PHYSIOLOGICAL TRAITS FOR TOLERANCE TO POST-ANTHESIS DROUGHT AND HEAT STRESS IN WHEAT

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Publications arising from this thesis

1. Tricker P. J., ElHabti A., Schmidt J., Fleury, D. 2018. The physiological and genetic basis of combined drought and heat tolerance in wheat. Journal of Experimental Botany, 69, 3195-3210. (Appendix)

2. El Habti A., Fleury D., Jewell N., Garnett T., Tricker P.J. High transpiration and water-soluble carbohydrates content in grains following combined drought and heat stress are associated with high grain weight in wheat. Formatted for Plant Physiology.

3. El Habti A., Corso D., Fleury D., Tricker P.J., Brodribb T. Hydraulic vulnerability segmentation in wheat protects the spike during drought. Formatted for New Phytologist.

Abbreviations

ABA	abscisic acid
ABARES	Australian Bureau of Agricultural and Resource Economics and Sciences
ABS	Australian Bureau of Statistics
ANOVA	analysis of variance
APX	ascorbate peroxidase
D	drought
DAA	days after anthesis
DAT	days after treatment
D&H	combined drought and heat stress
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
GR	glutathione reductase
HSD	Honest Significant Difference
IPCC	Intergovernmental Panel on Climate Change
ITC	International Trade Centre
ROS	reactive oxygen species
SOD	superoxide dismutase
VPD	vapour pressure deficit
WSC	water-soluble carbohydrates
WW	well-watered
WUE	water-use efficiency

Abstract

Drought and heat waves are projected to increase in frequency and severity in the light of a changing climate. Both stresses commonly occur simultaneously in wheat growing regions and challenge wheat production, especially during the grain filling stage when grain weight is being determined. Given the importance of wheat as a global food crop and its value as an export commodity, fluctuations in wheat productivity cause both social and economic issues. One of the strategies to alleviate such consequences is to continually develop wheat varieties with better performance in a dry and hot climate. This work aimed to identify novel physiological traits underlying wheat tolerance to the combination of drought and high temperatures to help identifying drought and heat-tolerant wheat varieties. To this end, the presented studies investigated four potential indicators of tolerance to combined drought and heat stress (D&H): plant water use, carbohydrate partitioning, xylem embolism and reactive oxygen species (ROS) modulation following combined drought and high temperature stress. Experiments were designed to mimic post-anthesis drought including a three-day heat stress.

The first experiment examined plant water use, carbohydrate partitioning and ROS response to combined drought and heat stress in eight wheat genotypes contrasting for grain weight under drought or combined drought and heat stress. Water-soluble carbohydrates (WSC) were mainly stored in the spike of modern varieties, unlike older varieties where most of the WSC were stored in the stem. Glucose and fructose concentrations in grains measured 12 days after anthesis were associated with total grain weight. Three-day heat stress differentially affected transpiration response to vapour pressure deficit, subsequently reducing daily water use in some genotypes while other genotypes were able to maintain water use. A genotypic difference in the modulation of the ROS scavenging system was observed following combined D&H; the activity of glutathione reductase, a ROS scavenging enzyme, was specifically reduced by combined D&H, while non-enzymatic antioxidant capacity was induced in the same genotypes. An *in planta* method was develop to quantify H_2O_2, one of the ROS, and oxidative stress *in vivo* using optical fibres coupled with probes that could detect physiological ROS concentrations.

Reduction in transpiration following D&H could reduce water flow to the spike, alter its hydraulic properties and subsequently the grain filling process. The second experiment investigated the hydraulic properties of the wheat spike following combined D&H. Hydraulic conductance, water potential and water flow to the spike were measured in two wheat varieties contrasting for grain weight under combined D&H. Reduced daily water use following combined D&H, used as a surrogate for transpiration, did not negatively affect water flow to the spike unlike leaf transpiration that was reduced by drought and combined D&H. This suggested differential regulation of transpiration under stress depending on the transpiring organ. Although there was a genotypic difference in the hydraulic conductance of the peduncle and water potential of the spike, these hydraulic properties were not associated with sap flow to the spike.

Xylem embolism could possibly explain reduction in whole plant transpiration following D&H. The third experiment explored xylem embolism in the peduncle and the flag leaf of one wheat genotype. Visualisation of cavitation using the optical method revealed a delayed embolism in the peduncle compared to the flag leaf. Vulnerability curves showed that the peduncle was more resistant to embolism than the flag leaf. This hydraulic vulnerability segmentation in wheat may protect the spike from variations in water availability in the rest of the plant.

The findings arising from this work contribute significantly to the body of knowledge on the physiological response of wheat plants to drought and to combined drought and heat stress and the differences between them. We investigated partitioning of water use and carbohydrates between tissues following stress and gained significant, new insights into tissue variability. We explored, in detail, potential physiological traits that could assist in the selection of wheat varieties well-adapted to a dry and hot environment.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Chapter 1:

Introduction

Wheat, a major crop

Wheat is one of the founder crops of agriculture (Zohary et al., 2012). Since its domestication, wheat has accompanied humankind, providing food and allowing farming, settlement and the development of human civilization. Today, wheat is a major staple food in many parts of the world and is consumed by over 2.5 billion people on a daily basis (Shiferaw et al., 2013). Wheat is the most widely cultivated cereal in the world. In 2017, wheat was grown on more than 218 million hectares and over 770 million tonnes of wheat were harvested worldwide (FAOSTAT, 2019). The main wheat-producing regions are the European Union, China, India, Russia, North America, Australia and Pakistan. Although most of the wheat produced is used for domestic consumption, the wheat trade represents 20% of the world's production and wheat is the most important cereal traded on international markets (ITC, 2019). In 2017, wheat exports were valued at about US $39 billion, the five main suppliers (USA, Russia, Australia, Canada and France) accounting for 64% of total exports. Besides its economic value, wheat has become part of our cultural heritage, influencing food habits, farming practices and social behaviour (Talhelm et al., 2014, Bjørnstad, 2016).

