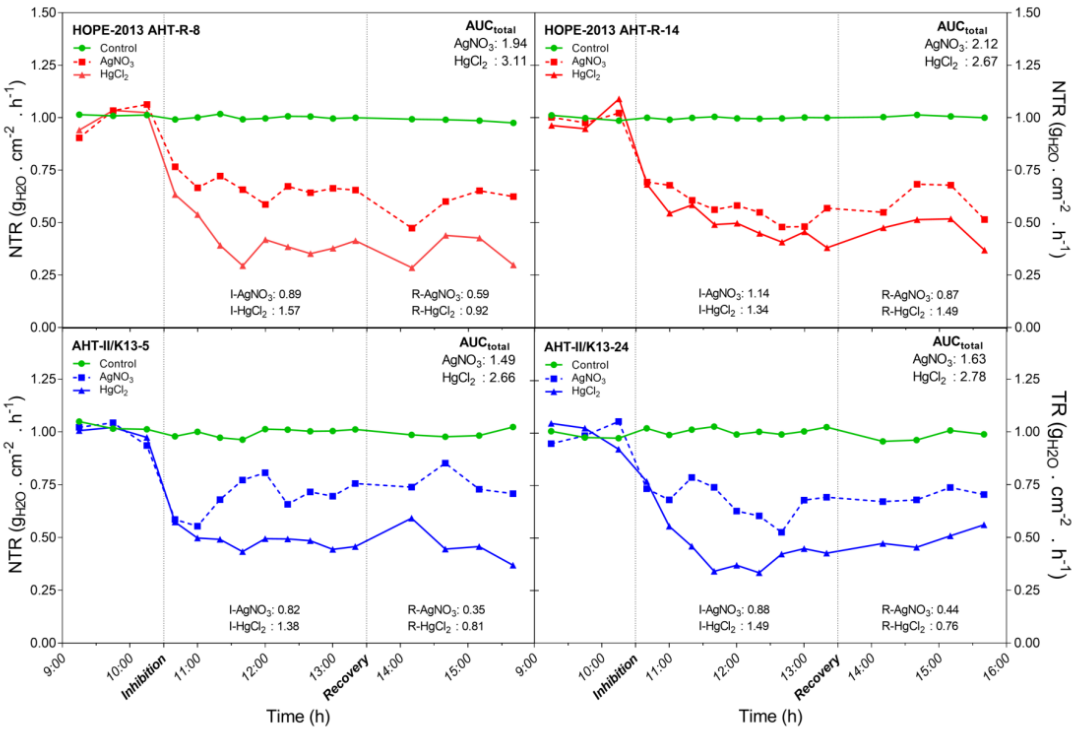
Vadez, V., Kholova, J., Zaman-Allah, M., and Belko, N. (2013). Water: The most important “molecular” component of water stress tolerance research. Funct. Plant Biol. 40, 1310–1322. doi:10.1071/FP13149.

Xu, C., Wang, M., Zhou, L., Quan, T., and Xia, G. (2013). Heterologous expression of the wheat aquaporin gene TaTIP2;2 compromises the abiotic stress tolerance of Arabidopsis thaliana. PLoS One 8, 1–10. doi:10.1371/journal.pone.0079618.

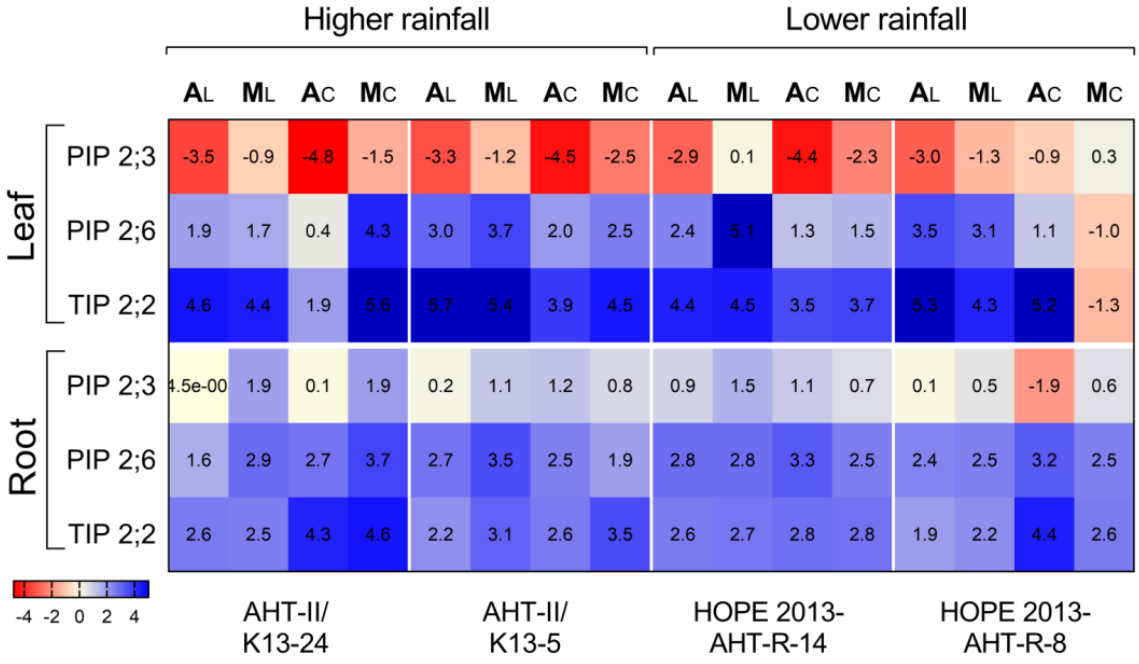
Zarrouk, O., Garcia-Tejero, I., Pinto, C., Genebra, T., Sabir, F., Prista, C., et al. (2016). Agricultural Water Management Aquaporins isoforms in cv. Touriga Nacional grapevine under water stress and recovery—Regulation of expression in leaves and roots. Agric. Water Manag. 164, 167–175. doi:10.1016/j.agwat.2015.08.013.

Zhang, J., Li, D., Zou, D., and Luo, F. (2013). A cotton gene encoding a plasma membrane aquaporin is involved in seedling development and in response to drought stress. Acta Biochim Biophys 45, 104–114. doi:10.1093/abbs/gms096.Advance.

Zhou, S., Hu, W., Deng, X., Ma, Z., Chen, L., Huang, C., et al. (2012). Overexpression of the Wheat Aquaporin Gene, TaAQP7, Enhances Drought Tolerance in Transgenic Tobacco. PLoS One 7. doi:10.1371/journal.pone.0052439.



SUPPLEMENTAL FIGURE 1S. Aquaporin inhibition in higher and lower rainfall genotypes.



SUPPLEMENTAL FIGURE 2S. Real-time PCR expression profiling of Aquaporin in leaf and root tissue relative to transpiration response to high VPD. The expression of over six Aquaporin´s mRNAs was profiled from a control point in the morning (M_L and M_C at 2KPa) and across constant VPD of 2KPa (A_L) and after VPD ladder of 2-4.5 KPa (A_H) in roots and leave of Pearl Millet, using a real-time PCR assay. The heatmap was generated by a log transformation of the real-time PCR data presented as ΔCT (C_T mRNA - C_T Reference RNA). For scheme of sampling points see Fig 3.

SUPPLEMENTAL TABLES

SUPPLEMENTAL TABLE 1S. Primes used in RT-qPCR assay. Protein ID and accession number are indicated, details are described elsewhere (Reddy *et al.*, 2015*a*,*b*)

Primer (Accession)	Protein name	Sequence F: forward and R: reverse
qPgPIP2;3 (XP_002461931)	Plasma membrane intrinsic protein	F: GTTCGCGGTTTTCATGGTC R: AGAAGATCCGGTGGTCATCC
qPgPIP2;6 (XP_002461936)	Plasma membrane intrinsic protein	F: GTGATCGGGTACAAGCACCA R: CGGTGCAGTAGACGAGGATG
qPgTIP2;2 (XP_002438430)	Tonoplast intrinsic protein	F: CTCCCTCAGGGCCTACGTC R: GCCGTCGCTCAACTTTCTG
qPgEF-1α (EF694165)	Elongation factor 1-alpha	F: AATGATCCGCTGCTGTAACAAG R: AGGCAATCTTGTCTGGGTTGTA
qPgEIF4A (EU856535)	Eukaryotic initiation factor 4A	F: ACTGAAAGAATGCGCAGCAA R: ACGAGTTGCACCAGACCTGA
qPgACP (KM105958)	Acyl carrier protein	F: AGCAACCAGTGCCACAAAGA R: GGAACTTGGAGGAGCCAGAA

Supplemental TABLE 2S. Aquaporin fold change variation in leaf and root tissues. Fold change shows the variation in expression of PIP 2;3. PIP2;6 and TIP 2;2 within afternoon (A) and morning (M) expression (upper panel), low (LR) and high (HR) rainfall zones (mid panel) and high (A_L) and (A_C, M_C and M_L) low VPD (bottom panel) expressed as a $\log_2$ transformation of $2^{-\Delta\Delta Ct}$, significant differences were assessed with LSD test (ns, *non-significant*; *,p<0.05; **,p<0.01 and ***,p<0.001). For experiment design see figure 3.

Fold change in expression	PIP 2;3		PIP 2;6		TIP 2;2	
Condition	Leaf	Root	Leaf	Root	Leaf	Root
Afternoon respect to morning.						
HR at ladder of VPD	-3.30***	-14.68***	-1.10 ns	-1.47**	1.04 ns	-1.18***
HR at constant low VPD	-2.10 ***	-2.073*	-2.86***	-1.06 ns	-1.74***	-1.17 ns
LR at ladder of VPD	-4.98***	-2.05 ns	-1.37*	-1.01 ns	1.10 ns	-1.08 ns
LR at constant low VPD	-2.69****	1.46**	4.99*	1.29**	3.62**	1.35*
Low rainfall respect to high rainfall.						
Ladder of VPD-A (high VPD)	**1.14***	**4.94 ns**	**1.22 ns**	**1.22 ns**	**-1.05 ns**	**-1.05 ns**
Ladder of VPD-M (low VPD)	1.72 ns	-1.45 ns	1.52*	-1.19 *	-1.12 ns	-1.15 ns
Constant low VPD-A	1.74*	1.46*	-1.01 ns	1.24**	1.49*	1.04 ns
Constant low VPD-M	2.03*	-2.07 ns	-14.45***	-1.11 ns	-4.22**	-1.52**
High VPD respect to low VPD						
HR on the afternoon (A_H/A_L)	**1.36 *** **	**-6.43 ns**	**2.03****	**-1.21 ns**	**1.77*****	**-1.45***
LR on the afternoon (A_H/A_L)	**-1.12 ns**	**1.13***	**2.52*****	**-1.23****	**1.12 ns**	**-1.59*****
HR on the morning (M_H/M_L)	1.95*	1.10 ns	-1.28 ns	1.14 ns	-1.02 ns	-1.44*
LR on the morning (M_H/M_L)	1.65 ns	1.57 ns	17.23*	1.06 ns	3.66*	-1.09 ns

DISCUSSION

La disciplina es la parte

más importante del éxito.

Truman Capote

Agriculture is one of the main activities over the world to produce food; the changes on climatic conditions may demand crops with higher abilities to buffer future drought environments (FAO, 2015). The production of more food per unit of water is an important feature for the society development and food supply. The water demand by economic sectors may enhance the scarcity of water for agriculture, so it is important to enhance the water use and the yield gains of the main crops over the world. Since the green revolution in Europe in 1970s and 1980s, breeding has played an important role producing more yielding crops, especially in cereals like wheat and pearl millet which are very important crops in either Mediterranean or Asian and African countries.

The breeding story of these crops may have an influence over their growth response to specific environments, such as high CO_2 concentration and water availability in the soil or the atmosphere. In this thesis, I have compared how breeding material having different breeding origin, either for historical reasons in the case of wheat or geographical reasons in the case of pearl millet, responded to a series of environmental conditions, at the physiological and molecular level. This included the response of wheat to different CO2 concentrations, the response of transpiration to increasing VPD conditions in pearl millet and wheat, the response of canopy development to different VPD conditions in pearl millet, the expression of genes related to water transport and photosynthetic activities, and finally the assessment of hydraulic features including functional analysis of aquaporins in water transport processes.