Wheat in a hot and dry climate

The wide adoption of wheat as a staple food is probably due to its adaptation to a broad range of weather conditions, allowing cultivation in regions where other crops cannot grow. Wheat can grow in altitudes from sea level to more than 3000 m above sea level, in locations where annual precipitations range from 250 to 1750 mm and growth temperatures range from 3°C to 32°C (Curtis, 2002). Wheat is more successfully grown between the latitudes of 30° and 60°N and 27° and 40°S; optimal temperatures for wheat growth range from 17°C to 23°C (Porter and Gawith, 1999) and the majority of wheat growing regions receive an average of between 375 and 875 mm of annual precipitation (Curtis, 2002). The broad growing range of wheat also means that the crop is often exposed to extreme weather conditions and wheat production is prone to fluctuations (Ray et al., 2013). In the last decades, wheat production was severely affected by drought and heat waves in many wheat-growing regions around the world, especially in arid and semi-arid areas, causing significant economic losses and social damage. In 2003, prolonged drought combined with high temperatures caused severe declines in wheat yields in major wheat producing countries such as France (-13%), Germany (-12%) and Italy (-19%), the loss to farmers reaching €1.15 billion (Munich Re Group, 2003). In China, average annual drought losses between 1986 and 2005 approached $4.2 billion, corresponding to a yield decrease of -2.5% (Lobell et al., 2011, Su et al., 2018), causing economic hardship in rural communities (Ye et al., 2012). Australia has experienced several devastating droughts, the Millennium Drought being an example. In 2002 and 2006, drought and heat waves decreased wheat production by 58% and 57% respectively compared to the previous years, resulting in economic losses of $0.7 and $0.9 billion in wheat exports, respectively (ABS 2004, 2006, ITC, 2019). Fluctuations in wheat production in major wheat exporting countries such as France, China and Australia immediately affect global prices, threatening food security and enhancing household, community and political instability (Asian Development Bank, 2013, Kalkuhl et al., 2016).

Current climate projections predict drought and heat waves will increase in intensity and frequency (Trenberth et al., 2013, Mbow et al., 2019). This is especially relevant for wheat growing regions where low rainfall and high temperatures occur simultaneously. It is estimated that global wheat yields will decline by 4.1% to 7.8% for each degree increase in temperature, depending on the region (Asseng et al., 2015, Liu et al., 2016, Zhao et al., 2017), and that drought will reduce wheat yields by 9% to 12% by the end of the century (Leng and Hall, 2019). In parallel, wheat demand is increasing due to the growing world population and to changing diets (ABARES, 2019). Consequently, worldwide wheat demand is predicted to exceed global wheat production by 2050 (Ray et al., 2013), highlighting the need for strategies to achieve long-term food security. Along with supportive institutional policies and economic, educational and social adaptation measures, agricultural research has been recognised as a central measure to mitigate the impact of climate change on crop production (FAO, 2017, de Coninck et al., 2018). As one of the scopes of agricultural research, wheat varietal improvement through breeding has been a major driver of yield increase, continually improving wheat productivity in challenging environments (Baenziger and DePauw, 2009, Passioura, 2010, Reynolds and Langridge, 2016).

Breeding for more tolerant wheat

Following wheat domestication, by which wheat morphology was modified to facilitate harvest and improve productivity, farmers developed landraces well adapted to local environments. The understanding of the principles of plant genetics and development of new breeding tools led to modern wheat breeding, replacing landraces with higher-yielding cultivars (Baenziger and DePauw, 2009). The incorporation of molecular markers and new hybridisation and statistical methods into breeding programs improved the choice of genetic resources and generation and assessment of genetic variation (Baenziger and DePauw, 2009). While genetic markers are helpful to identify plants with potential desirable traits, the expression of this potential is not guaranteed as it highly depends on the environment in which the plant grows (Sinclair, 2011). Trait-based breeding, also called physiological breeding, complements the molecular approach by selecting plants based on their physiological adaptation to specific environments (Reynolds et al., 2001). Commonly used physiological traits in breeding programs are sensitivity to photoperiod and reduced plant height. Differential sensitivity to photoperiod allows plants to adapt phenology to diverse environments in order to benefit from optimal conditions or to avoid adverse conditions (Worland, 1996). Shorter wheat plants have higher lodging resistance and better biomass partitioning to grains (Flintham et al., 1997). Other physiological traits have been recommended to improve grain yield. High transpiration efficiency, the ratio of carbon assimilation to water loss, is associated with improved grain yield in water-limited environments (Rebetzke et al., 2002, Richards et al., 2010). Leaf carbon isotopic composition, a proxy for transpiration efficiency, was used in a wheat breeding program to screen for high transpiration efficiency, which resulted in the release of Drysdale and Rees, two Australian wheat varieties with increased water use efficiency and higher yields compared to varieties grown at that time (Richards, 2006). Cooler canopy temperature in water-limited and warm environments can be associated with increased rooting depth, greater water use and higher yields (Reynolds et al., 2006, Olivares-Villegas et al., 2007, Lopes and Reynolds, 2010, Rattey et al., 2011) suggesting canopy temperature is a potential trait for high grain yield to be deployed in breeding programs (Deery et al., 2019).

Although these examples demonstrate the value of physiological traits for breeding improved varieties, lack of detailed understanding of the physiological basis of plant adaptation to abiotic stress has prevented further incorporation of physiological traits into breeding programs (Reynolds and Langridge, 2016). This is partly due to the lack of suitable tools to study traits other than the easily observable and measurable traits such as plant height, time to heading or greenness.

Today, available technology allows precision phenotyping at high temporal and spatial resolutions, making measurable traits that could not be measured previously. Examples are the continuous measurement of leaf elongation rate in maize using displacement transducers (Sadok et al., 2007), the monitoring of sucrose allocation during grain filling in barley using nuclear magnetic resonance (Melkus et al., 2011) and the quantification of root water uptake in wheat using X-ray computed tomography (Daly et al., 2018). Advances in precision phenotyping open new opportunities to uncover novel traits underlying tolerance to drought and heat stress and to achieve genetic gains through physiological breeding. Breeding for tolerance to drought and high temperature stress is an old challenge (Heyne and Brunson, 1940). Extensive research has been conducted to understand how wheat plants respond to environment, providing a wealth of knowledge on the physiological and genetic basis of drought or heat stress in wheat, focusing on single stresses.

Impact of drought and heat stress on wheat growth and development

Studies describing the impact of drought or heat stress consistently report the detrimental effect of both stresses on wheat productivity. The impact depends on the severity and duration of the stress, the sensitivity of the genotype to the stress and the developmental stage of the stressed plant. Accordingly, this review will consider physiological drought, defined as insufficient water availability to cover plants needs at a specific stage (Novák, 2009). Similarly, heat stress is defined as temperatures above the optimal temperatures required for growth at a specific stage (Wahid et al., 2007).

Drought and heat stress can occur at any developmental stage during wheat growth depending on the growing region, and their impact on plant morphology and development is the most well-known aspect as the effects are comparatively easily observable and quantifiable. Germination is dependent on soil moisture and temperature. A minimum grain water content of 35% to 45% is required for germination, below which germination does not start (Acevedo, 2002). Reduced soil water availability slows germination. Optimal temperatures for germination range from 12°C to 25°C, within which germination rate is accelerated by accumulated temperature (Acevedo, 2002, Bowden et al., 2008). Germination can occur between 4°C and 37°C (Acevedo, 2002). Delayed germination at high temperature is mediated via increased biosynthesis and sensitivity to abscisic acid (ABA) and reduced sensitivity to gibberellin in the embryo (Izydorczyk et al., 2018). Drought or heat stress during seedling establishment cause seedling mortality (Bowden et al., 2008). Soil temperatures of 33°C and 42°C reduced the number of wheat plants established by 18.6% and 71.5% respectively (Acevedo, 2002). Drought increases the phyllochron (Krenzer et al., 1991, Simane et al., 1993) and reduces leaf expansion and growth (Eastham et al., 1984), tillering (Rickman et al., 1983, Peterson et al., 1984) and root growth by reducing photosynthesis and transpiration (Batts et al., 1998). Heat stress shortens developmental stages by accelerating plant development (Slafer and Rawson, 1994). Both stresses ultimately reduce biomass production.

Decrease in rainfall and increase in temperature are common in many growing regions as wheat plants enter the reproductive stage. Low water availability and high temperatures around flowering affect meiosis and cause floret sterility, which reduces the potential number of grains (Dwivedi et al., 2017, Fábián et al., 2019). Heat stress increases grain-filling rate (Sofield et al., 1977, Wardlaw and Moncur, 1995) but reduces starch synthesis in developing grains, partly by affecting the thermo-sensitive soluble starch synthase (Rijven, 1986, Keeling et al., 1993, Jenner, 1994). Drought and heat stress reduce grain filling duration by accelerating senescence, and reduce assimilate availability and remobilisation to grains (Yang et al., 2000, Shirdelmoghanloo et al., 2016). Grain weight was reduced at a rate of 1.8% to 7.5% per degree above 15°C in a range of field trials (Wardlaw and Wrigley, 1994). As grain number and grain weight are the primary components of grain yield, drought and heat stress cause reductions in yield.

The effect of drought and heat stress on wheat growth is the consequence of the impact on wheat physiology and the underlying molecular and genetic basis. Drought and heat stress share some common response mechanisms, whereas other response mechanisms are antagonistic, constraining plants to adopt unique strategies when facing both stresses simultaneously.

Common responses to drought and high temperature in wheat

Several physiological traits are similarly negatively affected by drought and heat stress, although the underlying mechanisms may differ between the stresses. Carbon assimilation is reduced by drought and by heat stress through different mechanisms. Drought reduces carbon assimilation by reducing stomatal and mesophyll conductance to CO2 (Grassi and Magnani, 2005, Flexas et al., 2009, Galmés et al., 2013, Olsovska et al., 2016), whereas heat stress alters photosynthesis partly by decreasing the Rubisco activation state and by altering the electron transport capacity in thylakoids (Mathur et al., 2014, Perdomo et al., 2016, Sharkey, 2015, Perdomo et al., 2017, Singh and Thakur, 2018). Comparative studies on photosynthetic activity in the flag leaf and the spike of wheat under drought showed that photosynthesis of the spike was less sensitive to stress compared to photosynthesis in the flag leaf (Martinez et al., 2003, Abbad et al., 2004, Tambussi et al., 2005, Lou et al., 2018). The higher stability of photosynthetic capacity in the spike was explained by the higher relative water content of the spike and higher reactive oxygen species (ROS) scavenging enzymes in the spike (Abbad et al., 2004, Tambussi et al., 2005, Lou et al., 2018).

Similarly, chlorophyll content, an estimation of leaf photosynthetic capacity, is reduced by both drought and heat stress through different mechanisms. Drought during grain filling induces rapid remobilisation of nitrogen from leaves to grains through chlorophyll degradation (Hörtensteiner and Feller, 2002, Tian et al., 2013). Heat stress decreases chlorophyll content by damaging thylakoid membranes, the primary location of chlorophyll in the chloroplast (Ristic et al., 2007, Narayanan et al., 2016, Thomason et al., 2018). Reduced leaf chlorophyll content decreases assimilate production and limits assimilate availability for growth.