1 Wheat acclimation to elevated [CO_2] in the atmosphere

The early growth of wheat and its interaction with elevated [CO2] is important for buffering the future drought impacts in Mediterranean environments where major rainfall limitations and higher evapotranspiration during winter months may result in an early-season drought (Russo et al., 2015), with addition of an elevated [CO2] in

the atmosphere, in this scenario the response of the four wheat genotypes was relevant (chapter 2).

The elevated [CO_2] stimulated the plant growth and caused a downstream in the N content matching the transcript downregulation of Rubisco and GS2 enzymes. Moreover, the downstream expression of stress responsive genes suggests a lower oxidative damage, as well as the upregulation of GS1 that is related with N remobilization in the plant. The increment in plant biomass due to high [CO_2] did not compensate the penalties caused by the water stress, although there was genotypic variation in this response as reported in other studies (Ceccarelli et al., 1991). The aerial plant grow stimulation correlated with the root biomass increment as a positive effect of elevated [CO_2], this greater root growth may allow higher uptake of nutrients from the soil. The gene expression and correlations between both rubisco isoforms (RBCL and RBCS), GS1 and PEPC at transcript level elucidated a balanced coordination of N and C pathways under elevated [CO_2] and water stress. It highlights the Rubisco and GS functions in the plant responses to environmental changes, together with a higher and efficient use of nitrogen under this elevated [CO_2] in the atmosphere.

2 Transpiration responses to high VPD and root hydraulics in cereals

The improvement of water productivity is a need in agriculture, and the transpiration efficiency is assumed to represent its genetic part is a trait to be considerate in the breeding programs. This trait shows a large range of genotypic and environmental variations, which appears to be closely related to how transpiration responds to increasing evaporative demand. In this context, the water transport pathways plays a key role in the plant response to increasing evaporative demand, with root hydraulics conductivity and facilitation of water transport by aquaporins having a particular importance (Chapter 1, 3, 4 and 5).

In wheat and pearl millet (chapter 3 and 4) the transpiration response to high VPD of the genotypes revealed two groups, the ones who restricted the water lose and

others who did not restrict it when the VPD increased. The non-restrictive lines (NR) in wheat as well as pearl millet genotypes tended to be bred for lower rainfall zones and then did not limit their transpiration under high VPD. Those materials might have a special way to regulate the osmotic balance with no dehydration effect when the stomata is open; this characteristic was already reported in elite varieties of wheat (Schoppach and Sadok, 2012), and probably these pearl millet genotypes due to their history of evolution in sandy soils with erratic rainfalls might had developed this strategy for a rapid water uptake before the water is lost in the shallow soil either by evaporation or drainage. On the contrary, the restrictive (R) genotypes of wheat and the pearl millet tended to be bred for higher rainfall zones. These limited water losses under high VPD as reported in earlier studies (Kholová et al., 2010; Schoppach and Sadok, 2013) and postponed water use to later stages to avoid fixing carbon during periods of the day when the water cost to fix carbon is the highest (Vadez et al., 2013), agreeing other reports of limited transpiration in plants grown in cooler environments, as the case of genotypes bred in higher rainfall zone and wheat historically grown in Mediterranean conditions (Sermons et al., 2012). In pearl millet, the parental lines showed larger non limited transpiration response compared with the hybrids F1, this hybrid heterotic vigor may be a result of their breeding history of top crosses where one of the parental lines confer characters of adaptation to the environment (Yadav et al., 2009).

Moreover, in all the genotypes that were tested, either wheat or pearl millet, there was a genotypic variation in their transpiration as reported in previous studies of wheat and pearl millet (Kholova et al., 2016; Schoppach et al., 2017; Schoppach and Sadok, 2012, 2013), this genotypic variation may reflect a variable strategies of adaptation to an specific environment by adjusting the transpirative response and the stomata opening/closure. This better understanding of the physiological features of breeding material fitted to specific environments then opens the opportunity for a much more targeted breeding, towards the characteristics conferring specific adaptation, like the transpiration response to increasing VPD. Those stomata

features of transpiration measured gravimetrically and intrinsic water status measured by carbon isotope discrimination were also linked in this research.

3 Growth features linked to water use

Under increasing evaporative demand ("atmospheric drought") and progressive soil drying ("soil drought") the wheat and pearl millet genotypes decreased aerial and root biomass production as well as in grain yield (chapter 2, 3, 4 and 5).

The growth of the root and the shoot was coordinated and also responded in both directions (chapter 3): (i) the shoot growth responded to the photosynthetic and transpiration demand, and (ii) the root interacting over the nutrient and water uptake. Here the stomata closure/opening seemed to drive the water flux through the xylem which might be regulated by dehydrins and cell stress response genes (Laffray and Louguet, 1990), as well as driven by DREB transcription factors. Moreover in pearl millet (chapter 5) the root architecture and anatomical features were key aspects that influenced the highest water uptake in the nonrestrictive genotypes (lower rainfall), i.e. those plants had higher root hairs, tips and more metaxylem vessels which efficiency may conduct the accent of water and nutrients (Maurel, 1997; Richards and Passioura, 1989). Similarly the endodermis cells were thinner leading to a faster water flux in the cell-cell path where aquaporins may play a role as reported in wheat and rice (Sakurai-Ishikawa et al., 2011; Schoppach et al., 2014).

These interplay of shoot and root growth may also lead to economic strategies for N uptake, assimilation and remobilization and acquisition of carbon (Liu et al., 2010). The tiller development, accumulation in biomass and higher canopy developed by nonrestrictive or bred in lower rainfall zone genotypes, in opposition to the transpiration restrictive bred in higher rainfall zone ones in wheat and pearl millet respectively (chapter 2, 3, 4 and 5), was a strategy to compensate the spike or panicle losses in case of water stress (Kim et al., 2010; Van Oosterom et al., 2003).

The canopy development (Chapter 3 and 5) illustrated by the RGB vegetation indices in nonrestrictive plants (pearl millet plants bred in lower rainfall zone and non-restrictive wheat lines) showed greener and higher canopies, that suggest a higher nutrient and water flow together with higher CO_2 fixation to drive biomass production, accompanied with gene regulation, hydraulics and aquaporins as reported in different studies (Araus et al., 2013; Caldeira et al., 2014; Casadessús et al., 2007; Li et al., 2014; Postaire et al., 2010; Reymond et al., 2003; Robertson et al., 2016; Vicente et al., 2016) and also suggested higher water use efficiency (Farquhar and Richards, 1984).

Moreover, the growth under conditions of high VPD or drought constrained the leaf expansion, this may happen in both cereals which exhibited lower canopies under atmospheric water stress when grown in hotter environments with high evaporative demand (Caldeira et al., 2014; Reymond et al., 2003). These changes in growth were associated with water status parameters and the transpiration response patterns (chapter 4 and 5) in the genotypes tested (Acreche et al., 2008; Araus et al., 2013) The differences in restrictive and non-restrictive groups of wheat genotypes were clearly expressed in the yield production (chapter 3), the nonrestrictive lines had the highest yield in optimal growing conditions, whereas the restrictive lines only succeed when water was limited exhibiting their water saving capacity to enhance yield under water stress conditions (Belko et al., 2012; Kholová et al., 2010; Vadez et al., 2014).

4 Gene regulations

Complex associations in physiological traits driven by gene expression patterns (chapter 2 and 3) influenced the plant growth and yield production (Yousfi et al., 2016). In wheat the adaptation to a specific scenario was related with genes involved in the stress response (Kosová et al., 2014b). This study showed clear genotype-by-environment interaction between restrictive/nonrestrictive lines and higher/lower

yield scenarios. The genes that significantly regulated the plant behavior were: the transcription factors DREB1, DREB2, the dehydrins DNH16 and WCOR, and the cell stress markers SOD and CAT. Those genes influenced the transcript profile of the enzymes related with the primary metabolism of C (Rubisco, PEPC , PK) and N (GOGAT, GS1, GS2) as well as ATPase and aquaporins.

Under optimal growth conditions (chapter 3) where the yield production is high, an enhancement of DREB1 abundances corresponded to the better water conditions showed by the water status traits ($\delta^{13}C$ and g_s), this overexpression of DREB1 in non-restrictive lines played a key role driving the upregulation of enzymes involved in the remobilization of N (GOGAT and GS1), also influenced SOD which is a cellular stress marker that recognizes the overproduction of H_2O_2 in the cell, and enzymes related with the transformation of carbon skeletons (PK) as well as the aquaporins (TIP). Whereas in restrictive lines, the downregulation of DREB1 drove the under expression of both enzymes involved in the N assimilation and remobilization (GOGAT, GS1) as well as the aquaporin. In contrast, in this R+ lines the expression of the genes DREB2, GS2 and Rubisco was upregulated, which may indicate that the DREB2 transcription factor might give a rule a special signal under water stress conditions. Agreeing the DREB overexpression reports in in water limited environments (Engels et al., 2013; Zhao et al., 2016). In both cases DREB transcription factors seemed to recognize the water status and regulated the metabolism to optimize the yield gains as in nonrestrictive lines. This funding of DREB gene regulation agrees previous reports in wheat (Sheshadri et al., 2016; Yousfi et al., 2016).

The dehydrins (chapter 2 and 3) were less expressed in low yielding scenario, whereas in high yielding environments this dehydrins were overexpressed contrasting with previous studies which report its induction in drought conitions (Rampino et al., 2012). In nonrestrictive lines dehydrins expression was associated with enzymes of the C metabolism and traits related to biomass development, similarly in restrictive lines they were associated mainly with enzymes of N and C

metabolism, here also SOD expression was higher and played a protective role (Hassan et al., 2015). Those WCOR, DNH16 and SOD genes may have a protective behavior to assure the N remobilization and carbon fixation in response to the environmental conditions and controlling the stomata closure (Danyluk et al., 1996; Kosová et al., 2014a; Tsvetanov et al., 2000).

The regulation of the coordinated N and C metabolism enzymes (Chapter 2 and 3) reflect the high importance of the enzymes related to the N assimilation and remobilization (GS1 and GS2). In restrictive lines the N assimilation seemed to be the major strategy while the remobilization was inhibited; on the other hand, in nonrestrictive lines the N remobilization and translocation played the major role, with a lower investment of resources for Rubisco synthesis, this may suggest an efficient use and remobilization of nitrogen in the plant NUE (Carmo-Silva et al., 2015; Pang et al., 2014; Tian et al., 2015).

The Rubisco was over expressed in restrictive lines under high yielding conditions, whereas under low yielding conditions it was under expressed. The optimal conditions which led the higher yield of nonrestrictive lines may imply less need to increase the capacity for photosynthetic CO_2 fixation of non-restrictive lines, and could benefit plant growth by diversifying the high amount of N invested in Rubisco. In this sense Rubisco upregulation in restrictive lines together with GS2 overexpression may response to a higher demand of N supply to synthetize more Rubisco enzyme, which agrees previous reports about co-ordinated regulation of CO_2 fixation and N assimilation during grain filling in wheat (Nagy *et al.*, 2013; Komatsu *et al.*, 2014) and specially in durum wheat (Vicente *et al.*, 2015).

While under stress conditions, the down regulation of Rubisco in restrictive lines can lead to an improvement of biomass and grain yield due to lower N allocation in Rubisco synthesis, and greater investment in other limiting processes, as described for rice (Kanno *et al.*, 2017). While the Rubisco upregulation in nonrestrictive lines may agree with studies in wheat leaves where ABA signal is gattered by the stress

responsive genes resulting in higher Rubisco transcripts under water stress (Ashgari and Ebrahimzadeh, 2006; Budak *et al.*, 2013).

5 Water transport and hydraulics limitations

The water flow in the plants depends on the capacity of roots to uptake water and pass it by the root compartments for further axial water ascent, but there is a lack of universal rules for the water transport (Hodge, 2009). In chapter 5, the transpiration increased when the root system was pressurized, suggesting a root hydraulic limitation to water transport; in the hybrids bred in low rainfall zones this transpiration increase was higher suggesting a higher flux through the cell-cell radial path of the root (Suku et al., 2014) matching with the higher number of metaxylem vessels and lower hydraulic conductivity in these genotypes, as reported in grapevine and maize (Barrios-Masias et al., 2015; Caldeira et al., 2014). The opposite happened in the genotypes bred in higher rainfall zone. Moreover, the finding of higher PIP2;3 aquaporins expression in roots and leaves agreed with this same pattern (chapter 5) in genotypes bred in low rainfall zone, and the highly abundances of TIP 1:1 in nonrestrictive wheat lines (chapter 3) may confirm that aquaporins do a fine regulation in the root and leave hydraulics as reported in wheat and Arabidopsis (Almeida-Rodriguez et al., 2011). Hence the hydraulic features of these genotypes was closely related with their life history like reported in previous studies of chaparral communities (Pratt et al., 2010).