During the vegetative stage, when assimilate production exceeds the needs for plant growth, excess carbohydrates are stored in vegetative tissues such as the stem (Blum, 1998). In wheat, carbohydrates are mainly stored in the form of fructans in the stem and starch in leaves. After anthesis, fructans are hydrolysed to produce other soluble carbohydrates (WSC) such as glucose, fructose and sucrose, which are remobilised to grains to sustain grain filling if current carbon assimilation is limited by stress (Blum, 1998, Veenstra et al., 2017). Following drought or heat stress, reduced fructan content is accompanied by increased mobile sugar content in the stem during grain filling (Yang et al., 2000, Zhang et al., 2015, Shirdelmoghanloo et al., 2016). Mobile sugars such as fructose may also be remobilised to support deep root development to access water (Blum, 1998, Pinto and Reynolds, 2015). Root WSC were also shown to contribute to grain filling (Zhang et al., 2016). In the flag leaf, reduction of starch content and increased WSC content during grain filling were observed in wheat following heat stress (Dwivedi et al., 2017, Qaseem et al., 2019). In grains, drought and heat stress reduce WSC content due to altered carbohydrate metabolism. The thermosensitivity of soluble starch synthase reduces its activity under high temperature and reduces the conversion of sucrose to starch, leading to low starch content in developing grains (Bhullar and Jenner, 1986, Rijven, 1986, Keeling et al., 1993, Altenbach et al., 2003, Shah and Paulsen, 2003).

Both drought and heat stress increase cellular concentrations of reactive oxygen species (ROS) (Wang et al., 2010, Narayanan et al., 2015, Narayanan et al., 2016, Fan et al., 2018, Lou et al., 2018). High ROS concentration following stress is accompanied by an increase in activity of most of the ROS scavenging enzymes, namely glutathione reductase, superoxide dismutase, dehydroascorbate reductase and the antioxidant metabolites ascorbate and glutathione (Keleş and Öncel, 2002, Wang et al., 2010, Narayanan et al., 2015, Narayanan et al., 2016, Fan et al., 2018, Lou et al., 2018), resulting in a higher antioxidant capacity (Kumar et al., 2017). High ROS concentrations cause membrane and protein damage and lipid peroxidation. Both drought and heat stress cause cellular damage, but the damage caused by heat stress is more severe than by drought (Wang et al., 2010, Fan et al., 2018, Lou et al., 2018, Marček et al., 2019, Qaseem et al., 2019).

Despite the many studies on the wheat metabolome under drought and heat stress (Bowne et al., 2012, Ullah et al., 2017, Guo et al., 2018, Michaletti et al., 2018, Thomason et al., 2018, Wang et al., 2018, Impa et al., 2019, Marček et al., 2019, Yadav et al., 2019), there is no clear understanding on how both stresses affect the metabolome. Drought and heat stress in these studies differ with environmental conditions (field or controlled environment), the severity and duration of drought or heat stress, the level of tolerance of the genotypes, the tissue of interest (leaf, stem, spike), the developmental stage during which stress was applied and sampling time, making it difficult to compare the effect of drought or heat stress on the same metabolite. To date, there is no study that compared the stresses. Despite this limitation, a few metabolites were consistently affected by drought or heat stress across experiments. Both drought and heat stress increased leucine content in leaves at different developmental stages subjected to different stress intensity and duration (Bowne et al., 2012, Ullah et al., 2017, Guo et al., 2018, Michaletti et al., 2018, Thomason et al., 2018). This may reflect a higher energy demand supplied through glycolysis under drought or heat stress (Rollins et al., 2013). Drought and heat stress reduced glutarate content in leaves (Bowne et al., 2012, Michaletti et al., 2018, Thomason et al., 2018), which may indicate reduced nitrogen assimilation into amino acids (Hodges, 2002).

Contrasting mechanisms in response to drought and heat stress in wheat

While drought and heat stress have a similar impact on some aspects of wheat physiology, several mechanisms differ in response to drought or heat stress and may conflict. For instance, transpiration, one of the key components of wheat physiology and productivity is differentially affected by drought or heat stress. When water is available, soil water is absorbed by the roots, flows through the plant and is transpired via the stomata (Dixon, 1896). At high temperature, air and leaf temperatures rise and continuous transpiration allows evaporative cooling of the leaf, resulting in cooler canopies (Chaves et al., 2016). When the evaporative demand in the atmosphere exceeds water uptake through the roots, as it is the case during drought, stomata close to prevent water loss. Reduced stomatal conductance increases leaf temperature and alters heat-sensitive cellular processes such as photosynthesis. When both stresses are combined, plants face two conflicting mechanisms in response to the new stress: open stomata and allow evaporative cooling, or close stomata and save water.

ABA is a central regulator of stomatal aperture. Increased ABA in leaves following reduced air humidity induces stomatal closure (Buckley, 2019). In wheat, drought increased ABA concentration in the flag leaf, spike and floral tissues and xylem sap (Westgate et al., 1996, Izanloo et al., 2008, Yang et al., 2006, Weldearegay et al., 2012), whereas heat stress had no effect on ABA concentration (Weldearegay et al., 2012), which agrees with the lower transpiration due to stomata closure observed in wheat under drought but not under heat stress (Faghani et al., 2015). Low ABA concentration was associated with lower tissue water potential in wheat (Westgate et al., 1996).

Drought or heat stress differentially affect plant water status, a key aspect of whole plant physiology. While heat stress has no effect on leaf relative water content when water is ample, drought reduces tissue water content to different extents depending on genotypes

(Machado and Paulsen, 2001, Wardlaw, 2002, Wang et al., 2010, Zhang et al., 2010, Grigorova et al., 2011, Weldearegay et al., 2012). Water content in spike tissues is less sensitive to drought compared to leaves (Barlow et al., 1980, Tambussi et al., 2005). Ability to maintain a relatively high water content, and thereby cellular function, under drought has been attributed to better osmotic adjustment due to higher content of metabolites such as monosaccharides (sucrose, glucose and fructose) and organic acids (Tambussi et al., 2005, Bowne et al., 2012, Marček et al., 2019).

Root growth response to drought and heat stress is variable: while severe drought limits root growth, mild drought promotes root growth deep in the soil where water is available (Prasad et al., 2008). Heat stress decreases root growth as a result of reduced carbon partitioning to roots (Batts et al., 1998). Differential effect of drought or heat stress on root growth results in contrasting root distribution. Wheat plants grown under drought were characterised by deep roots, while roots of plants grown in hot irrigated conditions proliferated at the soil surface (Pinto and Reynolds, 2015).