6 Aquaporins expression and inhibition

In the context of water transport driven by the transpiration demand and influenced by the root hydraulics, the role of aquaporins is very important (chapter 3 and 5).

The inhibition of aquaporins by $HgCl_2$ or $AgNO_3$ caused a decrease in the transpiration of all genotypes due to the blocking effect of this metal compounds (Liu

et al., 2014), it agrees with negative effects of aquaporin inhibitors that cause decreases in the stomatal conductance, carbon isotope discrimination and downregulation of aquaporin transcripts (Lopes et al., 2013; Terashima and Ono, 2002). The plant exudates also decreased in plants that had been treated with aquaporin inhibitors, although more so in genotypes bred in lower rainfall zone. This may suggest that these hybrids depend more on the aquaporin-mediated pathways for water transport. This was confirmed with the high abundances of PIP 2;3 in their roots to drive the axial and radial water flow across membranes agreeing previous reports in wheat (Fricke et al., 2014).

The aquaporin gene expression (chapter 5) showed a circadian pattern which agrees with reports of PIP 1 and PIP2 abundances (Lopez et al., 2013), and enhances the water permeability besides maintaining the osmotic balance (Li et al., 2008; Maurel, 2007). In our study the PIP 2;6 was overexpressed by leaves of lower rainfall genotypes and under expressed in their roots, it may be related with the higher transpiration response under high VPD conditions of these genotypes corresponding to previous reports of PIP 2 families in rice and cotton (Sakurai-Ishikawa et al., 2011; Zhang et al., 2013). While in the case of PIP 2;3 which fits exactly with the transpiration and hydraulic profile of high and low rainfall genotypes may indicate the specific contribution of this PIP 2;3 aquaporin to the water flow as previous reports of aquaporin role and hydraulic properties (Katsuhara et al., 2003). This contrasting expression in roots and leaves of both genotypes bred in high and low rainfall zones, is probably an influence of the breeding story of these materials. Moreover TIP 2;2 seem to be expressed constitutively in roots and shoots as in Arabidopsis (Tolk et al., 2016), whereas TIP 1.1 in wheat (chapter 3) seemed to help with the water flux in the leaves being highly expressed in the nonrestrictive genotypes which had the highest yield production. Thus, the role of aquaporins could have a great influence on the water status of the plant facilitating the water transport in the vacuolar and cytosolic compartments through the cell-ell path.

7 Water transport strategies to enhance biomass and yield production in given environments

The future food demand together with the changes in the climate requires crops with higher efficiency of water use in order to produce higher yield and biomass. The water limitation and the elevated CO_2 concentration in the atmosphere cause changes in the physiological parameters of the plants, moreover the water stress led to decreases in yield and biomass production. This research showed that water flux were crucial for the nutrient transport in order to produce more yield and biomass, the assays in wheat and pearl millet showed that there was a close interplay between the C and N pathways which are regulated by DREB transcription factors, dehydrins and stress responsive genes. Furthermore, the aquaporins played a major role in the fine regulation of the water transport, but not all the aquaporin isoforms had the same expression pattern; probably the most notable aquaporins which enhanced the water flow are PIP 2;3 and TIP 1.1, their overexpression was related with higher production of biomass and yield respectively. The restriction of water losses under high evaporative demand reflected the transpiration efficiency of the plants. This agreed with most previous reports that this restriction is a successful strategy when water resources are limited but soil are such that it can store soil moisture and plant usually face long and gradual terminal water stress. The non-restriction of water lose seemed to be a better strategy in dry environments characterized by erratic rainfall and light soils subjected to high evaporation and drainage where the non-restriction came as a strategy of “use it or lose it”.

CONCLUSIONS

Todavía no se han levantado las vallas

que digan al talento "De aquí no pasas".

Ludwing Van Beethoven

The result of this Thesis illustrates for durum wheat and pearl millet the need to account the genotypic variability for a greater understanding of crop adaptation to climate change.

1. In the case of durum wheat, atmospheric [CO_2] may strongly affect the physiological and molecular response to water stress during vegetative growth. Moreover, the interactive effects of both [CO_2] and water regime depends on genotypic variability.

2. The ability of durum wheat lines to restrict or not restrict the transpiration in response to increasing to the vapour pressure may affect agronomical performance under a wide range of environmental conditions in the Mediterranean. And the regulation of water lose is a successful strategy when water source is limited, whereas the non-restrictive transpiration capacity is applicable to wetter environments to develop larger biomass and produce higher yield.

3. Genotypic differences in durum wheat to the response to VPD bring associated differences in the response of a wide range of genes, including transcription factors as well as genes involved in the carbon and nitrogen metabolism and plant water transport.

4. The pearl millet hybrids bred for lower and higher rainfall environments seem to have very contrasting performances in response to transpiration demand for the speed of water flux from the stem to the leaves, and in its root hydraulic conductivity. Furthermore, the inhibition of aquaporins seems to affect strongly the cell to cell (transmembrane) path as well as the simplastic and apoplastic paths.

5. In pearl millet, the different performance of the water flux through the plant may affect the biomass development. The roots may have a key role in this behaviour, controlling the water flux in different ways through variations in their hydraulic conductance.

6. In wheat, the regulation of primary metabolism (N and C) in the response to a specific environment at gene expression level seems regulated by DREB transcription factors and dehydrins. Moreover, in pearl millet the water status is strongly associated with the aquaporin expression in roots and leaves. Remarkably the expression of aquaporin PIP 2;3 in hotter environments is completely opposite in hybrids depending of their rainfall zone targeted by the breeding.

7. The combination of phenotyping and gene expression analysis is a useful approach to identify genotypic variability and its behaviour in response to different environments. The results obtained in this research give insights on the importance to include such traits as part of the breeding process for crop enhancement.

RESUMEN DE LA TESIS

El triunfo no está

en vencer siempre

sino en nunca desanimarse.

Napoleón

RESPUESTA DIFERENCIAL DE LÍNEAS HISTÓRICAS DE CEREALES FRENTE AL ESTRÉS HÍDRICO: FENOTIPO Y EXPRESIÓN GENÓMICA.

Resumen global

Los futuros cambios climáticos ocasionarán una subida de las temperaturas y déficit en la disponibilidad de agua para la agricultura. La sequía será el principal estrés en los próximos años, junto con una subida en la concentración de CO_2 en la atmósfera. En este contexto el estatus hídrico y el flujo de agua a través de la planta juegan un papel importante en la adaptación a un ambiente específico; así mismo, son importantes la eficiencia de transpiración, la hidráulica de raíces, las acuaporinas y la regulación genómica. Esta tesis se enmarca en las diferentes respuestas de los cereales frente al estrés hídrico tanto a nivel de fenotipo como de expresión genómica. Los cereales estudiados fueron: una colección post-revolución verde de 20 genotipos de trigo duro semi-enano (Triticum durum) y una colección de 40 combinaciones de híbridos F1 y parentales de mijo (Pennistum glaucum) adaptados en zonas de alto y bajo nivel de lluvias en India. Ambos cereales son cultivos de primer consumo y de importancia económica en las zonas mediterráneas como en Asia y África respectivamente. Se evaluaron la adaptación a altas concentraciones de CO_2, a niveles de estrés hídrico moderado y severo, la respuesta en ambientes calurosos o de alto déficit de presión de vapor (VPD) y la adaptación a zonas de alto y bajo nivel de lluvias. Se evaluaron parámetros de rendimiento y biomasa con técnicas clásicas y con técnicas de teledetección, parámetros de uso de agua como la respuesta transpirativa, conductancia estomática, déficit de la temperatura de dosel, conductividad hidráulica y la composición isotópica del carbono; así como la expresión a nivel de transcritos de genes asociados con la respuesta a estrés, al metabolismo primario (C y N), y las aquaporinas. En el estudionde de la respuesta a

las altas concentraciones de CO_2, encontramos variación genotípica y que el crecimiento en una atmósfera de alto CO_2 no compensa las perdidas ocasionadas por el estrés hídrico. Luego en los ensayos de respuesta transpiratoria a la alta VPD revelaron dos categorías de genotipos tanto en trigo como en mijo: genotipos que restringen la perdida de agua (que también fueron mejorados en zona de bajo nivel de lluvias) y genotipos que no la restringen o no son sensibles al aumento de temperatura. El crecimiento de estos dos ideotipos se evaluó a nivel de invernadero, hidropónico, en plataformas de crecimiento y a nivel de campo con dos regímenes hídricos; los resultados indicaron que los genotipos que no restringen la transpiración desarrollaron mayor biomasa aérea y raíces que los que no restringen, y que el crecimiento de las hojas y raíces es coordinado. Así mismo estos genotipos que no restringen la pérdida de agua tuvieron un rendimiento superior en ambientes bajo óptimas condiciones agronómicas, mientras que los genotipos que restringen la transpiración desarrollaron mayor producción de grano solo en condiciones de estrés hídrico. Por otro lado el perfil de transcritos evaluado en cámaras de crecimiento y en condiciones de campo mostró que los genes DREB ejercen un rol regulatorio sobre la expresión de los genes de enzimas relacionadas con el metabolismo de C y N, dehidrinas y acuaporinas; y que en los genotipos no restrictivos hay abundancia de transcritos del gen DREB1, los cuales regulan positivamente la expresión de Rubisco, GS1, DNH16 y TIP 1.1 involucrando una mejor re-movilización del N por la demanda que ejercería la Rubisco, también hay mayor presencia de acuaporinas activas y dehidrinas en respuesta al mejor estado hídrico de estos genotipos, todo esto conllevaría a un rendimiento más alto. Por otro lado, los genotipos restrictivos también estarían regulados por el gen DREB2 en coordinación con el gen DREB1 que regularon positivamente la GS2 indicando una mayor demanda de fijación de N, así como la sobreexpresión de Rubisco bajo condiciones de alto rendimiento. Luego, se evaluó el efecto de la presurización de raíces sobre la transpiración, la conductividad hidráulica y la expresión de acuaporinas bajo condiciones de alta y baja VPD, estos análisis evidenciaron que los genotipos mejorados en zonas de bajo nivel de lluvias incrementaron la transpiración

junto a una baja conductividad hidráulica, además de presentar mayor número de tubos de xilema, células endodérmicas más delgadas e incrementar los transcritos de la acuaporina PIP 2;3 en la raíz. Los genotipos mejorados en zonas de alta incidencia de lluvias mostraron un perfil totalmente opuesto. En conclusión, es importante tener en cuenta la variabilidad genética en la respuesta adaptativa al estrés hídrico. La estrategia de restringir la transpiración es exitosa solo en ambientes donde el recurso hídrico es limitado, mientras que el mantener el estoma abierto y no restringir la pérdida de agua es una estrategia exitosa que resulta en una mayor tasa de asimilación fotosintética y mejor balance osmótico para producir mayor rendimiento, esta estrategia se aplica a ambientes más húmedos. El flujo de agua es importante para el tránsito de nutrientes, este flujo axial es regulado por la conductividad hidráulica de las raíces y el flujo radial de agua involucra en parte a las vías apoplástica y simplástica, y es regulada finamente por las acuaporinas en la vía trans celular donde las acuaporinas PIP 2;3 y TIP 1.1 juegan un rol importante de incrementar el transporte de agua en la planta.

Objetivos

El objetivo principal de la tesis fue dilucidar mecanismos fisiológicos/moleculares que confieren adaptación a sequía en series históricas de cereales.

Los objetivos específicos fueron:

1. Comparar los mecanismos moleculares y fisiológicos que podrían conferir un mejor comportamiento al trigo duro bajo condiciones de estrés hídrico y ellevadas concentraciones de CO_2 atomosférico.

2. Evaluar la variabilidad genotípica de la transpiración de la planta en respuesta al deficit de presión de vapor en trigo duro, y los mecanismod

fisiológicos y moleculares involucrados en su potencial impacto sobre el rendimiento del grano y la adptación del cultivo a condiciones mediterrneas.

3. Comparar la respuesta transpiratoria y mecanismos fisiológicos involucrados en el desarrollos de las raices y hojas de mijo asociados con su história de mejoramiento.

4. Comparar la respuesta transpiratoria y mecanismos fisiológicos asociados con la expresión de genes de acuaporinas y su inhibición, y con la hidráulicaa de raices en mijo mejorado para ambientes con diferente nivel de lluvias.

La tesis se ha desarrollado en cinco capítulos, de los cuales presento un resumen a continuación:

Capítulo 1

El incremento de producción de alimentos por unidad de agua nunca ha sido tan importante como ahora. La demanda de agua por otros sectores económicos diferentes al agrario está ejercerciendo presión sobre este recurso menguante, llamando a un incremento de la productividad del agua en la agricultura. A este tema se le ha dado mucha prioridad en la agenda durante los últimos 30 años, pero con excepción de algunos pocos casos como el del trigo en Australia, la mejora de cultivos para eficiencia de uso de agua está poco desarrollada. En este capítulo revisamos las posibles estrategias para mejorar la eficiencia de la transpiración (TE), la cual es el componente genético de la eficiencia de uso de agua. Como TE es difícil de medir, especialmente en campo, las evaluaciones de TE se han basado mayormente en parámetros alternativos (que pueden sustituir a los primeros), lo que ha resultado probablemente en una sobre-dependencia en el empleo de dichos parámetros. Un nuevo método lisimétrico para evaluar TE de forma gravimétrica a lo

largo de ciclo de crecimiento del cultivo ha revelado una alta variación genética en cereales y leguminosas. Este método ha establecido claramente, a través de especies, regímenes hídricos y genotipos, una ausencia de relación entre TE y el total de agua utilizada. Esto desestima postulados anteriores referidos a que una alta TE puede conducir a un menor potencial de producción. Más interesante es el estrecho vínculo encontrado en varios cultivos entre estas diferencias de TE y los atributos de las plantas que les hacen restringir la perdida de agua bajo elevados déficits de presión de vapor. Estos parámetros proveen una nueva visión de la genética de TE, especialmente desde la perspectiva de la hidráulica de las plantas, donde probablemente están involucradas las acuaporinas. Además abren nuevas posibilidades para obtener ganancias genéticas en TE, vía mejoramiento basado en éste parámetro. Por ultimo, pero no menos importante, las pequeñas cantidades de agua utilizadas en periodos específicos del ciclo de crecimiento, como por ejemplo durante el llenado de grano, pueden ser críticas. El presente capítulo investiga la eficiencia del uso de agua en estos estadios críticos.

Capítulo 2

La interacción entre una elevada [CO_2] y estrés hídrico afectará la adaptación del trigo duro a los futuros escenarios climáticos para la cuenca mediterránea, incluyendo un aumento de la sequía durante las etapas inicales del cultivo. Este estudio evalua la interacción entre un aumento en [CO_2] y diferentes niveles de estrés hídrico, durante la primera parte del ciclo de crecimiento, sobre parámetros fisiológicos y expresión génica en cuatro variedades modernos de trigo duro. El aumento de [CO_2] promovió el crecimiento, pero redujo el contenido de N en los tejidos de la planta, lo que parece asociado a una regulación negativa de los genes de la Rubisco y asimilación de N y una regulación positiva de genes implicados en la remobilización de C y N, lo cual podría sugerir un incremento en la eficiencia de N. La restricción del riego limitó la estimulación de la biomasa de la planta bajo elevada

[CO_2], especialmente en condiciones de estrés hídrico severo, mientras la conductancia estomática y la firma isotópica de carbono revelaron una estrategia de evitar la pérdida de agua. El perfil de transcritos bajo estrés hídrico sugirió la inhibición de la fijación de C y asimilación de N. Sin embargo, la interacción de la elevada [CO_2] y el estrés hídrico dependieron del genotipo y de la severidad del estrés, especialmente para la expresión de genes de respuesta a la sequía como las dehidrinas, la catalasa y la súper oxido dismutasa. Los resultados sugieren cambios coordinados entre los caracteres fisiológicos y los niveles de transcritos, así como entre losniveles de transcritos asociados con el metabolismo del C y del N; indicando potenciales genes y parámetros que podrían ser utilizados como marcadores de vigor temprano en trigo duro ante futuros escenarios de cambio climático. Además el mayor crecimiento de la planta estuvo ligado a un incremento en el contenido de N y la expresión de genes relacionados con el metabolismo del N y la regulación negativa de genes relacionados con el sistema antioxidante. La combinación de elevada [CO_2] y el estrés hídrico severo fue altamente dependiente de la variabilidad genotípica, sugiriendo estrategias específicas para cada genotipo respecto a la adaptación a las condiciones ambientales.

Capítulo 3

La regulación de la transpiración parece ser un factor clave que afecta la eficiencia de la transpiración y la adaptación agronómica del trigo a las condiciones de limitación de agua propias de los ambientes mediterráneas. Hasta la fecha no hay estudios relacionados con este parámetro bajo condiciones de campo. En este estudio, la respuesta transpirativa frente al incremento del déficit de presión de vapor (VPD) de un conjunto de 20 variedades modernas (semi enanas), de trigo duro liberadas durante las cuatro décadas pasadas en España se estudió en condiciones controladas. La misma colección de genotipos se evaluó en campo, bajo un amplio

rango de condiciones de crecimiento mediterráneas, desde estrés hídrico severo hasta buenas condiciones agronómicas en ambientes mediterráneos.

El grupo de líneas con transpiración no restrictiva (NR) exhibió un mejor comportamiento que las líneas restrictivas (R) en términos de producción y biomasa particularmente en ambientes más húmedos, mientras que lo contrario ocurrió solo en el ensayo con estrés hídrico más severo. Excepto en este ensayo, en general las líneas NR exhibieron mejor estatus hídrico (conductancia estomática) y mayor biomasa verde (inferida mediante índices de vegetación) durante la etapa reproductora que las líneas R.

En ambas categorías de genotipos la respuesta a las condiciones de crecimiento parece asociada con la expresión de factores de transcripción que responden a la sequía (DREB) resultando en complejos y diferentes comportamientos de enzimas relacionadas con el metabolismo primario. Por lo tanto, la respuesta de los genotipos NR bajo condiciones de crecimiento razonables a buenas fue asociado con una mayor abundancia de transcritos de genes involucrados en el metabolismo del nitrógeno (GS1 y GOGAT) y carbono (Sub unidad mayor de la Rubisco), asi como en el transporte de agua (acuaporina TIP 1.1), probablemente asociadas a unas condiciones hídricas mejores. En conclusión, las variedades modernas de trigo duro varían en su respuesta a la pérdida de agua, excepto para ambientes con sequía severa, las líneas menos restrictivas a la transpiración parecer tener favorecida la captación y el transporte de agua y nutrientes, el intercambio gaseoso fotosintético y en consecuencia un mayor rendimiento. La respuesta transpirativa de las plantas a la VPD podría ser una característica a explorar en el futuro cuando se seleccionen genotipos mejor adaptados a condiciones hídricas específicas.

Capítulo 4

Bajo condiciones de elevado déficit de presión de vapor (VPD) y sequía edáfica, restringir la transpiración es una vía importante para incrementar la eficiencia del

uso de agua. La pregunta que planteamos en este artículo es que si la mejora de cultivos para ambientes agroecológicos en los que varía la pluviometría se ha basado en seleccionar caracteres que controlan el uso del agua por la planta. Estos caracteres se han medido en genotipos de mijo mejorados para zonas que varían en su nivel de precipitación (8 combinaciones de parentales e híbridos F_1, 18 híbridos F_1 y luego 40 híbridos F_1). En todos los casos, encontramos variaciones agro-ecológicas en la pendiente de la respuesta de la transpiración al incremento de la VPD, y diferencias entre las líneas parentales en la respuesta de la transpiración en la respuesta al secado del suelo entre las combinaciones de parentales e híbridos. Los híbridos adaptados a zonas de bajo nivel de precipitaciones tuvieron curvas de respuesta transpiratoria más altas que adaptados a zonas de niveles altos de precipitación, pero no mostraron ninguna variación en su respuesta al secado del suelo. Cuando crecieron en un ambiente de baja VPD dentro de invernadero, los genotipos adaptados a zonas de bajo nivel de precipitaciones mostraron menor área foliar y peso seco, hojas más gruesas, junto con más desarrollo de raíces y exudados que los genotipos adaptados a zonas de alto nivel de precipitaciones, pero no hubo diferencia en la longitud de raíz ni tampoco en el índice hoja-raíz en estos genotipos. Por el contrario, cuando crecieron a cielo abierto bajo condiciones de alta VPD, los híbridos adaptados a las zonas de baja pluviometría tuvieron las hojas más grandes, mayor número de hijuelos y de biomasa. Finalmente, bajo condiciones de secado del suelo los genotipos de las zonas de bajo nivel de lluvias acumularon menor biomasa que las adaptadas a zonas de alto nivel de lluvias, de la misma manera lo hicieron los parentales comparados con los híbridos. Estas diferencias en la respuesta transpiratoria y crecimiento claramente muestran que la mejora para diferentes zonas agroecológicas también implicó diferentes estrategias genotípicas de mejora en relación con los parámetros relativos al uso de agua por la planta.

Capítulo 5

Los parámetros de ahorro de agua son importantes para la adaptación al estrés hídrico en mijo. Estudios anteriores mostraron que estos parámetros podrían estar relacionados con el transporte de agua en el cilindro de la raíz, involucrando acuaporinas y su rol putativo influenciando la hidráulica de la planta. Existe variación genética para éstos parámetros, una variación que también depende de la historía del mejoramiento del cultivo. Este estudio confirma las diferencias en estos parámetros de ahorro de agua – la respuesta transpirativa al incremento de la VPD (deficit de presión de vapor) y crecimiento de la planta bajo condiciones de alta VPD- dependen de la história de mejoramiento de este cultivo. Luego analizamos las relaciones entre parámetros de ahorro de agua y las vias de transporte de agua en el cilindro de la raiz, primero probando la respuesta transpiratoria a la inhibición de acuaporinas, luego ensayando el efecto de la presurización en el sistema de raices sobre la respuesta transpirativa, y tambíen midiendo la conductividad hidráulica de la raiz en genotipos contrastantes. Luego ensayamos el perfil de transcritos de tres aquaporinas bajo condiciones de alta VPD. Este trabajo fue realizado en cuatro híbridos mejorados para zonas de la India con pluviometría contrastadas. Los híbridos mejorados para zonas de baja precipitación incrementaron su tasa de transpiración más que los mejorados para zonas de elevada pluviometría cuando el sistema de raices fue presurizado; este primer grupo también mostró baja conductividad hidráulica de raices. El crecimiento de las raices de hibridos mejorados para zonas de baja precipitación fue superior, exhibiendo ápices y pelos radiculares mayores, más vasos metaxilemáticos y celulas endodérmicas mas delgadas que los híbridos de zonas de alto pluviometría. De forma similar su crecimiento aéreo bajo condiciones de alta VPD también fue mayor. Los híbridos mejorados para zonas de baja pluviometría mostraron alta regulación positiva de PIP 2;3 en raices y regulación negativa en hojas que los híbridos de alto nivel de lluvias; ambos grupos exhibieron perfiles de transcritos similares para PIP 2;6 y TIP 2;2, y una disminución comparable en la transpiración tras la aplicación del inhibidor de acuaporinas, a

pesar de una tendecia no significativa de una mayor inhibición en los híbridos mejorados para baja pluviometría. Estas características sugieren que la mejora ha influido en la fisiología del transporte de agua en la planta, involucrando el desarrollo anatómico de la raiz y la dependencia de las acuaporinas en las vias de flujo de agua en las raices.

Conclusiones

Las conclusiones de la investigación en esta tesis doctoral fueron:

1. Para el trigo duro, la concentración de CO_2 atmosférico podría de afectar fuertemente la respuesta fisiológica y molecular al estrés hídrico durante el crecimiento vegetativo. Más los efectos interactivos de ambos: la concentración de CO_2 y el régimen hídrico dependen de la variabilidad genotípica.