At the metabolic level, although both drought and heat stress increase cellular ROS concentrations, their impact on some antioxidants contrasted in wheat. Peroxidase and superoxide dismutase activity and α-tocopherol content, an antioxidant, were increased under drought but decreased under heat stress (Keleş and Öncel, 2002, Wang et al., 2010, Fan et al., 2018).

Studies of the wheat proteome following drought or heat stress revealed that most of the differentially accumulated proteins following drought indicated an impairment of photosynthesis, carbohydrate and nitrogen metabolism and antioxidant capacity (Ford et al., 2011, Faghani et al., 2015, Deng et al., 2018, Michaletti et al., 2018). Differentially accumulated proteins following heat stress were related to altered biosynthesis and turnover of proteins, as evidenced by decreased amounts of heat-responsive proteins related to ribosomes and translation factors, but increased chaperones synthesis, e.g. heat-shock proteins (HSP), and oxidative stress response (Wang et al., 2018), two mechanisms that protect and limit protein damage.

Transcriptome studies in wheat revealed that heat-responsive genes largely exceed drought- or osmotic stress-responsive genes (Aprile et al., 2013, Liu et al., 2015). While half of the drought- or osmotic stress-responsive genes are in common with heat stress and include splicing factors, lipid transfer proteins, protease inhibitors and transcription factors (Aprile et al., 2013, Liu et al., 2015). Most heat-responsive genes are specific to heat stress and are related to chaperonins, carbohydrates and amino acids metabolism (Aprile et al., 2013, Liu et al., 2015).

Plant response to combined drought and heat stress

Wheat response to either drought or heat stress is relatively well studied. Surprisingly, considering its relevance to field conditions, less is known about wheat response to the combination of both stresses. Decreasing rainfall coupled with increasing temperatures often co-occur in wheat growing regions during the grain filling stage, a key sensitive period to environmental stress (Barnabás et al., 2008). Recent research is addressing the combination of these stresses since studies have shown that the combination is a unique stress rather than a simple addition of multiple stresses (Mittler, 2006). When response to drought or heat stress involves common mechanisms, the impact of the combined stresses on biological processes is likely to be more detrimental compared to each individual stress. However, when drought and heat stress responses differ, combined drought and heat stress (D&H) stress will require plants to respond to the stress that is most detrimental to vital processes, or to develop a unique response.

Combined D&H was more detrimental than individual stresses for leaf area, biomass accumulation, harvest index, spike fertility, grain number, and ultimately grain yield (Nicolas et al., 1984, Shah and Paulsen, 2003, Zhang et al., 2010, Prasad et al., 2011, Weldearegay et al., 2012, Fábián et al., 2019, Qaseem et al., 2019). Genotypic variation was found under combined D&H for biomass, grain number, individual grain weight and grain yield (Pradhan et al., 2012, Weldearegay et al., 2012, Fábián et al., 2019). Reduced grain yield was caused by a lower number of endosperm cells, lower grain filling rate and shorter grain filling duration, resulting in low individual grain weight (Nicolas et al., 1984, Altenbach et al., 2003, Shah and Paulsen, 2003, Prasad et al., 2011, Qaseem et al., 2019).

A recent study with combined D&H treatment was conducted on diverse wheat genotypes and combined agronomic with physiological and biochemical traits measurements (Qaseem et al., 2019). Results showed positive correlation of grain yield with chlorophyll contents, soluble carbohydrates contents and proline contents, and a negative correlation with growth duration (Qaseem et al., 2019). Another recent study focused on the causes of reduced fertility when plants were exposed to stress during gametogenesis (Fábián et al., 2019). Combined D&H affected pistil morphology and reduced pollen viability in a sensitive genotype by increasing the accumulation of ROS and reactive nitrogen species, and enhancing lipid peroxidation (Fábián et al., 2019). Overall, combined D&H exacerbates the effect of drought or heat stress on membrane damage (Wang et al., 2010, Grigorova et al., 2011, Qaseem et al., 2019).

At the physiological level, combined D&H severely impaired gas exchange in wheat by further reducing stomatal conductance compared to drought or heat stress alone and subsequently leaf transpiration and photosynthesis (Shah and Paulsen, 2003, Wang et al., 2010, Zhang et al., 2010, Prasad et al., 2011, Perdomo et al., 2016). Limited carbon assimilation was also caused by accelerated chlorophyll degradation (Wang et al., 2010, Prasad et al., 2011, Pradhan et al., 2012, Qaseem et al., 2019) and impairment of photosystem efficiency (Pradhan et al., 2012, Perdomo et al., 2016).

Interaction of drought with heat stress strongly affects water relations in wheat (Machado and Paulsen, 2001). Compared to individual drought or heat stress, combined D&H further reduced leaf water potential and relative water content (Machado and Paulsen, 2001, Wardlaw, 2002, Wang et al., 2010, Zhang et al., 2010¸ Grigorova et al., 2011, Weldearegay et al., 2012, Fábián et al., 2019, Qaseem et al., 2019). Combined D&H also increased leaf osmotic potential, which required higher osmotic adjustment as evidenced by enhanced accumulation of solutes such as proline, soluble carbohydrates and free amino acids (Machado and Paulsen, 2001, Wang et al., 2010, Qaseem et al., 2019).

ROS metabolism is very sensitive to combined D&H in wheat. Combined D&H increased singlet oxygen production rate and accumulation of hydrogen peroxide, resulting in a higher lipid peroxidation compared to individual drought or heat stress (Wang et al., 2010). In parallel, the ROS scavenging system was induced by combined D&H by increasing the activity of superoxide dismutase (Wang et al., 2010) and glutathione reductase (Keleş and Öncel, 2002), and by reducing the activity of ascorbate peroxidase, peroxidase and catalase (Keleş and Öncel, 2002, Wang et al., 2010). Ascorbate peroxidase activity under combined D&H was similar to drought, whereas peroxidase activity was similar to heat stress (Wang et al., 2010). Antioxidant metabolites glutathione and ascorbic acid were accumulated following combined D&H at a similar level to drought for glutathione and to a lesser extent than individual drought or heat stress for ascorbic acid (Wang et al., 2010).

In the transcriptome of durum wheat plants subjected to individual or combined D&H at booting stage, only 2.8 - 5.97% of the differentially expressed genes (DEG) under combined D&H were common with both drought- or heat stress-responsive genes. 0.94 - 2.14% were shared with drought, 36.09 - 74.31% were shared with heat stress, and 21.94 - 55.08% of the DEG were specific to combined D&H, depending on the level of tolerance of the genotype (Aprile et al., 2013). Similar observations were made on wheat seedlings subjected to heat stress, osmotic stress and combined heat and osmotic stress, where 24.49% of DEG under combined D&H were common with drought and heat stress, 6.53% were only shared with drought, 49.58% were shared with heat stress, and 19.46% of the DEG were specific to combined D&H, 6h after stress (Liu et al., 2015). Specifically induced genes following the combined stress were mostly related to RNA processing and epigenetic regulation of gene expression. These results highlight the heat dominant response when drought and heat stress are combined as well as the unique nature of combined D&H.

Physiological traits for tolerance to combined drought and heat stress

Given the unique nature of combined D&H, current knowledge of the mechanisms underlying wheat tolerance to individual drought or heat stress cannot be fully applied to their combination. Combining our advanced understanding of the mechanisms conferring tolerance to drought or heat stress and current knowledge of the impact of combined stresses on wheat productivity could allow better targeting of the mechanisms underlying wheat tolerance to combined D&H. Three traits of interest emerged from this review:

- Transpiration is an important aspect of plant water use and whole plant physiology. Transpiration is driven by evaporative demand of the atmosphere (Dixon, 1896). Genotypic differences in transpiration response to vapour pressure deficit (VPD) exist in wheat (Schoppach and Sadok, 2012, Schoppach et al., 2017). While transpiration is not restricted at high VPD in some genotypes, other genotypes restrict transpiration when a VPD threshold is reached. Restriction of transpiration at high VPD might be beneficial for crop production (Vadez et al., 2014). The conflicting response of transpiration to drought or heat stress implies that this trait is likely to be related to plant performance when both stresses are combined. Although explored in wheat plants subjected to drought, as far as we are aware, transpiration sensitivity to VPD following combined D&H had not been investigated. I hypothesised that the combination of drought and heat stress would be more detrimental to transpiration than drought only and would alter transpiration sensitivity to VPD.

- Grain filling is a complex process that combines assimilate transport to grains and biosynthesis of starch as the final product of stored carbohydrates. Successful grain filling requires assimilate availability for grain filling and functional biochemical pathways for conversion to starch. Drought and heat stress reduce assimilate production and deplete stored soluble carbohydrates, in addition to shortening the grain filling period. The additive, negative impact of drought and heat stress on grain filling suggests impairment of grain filling under combined D&H. However, genotypic variation exists for grain weight under combined stresses (Reynolds et al., 2015), implying the existence of tolerance mechanisms related to grain filling. Assimilate production for grain filling under stress may be less restricted in spike tissues where water status and photosynthesis are less sensitive to stress compared to leaves (Barlow et al., 1980, Lou et al., 2018, Tambussi et al., 2005). Accordingly, the spike may be a major contributor to the grain filling process under stress (Evans et al., 1972, Motzo and Giunta, 2002, Maydup et al., 2010, Maydup et al., 2014, Guo and Schnurbusch, 2016). Assessing carbohydrate partitioning in different plant tissues following combined D&H could provide insights into the major contributors of assimilates to grain filling under stress and allow identification of limiting factors for grain filling in harsh environments. When tissue water content is reduced due to a stressful growth environment, osmotic adjustment helps maintain a favourable tissue water status (Blum, 2017). This is partly achieved through accumulation of osmolytes such as soluble carbohydrates and some amino acids. Identification of metabolites' response to combined D&H is needed to understand how wheat plants adapt their metabolome to maintain favourable osmotic pressure under combined stresses.

- ROS metabolism and oxidative damage are well-studied stress responses at the biochemical and transcriptome levels in wheat as well as in other species (e.g. turfgrass (Jiang and Huang, 2000); *Arabidopsis* (Koussevitzky et al., 2008, Zandalinas et al., 2016); lotus (Signorelli et al., 2013); maize (Hussain et al., 2019); barley (Templer et al., 2017); citrus (Zandalinas et al., 2017a, Zandalinas et al., 2017b). Just as favourable water status is essential for the normal function of biological processes, ROS scavenging is essential to protect biological processes from oxidative damage. Modulation of ROS metabolism appears to be sensitive to stress stimuli, and results vary depending on stress intensity and duration. Moreover, while the response of ROS scavenging system to stress is well explored, there is little information about ROS content *per se*, as these molecules are highly unstable and difficult to measure (Queval et al., 2008, Smirnoff and Arnaud, 2019). The importance of ROS metabolism in plant response to stress, and particularly to combined stresses, incites the development of reliable tools to quantify oxidative response to stress.