2. La habilidad de las líneas de trigo duro de restringir o no restringir la transpiración en respuesta al incremento del déficit de presión de vapor podría afectar el comportamiento agronómico bajo un rango de ambientes mediterráneos. Y la regulación de la pérdida de agua es una estrategia exitosa cuando la fuente de agua es limitada, mientras que la capacidad de no restringir la transpiración es aplicable a ambientes más húmedos para desarrollar mayor biomasa y producir alto rendimiento.

3. Las diferencias genotípicas en trigo duro en la respuesta a la VPD refleja diferencias asociadas a un rango amplio de genes, incluyendo factores de transcripción , así como genes involucrados en el metabolismo de C y N y el transporte de agua en la planta.

4. Los híbridos de mijo mejorados para ambientes de bajo nivel de lluvias parecen tener comportamientos contrastantes en respuesta a la demanda tranpiratoria que rige la velocidad del flujo hídrico del tallo hacia las hojas , y la conductividad hidraúlica de las raices. Además, la inhibición de las acuaporinas parece afectar furtemente a la via transmembarana célula a célula aspi como a las vias apoplástica y simplática.

5. En mijo, los diversos patrones de flujo de agua en la planta podríasn afectar el desarrollo d ela biomasa. Las raices tendrpian un rol clave en este comportamiento,, controlando de diferentes maneras el flujo hídrico con variaciones en su conductividad hidráulica.

6. En el caso de trigo la regulación del metabolismo primario (C y N) en la respuesta a ambientes específicos a nivel de expresión de genes parece estar regulada por los factors de transcripción de respuesta a la deshidratación "DREB" junto con las dehidrinas. Además, en mijo el estado hídrico de la planta está fuertemente asociado con la expresión de acuaporinas en las raices y hojas., En especial la expresión de la acuaporina PIP 2;3 en ambientes calurosos es totalmente en hpibridos dependiendo del nivel de lluvias de la zona para la que fueron seleccionados en la mejora.

7. La combinación del análisis de fenotipado y expresión de genes es un enfoque util para identificar la variabilidad genotípica y su comportamiento en repuesta a diferentes ambientes. Los resultados obtenidos en esta investigación abren la oportunidad de incluir estas características agronómicas en els proceso de selección para la mejora de cultivos.

Informe de los directores de la tesis

El Dr. José Luis Araus y el Dr. Vincent Vadez como directores de la tesis titulada: "Respuesta diferencial de líneas históricas de cereales frente al estrés hídrico y expresión genómica"- ¨Differential responses of historical cereal lines to water stress: Phenotype and Gene expression" que ha sido desarrollada por la estudiante de doctorado Susan Mery Medina Canzio.

INFORMAN sobre el factor de impacto y la participación de la estudiante doctoral en los artículos incluidos en la tesis doctoral.

Capítulo 1. Artículo "Eficiencia de transpiración: nuevas ideas de una vieja historia" - "*Transpiration efficiency: new insights into an old story*" publicada en la revista "Journal of Experimental Botany" cuyo factor de impacto fue 5.526 en 2014. En esta revisión trata sobre los esfuerzos para aprovechar la eficiencia transpirativa (TE, del inglés transpiration efficiency) como un carácter a considerar en la mejora genética para una mayor eficiencia del uso de agua. Como TE es difícil de medir, se discute un nuevo método para evaluarla gravimétricamente durante el ciclo entero del cultivo. Esta metodología provee tanto una nueva visión dentro de la mejora genética de TE, como ayuda a investigar cómo influye la hidráulica de las plantas, via acuaporinas, para lograr ganancias genéticas en TE. También puede ayudar a comprender mejor la posible relación entre diferencias en TE y la capacidad de las plantas de restringir la transpiración bajo condiciones de alta demanda evaporativa (déficid de presión de vapor, VPD). Este artículo fue una revisión donde la contribución de la estudiante doctoral ha sido proveer la primera evidencia tangible sobre el estrecho vínculo entre la capacidad de restringir la conductancia estomática bajo el incremento de la VPD y altos valores de eficiencia de transpiración. Luego la estudiante doctoral estuvo involucrada en continuar esta actividad, analizando la expresión de genes en algunas de las líneas de mijo que contrastan en su respuesta transpiratoria al incremento de la VPD (los resultados adicionales de este trabajo son actualmente objeto de revisión en un artículo enviado a la revista "*Plant Cell and Environment*". Además de esto, la estudiante doctoral ha proveído más evidencia del estrecho

vínculo con el funcionamiento de las acuaporinas. Por lo tanto la revisión donde la estudiante doctoral ha colaborado, se ha desarrollado en gran medida a nivel teórico. La calidad de las bases de datos generadas por la estudiante de doctorado ha influenciado profundamente la manera en que la revisión ha sido escrita, especialmente en términos de futuras propuestas de investigación. Esto es parte de los conceptos e hipótesis que la estudiante doctoral ha aplicado luego en los capítulos 4 y 5 de la tesis.

Capítulo 2. Artículo "Efectos interactivos de la elevada [CO_2] y el estrés hídrico sobre parámetros fisiológicos y expresión de genes durante el crecimiento vegetativo de cuatro genotipos de trigo duro" – "*Interactive Effects of Elevated [CO_2] and Water stress on physiological traits and gene expression during vegetative growth in four durum wheat genotypes*" - publicado en la revista "Frontiers in Plant Science" que tuvo un índice de impacto de 4.495 en 2016. En este estudio se investigaron los efectos interactivos de la elevada concentración de CO_2 ([CO_2]) junto con el estrés hídrico moderado y severo durante la primera parte del ciclo de crecimiento, sobre parámetros fisiológicos y de expresión génica en cuatro variedades modernas de trigo duro. Los resultados de este estudio mostraron que el incremento del desarrollo de la planta estaba estrechamente ligado al aumento del contenido de nitrógeno (N) junto con una alta expresión de genes relacionados con el metabolismo del N y una baja expresión de genes relacionados con el sistema antioxidante de la planta. Por lo tanto, la respuesta frente a la combinación de ambos factores, [CO_2] y estrés hídrico, dependen básicamente de la variabilidad genotípica, lo cual sugeriría que las estrategias de adaptación a los ambientes ensayados son genotipo-específicas. La estudiante doctoral realizó la parte experimental y el análisis de los datos en este estudio, mostrando en todo momento gran dedicación y responsabilidad.

Capítulo 3. Artículo "La respuesta transpirativa a la VPD de la planta está associada a las difernecias de rendimiento y expresión génica en trigo duro" - "Plant-transpiration response to VPD is associated to differential yield performance and gene expression in durum wheat". El artículo será enviado a la revista "*Journal of Experimental and environmental Botany*" cuyo factor de impacto es 4.369. Este estudio compara las respuestas agronómicas, fisiológicas y de expresión de genes de 20 variedades comerciales modernas de trigo duro (variedades semi-enanas), las cuales fueron liberadas durante las cuatro décadas pasadas en España. Estas variedades fueron agrupadas en base a la respuesta transpiratoria de la planta entera ante el incremento de la VPD, donde se identificaron diferentes categorías de genotipos: los restrictivos (medianamente restrictivos y muy restrictivos) y los no restrictivos. Estas mismas líneas de trigo se evaluaron en condiciones de campo durante dos campañas consecutivas, en diferentes sitios con condiciones de riego diversas, contabilizando un amplio rango de condiciones ambientales de crecimiento: desde 2816 Tn ha^{-1} hasta 7194 Tn ha^{-1}, respectivamente. Las diferencias entre los genotipos restrictivos y no restrictivos a la transpiración fueron más evidentes en condiciones de medio y alto rendimiento, mientras que entre los sub-grupos de genotipos no restrictivos no se encontraron diferencias significativas. Las líneas no-restrictivas mostraron mayor rendimiento y biomasa que las líneas restrictivas bajo condiciones de medio y alto rendimiento. Este mayor rendimiento estuvo asociado con altos niveles de transcritos de los genes involucrados en el metabolismo del N tales como GS1 y GOGAT, así como de genes involucrados en el metabolismo del carbono (Rubisco) y también las acuaporinas. En este grupo de plantas la estrategia de restringir la transpiración solo fue observada en condiciones de sequía severa donde los rendimientos fueron más bajos. Por lo tanto, las variedades modernas de trigo difieren en su respuesta a la pérdida de agua, la cual fue regulada a nivel fisiológico y a nivel transcriptómico por los factores de transcripción DREB. Estos resultados son muy novedosos ya que son la primera evidencia directa que relaciona en trigo la respuesta de la transpiración de la planta frente al VPD con su comportamiento agronómico. Además este estudio sienta las

bases para la selección de genotipos de trigo que estén adaptados a un ambiente deseado respecto a la disponibilidad de agua. La estudiante doctoral ha llevado el estudio y la parte experimental con dedicación y esfuerzo, luego ha mostrado responsabilidad en el análisis de los resultados y ha escrito el borrador del manuscrito.

Capítulo 4. Artículo "*Respuesta de la transpiración y crecimiento en líneas parentales e híbridos de mijo mejorados para ambientes contrastantes en su nivel de precipitaciones*" - "*Transpiration response and growth in pearl millet parental lines and hybrids bred for contrasting rainfall environments*" ha sido enviado a la revista "*Environmental and Experimental Botany*" cuyo factor de impacto es 4.298. Este estudio compara la respuesta transpiratoria bajo condiciones de alta demanda evaporativa (aire caliente y seco) y secado del suelo, donde la restricción de la transpiración es una importante vía hacia las ganancias en la eficiencia del uso del agua. El estudio compara híbridos y líneas de parentales que fueron mejoradas para diferentes zonas agro-ecológicas de India que varían en la cantidad de lluvias que reciben. Este artículo dilucidó si la mejora para ambientes específicos (zonas que varían en el nivel de lluvias) que difieren en la demanda evaporativa han seleccionado parámetros que controlen el uso de agua de la planta, medido en mijo (híbridos y parentales). La estudiante doctoral ha concebido el estudio y realizado el trabajo experimental relacionado. Al realizar este trabajo, la estudiante doctoral ha mostrado gran dedicación para generar bases de datos de alta calidad – sus habilidades experimentales son muy sobresalientes y más contando que Susan no tenía experiencia previa en la clase de parámetros que ella se proponía evaluar. La doctoranda también ha manejado con madurez y habilidad experimentos a diferentes niveles en la organización de la planta (desde hidráulica de raíces hasta repuesta transpirativa de toda la planta). Los resultados obtenidos de este trabajo son extremadamente interesantes y abren un ámbito para la selección de mijo mejor adaptado a las diversas zonas agroecológicas en India.

Capítulo 5. Artículo “Patrones de flujo de agua y dinámica de acuaporinas: desde la demanda transpirativa hasta la hidráulica de raices en híbridos de mijo“, artículo en preparación para su publicación. Este estudio compara las respuestas transpirativas a la alta VPD, la respuesta transpirativa a los inhibidores de acuaporinas y su consecuencia en la conductancia hidráulica de diferentes variedades de mijo mejorado para diferentes zonas agro-ecológicas de India que varían en su nivel de lluvias. Esto persigue analizar posibles diferencias en la expresión de varias acuaporinas en híbridos de mijo mejorados para zonas de alto y bajo nivel de precipitaciones. Este trabajo ha mostrado nuevamente un estrecho vínculo entre la respuesta transpirativa al incremento de VPD y el nivel en que la transpiración se inhibe cuando se aplica el inhibidor, mostrando que los híbridos adaptados en zonas de bajo nivel de lluvias son más dependientes de las vías dependientes de las acuaporinas para el transporte de agua. La estudiante doctoral ha concebido y llevado a cabo el trabajo experimental, mostrando gran iniciativa en las medidas a realizar, un adecuado planeamiento experimental en términos logísticos, e independencia realizando todo el trabajo. En todas estas tareas la doctoranda ha mostrado habilidades experimentales sobresalientes. Ella también ha generado muy buenos datos anatómicos de raíces, referentes a la abundancia de vasos en el metaxilema así como de tamaño y forma de las células de la endodermis. Dichos datos han mostrado diferencias significativas entre híbridos adaptados a zonas de alto y bajo nivel de lluvias,

.