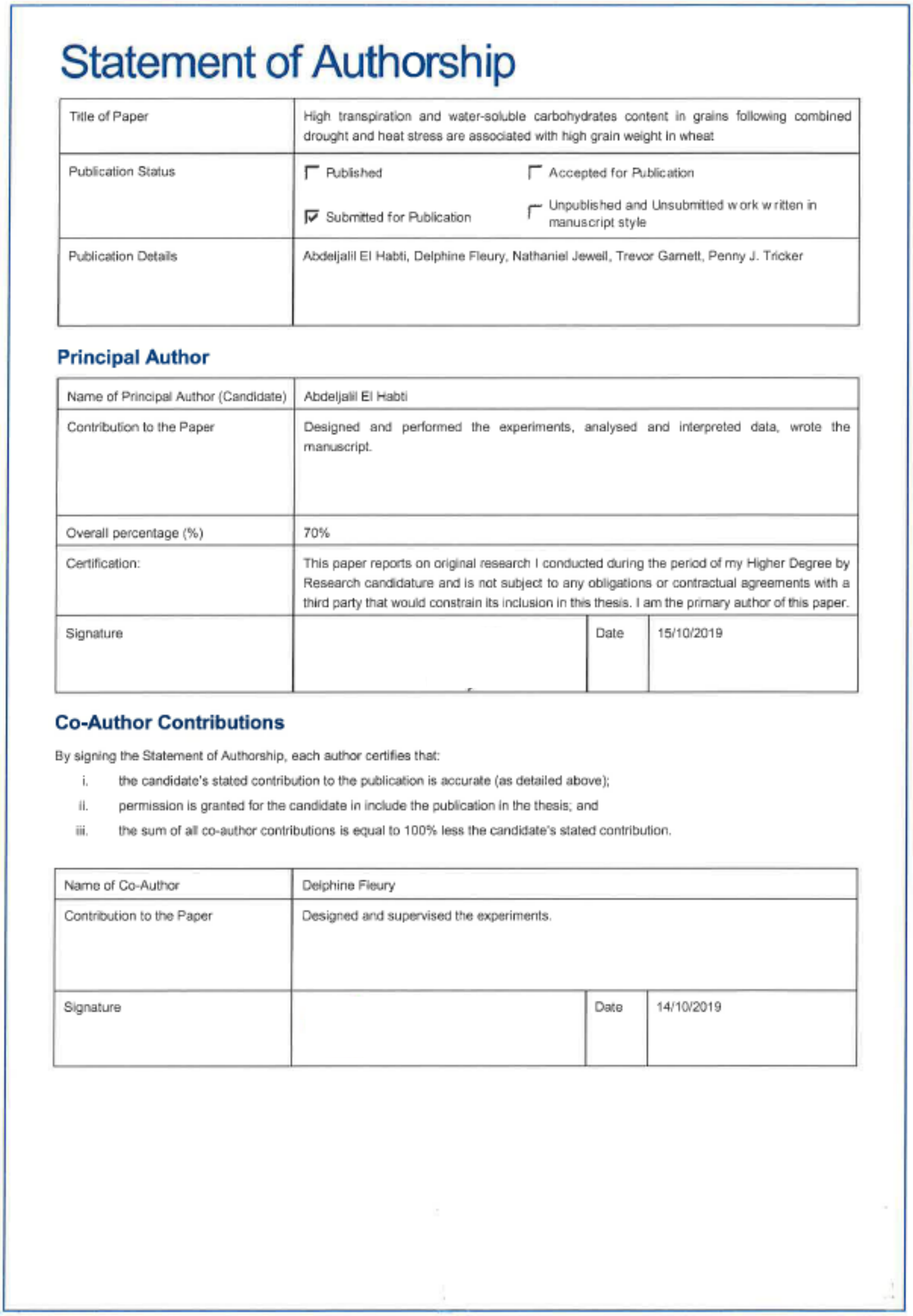
The aim of this work was to uncover novel physiological traits underlying tolerance to combined D&H in wheat. Using wheat genetic diversity and combining physiological analysis with recently available technology, experiments were conducted with the following objectives:

- To identify genotypes contrasting for grain weight under combined D&H (Chapter 2).
- To quantify plant water use response to drought and combined D&H (Chapter 2).
- To investigate carbohydrates partitioning in the main tiller and to determine the contribution of different plant tissues to grain filling (Chapter 2).
- To identify genotypic variation in plant water use and carbohydrate partitioning (Chapter 2).
- To investigate the hydraulic properties of the stem and the spike supporting favourable water status of the spike (Chapter 3 and 4).
- To identify genotypic variation in ROS scavenging system and develop non-destructive tools to measure oxidative stress in wheat plants (Chapter 5).

Chapter 2:

Higher transpiration and water-soluble carbohydrates content in grains following combined drought and heat stress are associated with high grain weight in wheat

Statement of authorship



Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Abdeljalil El Habti		
Contribution to the Paper	Designed and performed the experiments, analysed and interpreted data, wrote the manuscript.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	15/10/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Delphine Fleury		
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Contribution to the Paper	Designed and supervised the experiments, edited the manuscript.		
Signature		Date	17/10/19

Short title: Water use and carbohydrates availability

Higher transpiration and water-soluble carbohydrates content in grains following combined drought and heat stress are associated with high grain weight in wheat

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One sentence summary: Higher total grain weight at harvest is related to transpiration and water-soluble content in grains following drought and heat stress

Author contributions: A.E., D.F., T.G. and P.J.T. designed the experiments; A.E. performed most of the experiments and analysed and interpreted data; N.J. analysed and interpreted data; all authors contributed to drafting the manuscript.

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Abstract

Wheat production is increasingly challenged by simultaneous drought and heatwaves. We assessed the effect of both stresses combined on whole plant water use and carbohydrates partitioning in eight wheat genotypes that showed contrasting tolerance. Plant water use was monitored throughout growth and water-soluble carbohydrates (WSC) and starch were measured following a three-day heat treatment during drought. WSC were predominantly allocated to the spike in modern varieties, whereas the stem contained most WSC in older genotypes. Combined drought and heat stress increased WSC partitioning to the spike in older genotypes but not in the modern varieties. Glucose and fructose concentrations in grains measured 12 days after anthesis were associated with total grain weight at harvest in the main spike. At the whole plant level, combined drought and heat stress differentially altered daily water use and transpiration response to vapour pressure deficit during grain filling, compared to drought only. Final grain yield was increasingly associated with aboveground biomass and total water use with increasing stress intensity. Ability to maintain transpiration, especially following combined drought and heat stress, appears beneficial for maintaining wheat productivity.