Finalmente cabe destacar que Susan ha desarrollado su doctorado en una configuración compleja, trabajando en dos cereales distintos y en dos condiciones ambientales muy diferentes, así como en un amplio abanico de disciplinas, que abarcan desde la expresión genómica hasta la fisiología de toda la planta. En ICRISAT ella se ha adaptado rápidamente al nuevo ambiente cultural y ha prosperado en desarrollar su trabajo experimental con un alto grado de profesionalidad. En la

Universidad de Barcelona ella ha trabajado básicamente en un equipo multidisciplinario y pluricultural (incluyendo estudiantes e investigadores de España, EEUU, Colombia, Egipto, Túnez y China). Ella ha traído nuevas habilidades al laboratorio, por ejemplo el automatizar algunos de los procesos de recolección de datos, y también sobre aspectos logísticos de recolección de exudados de xilema o muestreos de campo y otros análisis de isótopos estables. Como se ha detallado antes, Susan tiene buenas habilidades experimentales y esto ha impactado su trabajo en el laboratorio. Durante su trabajo ella ha mostrado la capacidad de trabajar simultáneamente a varios niveles, tanto con plantas creciendo en sistemas hidropónicos en invernaderos y cámaras de crecimiento o en condiciones de campo. Susan ha mostrado excelente integración en el grupo y es una persona fácil de relacionarse con los demás. Susan ha madurado mucho en estos años, y mientras aún necesita trabajar más los aspectos de redacción, ella tiene la madurez y experiencia para liderar de forma independiente una investigación. Hay áreas de trabajo muy claras en que ella sobresale y donde se puede expandir en el futuro.

REFERENCES

Ser capaz de morir por una idea

no es grandeza.

La grandeza es tener la idea.

Noel Clarasó

Acreche MM, Briceño-Félix G, Sánchez JAM, Slafer GA. 2008. Physiological bases of genetic gains in Mediterranean bread wheat yield in Spain. European Journal of Agronomy 28, 162–170.

Almeida-Rodriguez AM, Hacke UG, Laur J. 2011. Influence of evaporative demand on aquaporin expression and root hydraulics of hybrid poplar. Plant, Cell and Environment 34, 1318–1331.

Andre C, Froehlich JE, Moll MR, Benning C. 2007. A Heteromeric Plastidic Pyruvate Kinase Complex Involved in Seed Oil Biosynthesis in Arabidopsis. The Plant Cell 19, 2006–2022.

Aranjuelo I, Cabrera-Bosquet L, Morcuende R, Avice JC, Nogues S, Araus JL, Martenez-Carrasco R, Perez P. 2011. Does ear C sink strength contribute to overcoming photosynthetic acclimation of wheat plants exposed to elevated CO_2? Journal of Experimental Botany 62, 3957–3969.

Araus JL, Cabrera-Bosquet L, Serret MD, Bort J, Nieto-Taladriz MT. 2013. Comparative performance of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ for phenotyping durum wheat adaptation to a dryland environment. Functional Plant Biology 40, 595.

Araus JL, Slafer GA, Reynolds MP, Royo C. 2002. Plant breeding and drought in C3 cereals: What should we breed for? Annals of Botany 89, 925–940.

Araus JL, Slafer G a., Royo C, Serret MD. 2008. Breeding for yield potential and stress adaptation in cereals. Critical Reviews in Plant Sciences 27, 377–412.

Aroca R, Porcel R, Ruiz-Lozano JM. 2012. Regulation of root water uptake under abiotic stress conditions. Journal of Experimental Botany 63, 43–57.

Ashgari R, Ebrahimzadeh H. 2006. Droughtstress increases the expression of wheat leaf Ribulose - 1, 5-bisphosphate carbolylase/oxygenase protein. Iranian Journal of Science & Technology 30, 1–7.

Barrios-Masias FH, Knipfer T, McElrone AJ. 2015. Differential responses of grapevine rootstocks to water stress are associated with adjustments in fine root hydraulic physiology and suberization. Journal of Experimental Botany 66, 6069–6078.

Belko N, Zaman-Allah M, Cisse N, Diop NN, Zombre G, Ehlers JD, Vadez V. 2012. Lower soil moisture threshold for transpiration decline under water deficit correlates with lower canopy conductance and higher transpiration efficiency in drought-tolerant cowpea. Functional Plant Biology 39, 306–322.

Belko N, Zaman-Allah M, Diop NN, Cisse N, Zombre G, Ehlers JD, Vadez V. 2013. Restriction of transpiration rate under high vapour pressure deficit and non-limiting water conditions is important for terminal drought tolerance in cowpea. Plant Biology 15, 304–316.

Bencze S, Bamberger Z, Janda T, Balla K, Varga B, Bedo Z, Veisz O. 2014. Physiological response of wheat varieties to elevated atmospheric CO2 and low water supply levels. Photosynthetica 52, 71–82.

Bloemen J, Teskey RO, McGuire MA, Aubrey DP, Steppe K. 2016. Root xylem CO_2 flux: an important but unaccounted-for component of root respiration. Trees - Structure and Function 30, 343–352.

Blum A. 2011. Plant breeding for water-limited environments (Springer, Ed.).

Blum A. 2013. Genomics for drought resistance – getting down to earth. Functional Plant Biology online, 1–8.

Bort J, Belhaj M, Latiri K, Kehel Z, Araus JL. 2014. Comparative performance of the stable isotope signatures of carbon, nitrogen and oxygen in assessing early vigour and grain yield in durum wheat. Journal of Agricultural Science 152, 408–426.

Budak H, Kantar M, Yucebilgili Kurtoglu K. 2013. Drought tolerance in modern and wild wheat. The Scientific World Journal 2013, 1–16.

Cabrera-Bosquet L, Molero G, Bort J, Nogues S, Araus JL. 2007. The combined effect of constant water deficit and nitrogen supply on WUE, NUE and $\delta^{13}C$ in durum wheat potted plants. Annals of Applied Biology 151, 277–289.

Caldeira CF, Bosio M, Parent B, Jeanguenin L, Chaumont F, Tardieu F. 2014. A Hydraulic Model Is Compatible with Rapid Changes in Leaf Elongation under Fluctuating Evaporative Demand and Soil Water Status. Plant Physiology 164, 1718–1730.

Calsa T, Figueira A. 2007. Serial analysis of gene expression in sugarcane (Saccharum spp.) leaves revealed alternative C4 metabolism and putative antisense transcripts. Plant Molecular Biology 63, 745–762.

Carmo-Silva E, Scales JC, Madgwick PJ, Parry MAJ. 2015. Optimizing Rubisco and its regulation for greater resource use efficiency. Plant, Cell and Environment 38, 1817–1832.

Casadessús J, Kaya Y, Bort J, et al. 2007. Using vegetation indices derived from conventional digital cameras as selection criteria for wheat breeding in water-limited environments. Annals of Applied Biology 150, 227–236.

Ceccarelli S, Acevedo E, Grando S. 1991. Breeding for yield stability in unpredictable environments: single traits, interaction between traits, and architecture of genotypes. Euphytica 56, 169–185.

Ceccarelli S, Grando S, Maatougui M, et al. 2010. Plant breeding and climate changes. The Journal of Agricultural Science 148, 627–637.

Chaumont F, Moshelion M, Daniels MJ. 2005. Regulation of plant aquaporin activity. Biology of the cell / under the auspices of the European Cell Biology Organization 97, 749–764.

Choudhary S, Mutava RN, Shekoofa A, Sinclair TR, Prasad PVV. 2013. Is the stay-green trait in sorghum a result of transpiration sensitivity to either soil drying or vapor pressure deficit? Crop Science 53, 2129–2134.

Clarkson DT. 2000. Root hydraulic conductance: diurnal aquaporin expression and the effects of nutrient stress. Journal of Experimental Botany 51, 61–70.

Comas LH, Becker SR, Cruz VM V, Byrne PF, Dierig D. 2013. Root traits contributing to plant productivity under drought. Frontiers in plant science 4, 1-16.

Danyluk J, Carpentier E, Sarhan F. 1996. Identification and characterization of a low temperature regulated gene encoding an actin-binding protein from wheat. FEBS Lett 389, 324–327.

Dawson TE, Mambelli S, Plamboeck AH, Templer PH, Tu KP. 2002. Stable Isotopes in Plant Ecology. Annual Review of Ecology and Systematics 33, 507–559.

Deak KI, Malamy J. 2005. Osmotic regulation of root system architecture. The Plant journal : for cell and molecular biology 43, 17–28.

Elazab A, Molero G, Serret MD, Araus JL. 2012. Root traits and δ13C and δ18O of durum wheat under different water regimes. Functional Plant Biology 39, 379.

Engels C, Fuganti-Pagliarini R, Marin SRR, Marcelino-Guimarães FC, Oliveira MCN, Kanamori N, Mizoi J, Nakashima K, Yamaguchi-Shinozaki K, Nepomuceno AL. 2013. Introduction of the rd29A:AtDREB2A ca gene into soybean (Glycine max L. Merril) and its molecular characterization in leaves and roots during dehydration. Genetics and Molecular Biology 36, 556–565.

FAO. 2015. Change Climate Change.

Farquhar G, Richards RA. 1984. Isotopic Composition of Plant Carbon Correlates With Water-Use Efficiency of Wheat Genotypes. Australian Journal of Plant Physiology 11, 539–532.

Fletcher AL, Sinclair TR, Hartwell allen J. 2008. Vapor deficit effects on leaf area expansion and transpiration of soybean subjected to soil drying. Proceedings 67, 15–20.

Forrest KL, Bhave M. 2007. Major intrinsic proteins (MIPs) in plants: A complex gene family with major impacts on plant phenotype. Functional and Integrative Genomics 7, 263–289.

Fricke W, Bijanzadeh E, Emam Y, Knipfer T. 2014. Root hydraulics in salt-stressed wheat. Functional Plant Biology 41, 366–378.

Gahlaut V, Jaiswal V, Kumar A, Gupta PK. 2016. Transcription factors involved in drought tolerance and their possible role in developing drought tolerant cultivars with emphasis on wheat (Triticum aestivum L.). Theoretical and Applied Genetics 129, 2019–2042.

Ghanem ME, Marrou H, Sinclair TR. 2015. Physiological phenotyping of plants for crop improvement. Trends in Plant Science.

Gholipoor M, Prasad PVV, Mutava RN, Sinclair TR. 2010. Genetic variability of transpiration response to vapor pressure deficit among sorghum genotypes. Field Crops Research 119, 85–90.

Guo Z, Slafer GA, Schnurbusch T. 2016. Genotypic variation in spike fertility traits and ovary size as determinants of floret and grain survival rate in wheat. Journal of Experimental Botany 67, 4221–4230.

Habash DZ, Baudo M, Hindle M, et al. 2014. Systems responses to progressive water stress in durum wheat. PLoS ONE 9, 1–21.

Habash DZ, Kehel Z, Nachit M. 2009. Genomic approaches for designing durum wheat ready for climate change with a focus on drought. Journal of Experimental Botany 60, 2805–2815.

Hassan NM, El-Bastawisy ZM, El-Sayed AK, Ebeed HT, Nemat Alla MM. 2015. Roles of dehydrin genes in wheat tolerance to drought stress. Journal of Advanced Research 6, 179–188.

Ho MD, Rosas JC, Brown KM, Lynch JP. 2005. Root architectural tradeoffs for water and phosphorus acquisition. Functional Plant Biology 32, 737–748.

Hodge A. 2009. Root decisions. Plant, Cell and Environment 32, 628–640.

Hose E, Clarkson DT, Steudle E, Schreiber L, Hartung W. 2001. The exodermis: a variable apoplastic barrier. Journal of experimental botany 52, 2245–2264.

Hove RM, Ziemann M, Bhave M. 2015. Identification and expression analysis of the barley (Hordeum vulgare L.) aquaporin gene family. PLoS ONE 10, e0128025.

Hu H, Xiong L. 2014. Genetic engineering and breeding of drought-resistant crops. Annual Review of Plant Biology 65, 715–741.

Hub JS, Aponte-Santamaría C, Grubmü H, De BL. 2010. Voltage-Regulated Water Flux through Aquaporin Channels In Silico. Biophysj 99, L97–L99.

Huseynova IM, Aliyeva DR, Mammadov AC, Aliyev JA. 2015. Hydrogen peroxide generation and antioxidant enzyme activities in the leaves and roots of wheat cultivars subjected to long-term soil drought stress. Photosynthesis Research 125, 279–289.

IGC. 2017. Cerelas Market report 2016-2017.

IPCC. 2014. Summary for Policymakers.

Javot H, Maurel C. 2002. The role of aquaporins in root water uptake. Annals of Botany 90, 301–313.

Kaldenhoff R, Ribas-Carbo M, Sans JF, Lovisolo C, Heckwolf M, Uehlein N. 2008. Aquaporins and plant water balance. Plant, Cell and Environment 31, 658–666.

Kanno K, Suzuki Y, Makino A. 2017. A small decrease in rubisco content by individual suppression of RBCS genes leads to improvement of photosynthesis and greater biomass production in rice under conditions of elevated CO2. Plant and Cell Physiology 58, 635–642.

Katerji N, Mastrorilli M, Rana G. 2008. Water use efficiency of crops cultivated in the Mediterranean region: Review and analysis. European Journal of Agronomy 28, 493–507.

Katsuhara M, Koshio K, Shibasaka M, Hayashi Y, Hayakawa T, Kasamo K. 2003. Over-expression of a Barley Aquaporin Increased the Shoot/Root Ratio and Raised Salt Sensitivity in Transgenic Rice Plants. Plant and Cell Physiology 44, 1378–1383.

Kholová J, Hash CT, Kumar PL, Yadav RS, Koová M, Vadez V. 2010. Terminal drought-tolerant pearl millet [Pennisetum glaucum (L.) R. Br.] have high leaf ABA and limit transpiration at high vapour pressure deficit. Journal of Experimental Botany 61, 1431–1440.

Kholová J, Nepolean T, Tom Hash C, Supriya A, Rajaram V, Senthilvel S, Kakkera A, Yadav R, Vadez V. 2012. Water saving traits co-map with a major terminal drought tolerance quantitative trait locus in pearl millet [Pennisetum glaucum (L.) R. Br.]. Molecular Breeding 30, 1337–1353.

Kholová J, Vadez V. 2013. Water extraction under terminal drought explains the genotypic differences in yield, not the anti-oxidant changes in leaves of pearl millet (Pennisetum glaucum). Functional Plant Biology 40, 44–53.

Kholova J, Zindy P, Mallayee S, et al. 2016. Component traits of plant water use are modulated by vapor pressure deficit in pearl millet [Pennisetum glaucum (L.) R. Br.]. Functional Plant Biology, 423–437.

Kim HK, Van Oosterom E, Dingkuhn M, Luquet D, Hammer G. 2010. Regulation of tillering in sorghum: Environmental effects. Annals of Botany 106, 57–67.

Komatsu S, Kamal AHM, Hossain Z. 2014. Wheat proteomics: proteome modulation and abiotic stress acclimation. Frontiers in Plant Science 5, 1-20.

Kosová K, Vitámvás P, Prášil IT. 2014a. Wheat and barley dehydrins under cold, drought, and salinity - what can LEA-II proteins tell us about plant stress response? Frontiers in Plant Science 5, 1–6.

Kosová K, Vítámvás P, Prášil IT. 2014b. Proteomics of stress responses in wheat and barley-search for potential protein markers of stress tolerance. Frontiers in plant science 5, 1–14.

Kruse E, Uehlein N, Kaldenhoff R. 2006. The aquaporins. Genome biology 7, 206.

Kumar S, Sunish Kumar Sehgal B, Uttam Kumar B, et al. 2012. Genomic characterization of drought tolerance-related traits in spring wheat. Euphytica 186, 265–276.

Laffray D, Louguet P. 1990. Stomatal responses and drought resistance. Bulletin de la Societe Botanique de France 137, 47–60.

Langridge P, Reynolds MP. 2015. Genomic tools to assist breeding for drought tolerance. Current Opinion in Biotechnology 32, 130–135.

Lian HL, Yu X, Ye Q, Ding XS, Kitagawa Y, Kwak SS, Su WA, Tang ZC. 2004. The role of aquaporin RWC3 in drought avoidance in rice. Plant and Cell Physiology 45, 481–489.

Li G, Santoni V, Maurel C. 2014. Plant aquaporins: Roles in plant physiology. Biochimica et Biophysica Acta - General Subjects 1840, 1574–1582.

Li Y, Ye W, Wang M, Yan X. 2009. Climate change and drought: a risk assessment of crop-yield impacts. Climate Research 39, 31–46.

Li GW, Zhang MH, Cai WM, Sun WN, Su WA. 2008. Characterization of OsPIP2;7, a water channel protein in rice. Plant and Cell Physiology 49, 1851–1858.

Liu G, Freschet GT, Pan X, Cornelissen JHC, Li Y, Dong M. 2010. Coordinated variation in leaf and root traits across multiple spatial scales in Chinese semi-arid and arid ecosystems. New Phytologist 188, 543–553.

Liu EK, Mei XR, Yan CR, Gong DZ, Zhang YQ. 2016. Agricultural Water Management Effects of water stress on photosynthetic characteristics, dry matter translocation and WUE in two winter wheat genotypes. Agricultural Water Management 167, 75–85.

Liu P, Yin L, Deng X, Wang S, Tanaka K, Zhang S. 2014. Aquaporin-mediated increase in root hydraulic conductance is involved in silicon-induced improved root water uptake under osmotic stress in Sorghum bicolor L. Journal of Experimental Botany 65, 4747–4756.

Long SP, Ainsworth EA, Leakey ADB, Nösberger J, Ort DR. 2006. Food for thought: lower-than-expected crop yield stimulation with rising CO2 concentrations. Science (New York, N.Y.) 312, 1918–21.

Lopes MS, Araus JL, Van Heerden PDR, Foyer CH. 2011. Enhancing drought tolerance in C 4 crops. Journal of Experimental Botany 62, 3135–3153.

Lopes MS, Iglesia-Turiño S, Cabrera-Bosquet L, Serret MD, Bort J, Febrero A, Araus JL. 2013. Molecular and physiological mechanisms associated with root exposure to mercury in barley. Metallomics 5, 1305–1315.

Lopez D, Venisse JS, Fumanal B, Chaumont F, Guillot E, Daniels MJ, Cochard H, Julien JL, Gousset-Dupont A. 2013. Aquaporins and leaf hydraulics: Poplar sheds new light. Plant and Cell Physiology 54, 1963–1975.

Loque D, Ludewig U, Yuan L, Wire N Von. 2005. Tonoplast Facilitate NH 3 Transport into the Vacuole 1. Society 137, 671–680.

Ludevid D, Höfte H, Himelblau E, Chrispeels MJ. 1992. The Expression Pattern of the Tonoplast Intrinsic Protein gamma-TIP in Arabidopsis thaliana Is Correlated with Cell Enlargement. Plant physiology 100, 1633–9.

Lynch JP. 2013. Steep, cheap and deep: An ideotype to optimize water and N acquisition by maize root systems. Annals of Botany 112, 347–357.

Manga VK, Kumar A. 2011. Cultivar Options for Increasing Pearl Millet Productivity in Arid Regions. Indian Journal of Fundamental and Applied Life Sciences 1, 200–208.

Marschner P. 2011. Marschner's Mineral Nutrition of Higher Plants: Third Edition.

Martin B, Thorstenson YR. 1988. Stable Carbon Isotope Composition (deltaC), Water Use Efficiency, and Biomass Productivity of Lycopersicon esculentum, Lycopersicon pennellii, and the F(1) Hybrid. Plant Physiol 88, 213–217.

Martins MQ. b, Rodrigues WP. c, Fortunato AS., et al. 2016. Protective response mechanisms to heat stress in interaction with high [CO_2] conditions in coffea spp. Frontiers in Plant Science 7, 1–18.

Maurel C. 1997. Aquaporins and Water Permeability of Plant Membranes. Annual Review of Plant Physiology and Plant Molecular Biology 48, 399–429.

Maurel C. 2007. Plant aquaporins: Novel functions and regulation properties. FEBS Letters 581, 2227–2236.

Maurel C, Simonneau T, Sutka M. 2010. The significance of roots as hydraulic rheostats. Journal of Experimental Botany 61, 3191–3198.

McKersie B. 2015. Planning for food security in a changing climate. Journal of Experimental Botany 66, 3435–3450.

Medina S, Vicente R, Amador A, Araus JL. 2016. Interactive effects of elevated [CO_2] and water stress on physiological traits and gene expression during vegetative growth in four durum wheat genotypes. Frontiers in Plant Science 7, 1–17.

Monneveux P, Rekika D, Acevedo E, Merah O. 2006. Effect of drought on leaf gas exchange, carbon isotope discrimination, transpiration efficiency and productivity in field grown durum wheat genotypes. Plant Science 170, 867–872.

Morison JI, Baker NR, Mullineaux PM, Davies WJ. 2008. Improving water use in crop production. Philosophical Transactions of the Royal Society B 363, 639–658.

Mwadzingeni L, Shimelis H, Dube E, Laing MD, Tsilo TJ. 2016. Breeding wheat for drought tolerance: Progress and technologies. Journal of Integrative Agriculture 15, 935–943.

Nagy Z, Németh E, Guóth A, Bona L, Wodala B, Pécsváradi A. 2013. Metabolic indicators of drought stress tolerance in wheat: Glutamine synthetase isoenzymes and Rubisco. Plant Physiology and Biochemistry 67, 48–54.

Nardini A, Salleo S. 2005. Water stress-induced modifications of leaf hydraulic architecture in sunflower: Co-ordination with gas exchange. Journal of Experimental Botany 56, 3093–3101.

Niemietz CM, Tyerman SD. 2002. New potent inhibitors of aquaporins: Silver and gold compounds inhibit aquaporins of plant and human origin. FEBS Letters 531, 443–447.

Van Oosterom EJ, Bidinger FR, Weltzien ER. 2003. A yield architecture framework to explain adaptation of pearl millet to environmental stress. Field Crops Research 80, 33–56.

Pang J, Palta JA, Rebetzke GJ, Milroy SP. 2014. Wheat genotypes with high early vigour accumulate more nitrogen and have higher photosynthetic nitrogen use efficiency during early growth. Functional Plant Biology 41, 215–222.

Parry M, Rosenzweig C, Livermore M. 2005. Climate Change, Global Food Supply and Risk of Hunger. Philosophical Transactions of the Royal Society B: Biological Sciences 360, 2125–2138.

Péret B, Li G, Zhao J, et al. 2012. Auxin regulates aquaporin function to facilitate lateral root emergence. Nature cell biology 14, 991–8.

Postaire O, Tournaire-Roux C, Grondin A, Boursiac Y, Morillon R, Schäffner AR, Maurel C. 2010. A PIP1 aquaporin contributes to hydrostatic pressure-induced water transport in both the root and rosette of Arabidopsis. Plant physiology 152, 1418–30.

Pratt RB, North GB, Jacobsen AL, Ewers FW, Davis SD. 2010. Xylem root and shoot hydraulics is linked to life history type in chaparral seedlings. Functional Ecology 24, 70–81.

Quigley F, Rosenberg JM, Shachar-Hill Y, Bohnert HJ. 2001. From genome to function: the Arabidopsis aquaporins. Genome Biology 3, 1–17.

Rai KN, Gupta SK, Govindaraj M, Yadav HP. 2015. Pearl Millet Improvement for enhanced productivity-strategies and impact. Indian Farming 62.

Rampino P, Mita G, Fasano P, Borrelli GM, Aprile A, Dalessandro G, De Bellis L, Perrotta C. 2012. Novel durum wheat genes up-regulated in response to a combination of heat and drought stress. Plant Physiology and Biochemistry 56, 72–78.

Ray DK, Mueller ND, West PC, Foley JA. 2013. Yield Trends Are Insufficient to Double Global Crop Production by 2050. PLoS ONE 8, 1-8.

Reymond M, Muller B, Leonardi A, Charcosset A, Tardieu F. 2003. Combining Quantitative Trait Loci Analysis and an Ecophysiological Model to Analyze the Genetic Variability of the Responses of Maize Leaf Growth to Temperature and Water Deficit. Plant Physiology 131, 664–675.

Reynolds MP, Mujeeb-Kazi A, Sawkins M. 2005. Prospects for Utilising Plant-Adaptive Mechanisms to Improve Wheat and other Crops in Drought- and Salinity-prone Environments. Annals of Applied Biology 146, 239–259.

Richards RA, Passioura JB. 1989. A breeding program to reduce the diameter of the major xylem vessel in the seminal roots of wheat and its effect on grain yield in rain-fed environments. Australian Journal of Agricultural Research 40, 943–950.

Robertson M, Kirkegaard J, Rebetzke G, Llewellyn R, Wark T. 2016. Prospects for yield improvement in the Australian wheat industry: a perspective. Food and Energy Security 5, 1–16.

Rostamza M, Richards RA, Watt M. 2013. Response of millet and sorghum to a varying water supply around the primary and nodal roots. Annals of Botany 112, 439–446.

Russo AC, Gouveia CM, Trigo RM, Liberato MLR, DaCamara CC. 2015. The influence of circulation weather patterns at different spatial scales on drought variability in the Iberian Peninsula. Frontiers in Environmental Science 3, 1+.

Sade N, Shatil-Cohen A, Attia Z, Maurel C, Boursiac Y, Kelly G, Granot D, Yaaran A, Lerner S, Moshelion M. 2014. The role of plasma membrane aquaporins in regulating the bundle sheath-mesophyll continuum and leaf hydraulics. Plant Physiology 166, 1609–20.

Sakurai-Ishikawa J, Murai-Hatano M, Hayashi H, Ahamed A, Fukushi K, Matsumoto T, Kitagawa Y. 2011. Transpiration from shoots triggers diurnal changes in root aquaporin expression. Plant, Cell and Environment 34, 1150–1163.

Salekdeh GH, Reynolds M, Bennett J, Boyer J. 2009. Conceptual framework for drought phenotyping during molecular breeding. Trends in Plant Science 14, 488–496.

Sanchez-Bragado R, Elazab A, Zhou B, Serret MD, Bort J, Nieto-Taladriz MT, Araus JL. 2014. Contribution of the ear and the flag leaf to grain filling in durum wheat inferred from the carbon isotope signature: Genotypic and growing conditions effects. Journal of Integrative Plant Biology 56, 444–454.

Sanchez-Garcia M, Royo C, Aparicio N, Martín-Sánchez JA, Álavaro F. 2013. Genetic improvement of bread wheat yield and associated traits in Spain during the 20th century. The Journal of Agricultural Science 151, 105–118.

Savage DF, Stroud RM. 2007. Structural Basis of Aquaporin Inhibition by Mercury. Journal of Molecular Biology 368, 607–617.

Schoppach R, Fleury D, Sinclair TR, Sadok W. 2017. Transpiration sensitivity to evaporative demand across 120 years of breeding of australian wheat cultivars. Journal of Agronomy and Crop Science 203, 219–226.

Schoppach R, Sadok W. 2012. Differential sensitivities of transpiration to evaporative demand and soil water deficit among wheat elite cultivars indicate different strategies for drought tolerance. Environmental and Experimental Botany 84, 1–10.

Schoppach R, Sadok W. 2013. Transpiration sensitivities to evaporative demand and leaf areas vary with night and day warming regimes among wheat genotypes. Functional Plant Biology 40, 708–718.

Schoppach R, Wauthelet D, Jeanguenin L, Sadok W. 2014. Conservative water use under high evaporative demand associated with smaller root metaxylem and limited trans-membrane water transport in wheat. Functional Plant Biology 41, 257-269

Segal E, Kushnir T, Mualem Y, Shani U. 2008. Water uptake and hydraulics of the root hair rhizosphere. Vadose Zone J. 7, 1027–1034.

Sermons SM, Seversike TM, Sinclair TR, Fiscus EL, Rufty TW. 2012. Temperature influences the ability of tall fescue to control transpiration in response to atmospheric vapour pressure deficit. Functional Plant Biology 39, 979–986.

Sheshadri SA, Nishanth MJ, Simon B. 2016. Stress-mediated cis-lement transcription factor interactions interconnecting primary and specialized metabolism in planta. Frontiers in Plant Science 7, 1–23.

Sinclair TR, Rufty TW. 2012. Nitrogen and water resources commonly limit crop yield increases, not necessarily plant genetics. Global Food Security 1, 94–98.

Sinha SK, Rani M, Bansal N, Gayatri, Venkatesh K, Mandal PK. 2015. Nitrate Starvation Induced Changes in Root System Architecture, Carbon:Nitrogen Metabolism, and miRNA Expression in Nitrogen-Responsive Wheat Genotypes. Applied Biochemistry and Biotechnology 177, 1299–1312.

Slafer GA, Araus JL, Royo C, Moral LF. 2005. Promising eco-physiological traits for genetic improvement of cereal yields in Mediterranean environments. Annals of Applied Biology 146, 61–70.

Soriano JM, Villegas D, Aranzana MJ, García Del Moral LF, Royo C. 2016. Genetic structure of modern durum wheat cultivars and mediterranean landraces matches with their agronomic performance. PLoS ONE 11, 1-19

Steudle E. 2000. Water uptake by plant roots: an integration of views. Plant and Soil 226, 45–56.

Steudle E, Peterson C. 1998. How does water get through roots ? Journal of Experimental Botany 49, 775–788.

Stitt M, Krapp. 1999. The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. Plant, Cell and Environment 22, 553–621.

Sugiyama T, Goto Y, Akazawa T. 1968. Pyruvate Kinase Activity of Wheat Plants Grown under Potassium Deficient Conditionsi. 43, 730–734.

Suku S, Knipfer T, Fricke W. 2014. Do root hydraulic properties change during the early vegetative stage of plant development in barley (Hordeum vulgare)? Annals of Botany 113, 385–402.

Sutka MR, Manzur ME, Vitali VA, Micheletto S, Amodeo G. 2016. Evidence for the involvement of hydraulic root or shoot adjustments as mechanisms underlying water deficit tolerance in two Sorghum bicolor genotypes. Journal of Plant Physiology 192, 13–20.

Taiz L, Zeiger E. 2010. Plant Physiology, Fifth Edition. Cell 1, 782.

Tambussi E a, Bort J, Guiamet JJ, Araus JL, Nogu S. 2007. The Photosynthetic Role of Ears in C3 Cereals: Metabolism , Water Use Efficiency and Contribution to Grain Yield. Critical Reviews in Plant Sciences 26, 1–16.

Tardieu F, Granier C, Muller B. 2011. Water deficit and growth. Co-ordinating processes without an orchestrator? Current Opinion in Plant Biology 14, 283–289.

Tardieu F, Parent B, Caldeira CF, Welcker C. 2014. Genetic and physiological controls of growth under water deficit. Plant physiology 164, 1628–35.

Terashima I, Ono K. 2002. Effects of HgCl(2) on CO(2) dependence of leaf photosynthesis: evidence indicating involvement of aquaporins in CO(2) diffusion across the plasma membrane. Plant & cell physiology 43, 70–78.

Thomsen HC, Eriksson D, Møller IS, Schjoerring JK. 2014. Cytosolic glutamine synthetase: a target for improvement of crop nitrogen use efficiency? Trends in Plant Science 19, 656–663.

Tian H, Fu J, Drijber RA, Gao Y. 2015. Expression patterns of five genes involved in nitrogen metabolism in two winter wheat (Triticum aestivum L.) genotypes with high and low nitrogen utilization efficiencies. Journal of Cereal Science 61, 48–54.

Tolk JA, Evett SR, Xu W, Schwartz RC. 2016. Constraints on water use efficiency of drought tolerant maize grown in a semi-arid environment. Field Crops Research 186, 66–77.

Toscano P, Gioli B, Genesio L, et al. 2014. Durum wheat quality prediction in Mediterranean environments: From local to regional scale. European Journal of Agronomy 61, 1–9.

Trnka M, Rötter RP, Ruiz-Ramos M, Kersebaum KC, Olesen JE, Žalud Z, Semenov MA. 2014. Adverse weather conditions for European wheat production will become more frequent with climate change. Nature Climate Change 4, 637–643.

Tsvetanov S, Atanassov A, Nakamura C. 2000. Gold responsive gene/protein families and cold/freezing tolerance in cereals. Biotechnology & Biotechnological Equipment 14, 3–11.

Vadez V. 2014. Root hydraulics: The forgotten side of roots in drought adaptation. Field Crops Research 165, 15–24.

Vadez V, Kholová J, Hummel G, Zhokhavets U, Gupta SK, Hash CT. 2015. LeasyScan: A novel concept combining 3D imaging and lysimetry for high-throughput phenotyping of traits controlling plant water budget. Journal of Experimental Botany 66, 5581–5593.

Vadez V, Kholova J, Medina S, Kakkera A, Anderberg H. 2014. Transpiration efficiency: New insights into an old story. Journal of Experimental Botany 65, 6141–6153.

Vadez V, Kholová J, Yadav RS, Hash CT. 2013. Small temporal differences in water uptake among varieties of pearl millet (Pennisetum glaucum (L.) R. Br.) are critical for grain yield under terminal drought. Plant and Soil 371, 447–462.

Vadez V, Krishnamurthy L, Hash CT, Upadhyaya HD, Borrell AK. 2011. Yield, transpiration efficiency, and water-use variations and their interrelationships in the sorghum reference collection. Crop and Pasture Science 62, 645–655.

Vadez V, Rao S, Kholova J, Krishnamurthy L, Kashiwagi J, Ratnakumar P, Sharma K, Bhatnagar-Mathur P, Basu P. 2008. Roots research for legume tolerance to drought: quo vadis? Journal of Food Legumes 21, 77–85.

Vicente R, Martínez-Carrasco R, Pérez P, Morcuende R. 2016a. An association network reveals co-regulation of carbon and nitrogen metabolism-related parameters in durum wheat grown under different environmental conditions. New Biotechnology 33, 414.

Vicente R, Pérez P, Mart??nez-Carrasco R, Feil R, Lunn JE, Watanabe M, Arrivault S, Stitt M, Hoefgen R, Morcuende R. 2016b. Metabolic and transcriptional analysis of durum wheat responses to elevated CO2 at low and high nitrate supply. Plant and Cell Physiology 57, 2133–2146.

Vicente R, Pérez P, Martínez-Carrasco R, Usadel B, Kostadinova S, Morcuende R. 2015. Quantitative RT–PCR platform to measure transcript levels of C and N metabolism-related genes in durum wheat: Transcript profiles in elevated [CO2] and high temperature at different levels of N supply. Plant and Cell Physiology 56, 1556–1573.

Watt M, Magee LJ, McCully ME. 2008. Types, structure and potential for axial water flow in the deepest roots of field-grown cereals. New Phytologist 178, 135–146.

Xu Z, Zhou G, Shimizu H. 2010. Plant responses to drought and rewatering. Plant signaling & behavior 5, 649–54.

Yadav OP, Bidinger FR, Singh D V. 2009. Utility of pearl millet landraces in breeding dual-purpose hybrids for arid zone environments of India. Euphytica 166, 239–247.

Yousfi S, Márquez AJ, Betti M, Araus JL, Serret MD. 2016. Gene expression and physiological responses to salinity and water stress of contrasting durum wheat genotypes. Journal of Integrative Plant Biology 58, 48–66.

Zaman-Allah M, Jenkinson DM, Vadez V. 2011. A conservative pattern of water use, rather than deep or profuse rooting, is critical for the terminal drought tolerance of chickpea. Journal of Experimental Botany 62, 4239–4252.

Zhang J, Li D, Zou D, Luo F. 2013. A cotton gene encoding a plasma membrane aquaporin is involved in seedling development and in response to drought stress. Acta Biochim Biophys 45, 104–114.

Zhang X, Liu S, Takano T. 2008. Overexpression of a mitochondrial ATP synthase small subunit gene (AtMtATP6) confers tolerance to several abiotic stresses in Saccharomyces cerevisiae and Arabidopsis thaliana. Biotechnology Letters 30, 1289–1294.

Zhang M, Ma D, Ma G, Wang C, Xie X, Kang G. 2017. Responses of glutamine synthetase activity and gene expression to nitrogen levels in winter wheat cultivars with different grain protein content. Journal of Cereal Science 74, 187–193.

Zhao P, Liu P, Yuan G, Jia J, Li X, Qi D, Chen S, Ma T, Liu G, Cheng L. 2016. New Insights on Drought Stress Response by Global Investigation of Gene Expression Changes in Sheepgrass (Leymus chinensis). Frontiers in plant science 7, 1-18.

No te rindas que la vida es eso,

continuar el viaje,

perseguir tus sueños,

destrabar el tiempo,

correr los escombros y destapar el cielo.

Mario Benedetti

Do not give up because life is that, continue the journey,
follow your dreams, unlock time, run the debris and touch the sky.


Universitat de Barcelona