Introduction

Recent decades have witnessed severe drought and heatwaves worldwide, including in major wheat producing regions such as India, the U.S.A., Russia, Western Europe and Australia. These climatic conditions have a significant impact on global wheat production, with dramatic social and economic consequences (van Dijk et al., 2013). Current climate projections predict drought and heatwaves will become more common and more intense in the future (IPCC, 2014; Rosenzweig et al., 2014; Asseng et al., 2015). One way to limit the impact of weather variability on productivity is to develop wheat varieties better adapted to the changing climate. This can be assisted by understanding the mechanisms underlying plants' responses to complex stresses so as to identify the traits that characterise stress tolerant varieties for breeding.

A small number of studies document the impact of combined drought and high temperature on wheat productivity and biological processes, especially during the reproductive developmental stage (reviewed in Tricker et al., 2018). The effect of combined stresses is more detrimental than the effect of an individual stress (Mittler, 2006). Both drought and high temperatures reduce expansive growth, accelerate flowering and shorten grain filling duration, resulting in a low grain set, size and weight (Asana and Williams, 1965; Hochman, 1982; Saini and Aspinall, 1982; Pradhan et al., 2012; Weldearegay et al., 2012). In combination, drought and high temperature impair the photosynthetic system, reduce stomatal conductance and gas exchange, and disrupt plants' water relations (Machado and Paulsen, 2001; Shah and Paulsen, 2003; Prasad et al., 2011). These additive alterations of morphological, physiological and cellular processes result in severe reductions in final grain weight.

Although the major impact of combined drought and high temperature on wheat productivity is well described, there is scarce information on the mechanisms that determine the ability to maintain grain weight in these unfavourable environments. Wheat harvested grain mass consists of 85 % carbohydrates, of which ~80 % is starch (Bidinger et al., 1977; Dale and Housley, 1986; Stone and Morell, 2009). During grain filling, water-soluble carbohydrates (WSC) are delivered to grains either from current photosynthesis in photosynthesising organs or from remobilisation of WSC stored during the vegetative stage (Borrell et al., 1989; Kobata et al., 1992; Schnyder, 1993; Ehdaie et al., 2006). Abiotic stress after anthesis can limit gas exchange and damage the photosynthetic system, in which case stored carbohydrates become a major source of carbon for grain filling (Blum, 1998). In addition to the contribution from stem reserves, spike organs, especially awns, are thought to contribute to the grain filling process due to their active photosynthesis, especially in dry environments (Thorne 1966, Evans et al., 1972; Johnson and Moss, 1976; Motzo and Giunta, 2002; Guo and Schnurbusch, 2016; Rebetzke et al., 2016).

Carbohydrate synthesis and transport are closely related to water movements in plants. Open stomata are necessary for carbon capture and plants trade off between maximising carbon assimilation and limiting water loss through transpiration under adverse conditions such as drought. Carbohydrate transport via the phloem and distribution throughout the plant relies on water exchange with adjacent xylem (Hölttä et al., 2009), and the impact of water shortage on xylem water transport also impairs phloem function (Sevanto, 2018). Soluble carbohydrates also play an important role during drought by acting as compatible osmolytes to maintain cell turgor and favourable plant water status, thereby sustaining biological processes and soil water uptake (Blum, 2017). Maintaining plant hydration and enhancing carbohydrate remobilisation to grains are considered key factors for crop productivity in limiting environments (Blum, 2006), and the interplay between plant water relations and carbohydrate metabolism and distribution highlights the importance of studying both mechanisms together.

In this work, we describe the impact of combined drought and heat stress on whole plant water use and carbohydrate partitioning during grain filling in diverse wheat genotypes. We hypothesised that the combination of both stresses would alter plant water use and carbohydrate partitioning in the stem and spike, and that WSC availability would be a limiting factor for optimal grain weight under combined drought and heat stress.

Material and methods

2016 experiment

Plants and growth conditions

Eight bread wheat (*T. aestivum* L.) genotypes were selected from a diverse panel of 534 wheat accessions from 44 countries described in Garcia et al. (2019). The diversity panel was previously subjected to post-anthesis drought and combined drought and heat stress in a pilot experiment and evaluated for plant total grain weight (yield) at harvest (data not shown). The selected genotypes contrasted for grain weight following drought or combined drought and heat stress, and consisted of three Australian older varieties (Currawa, Koda, Mendos), three Australian more modern commercial varieties (Frame, Young, Gladius), one synthetic line from CIMMYT (Synthetic W7984) and one landrace from Ethiopia (Odessa ES19565) (Table 1). The selected genotypes were released between 1912 and 2007 (Supp. Table S1). In this study, Frame, Gladius and Young were considered as modern genotypes; the remaining genotypes were considered as older genotypes.

Single seeds were sown in 40 cm x 15 cm round pots containing 8.2 kg of a mixture of 1:1:1 (v/v/v) clay/loam:UC Davis mix:cocopeat mix. Seeds were sown on 11 August

2016, late winter in the southern hemisphere. From 13 days after sowing (DAS) until the end of the experiment, plants were grown in a glasshouse (34°58'17.8"S, 138°38'23.4"E) on a gravimetric platform (Droughtspotter, Phenospex, Heerlen, The Netherlands) that automatically irrigated to the pre-defined pot weight and recorded weights and water added (details in *Water use and transpiration* below). The 168 pots were randomized to 168 Droughtspotter cells using a factorial, randomized complete block design, such that each block comprised one replicate of each Genotype–Treatment combination, except in three blocks that contained one empty pot each to estimate soil evaporation. The three treatment groups comprised well-watered (WW), drought (D) and combined drought & heat stress (D&H). In particular, all plants were well-watered (soil water potential = - 0.3 MPa, gravimetric soil water content = 20 % (g/g)) and grown in temperate conditions (22 °C/ 15 °C day/ night) until anthesis of the main spike. Anthesis date was the first day anthers were observed on the main spike. One third of the plants (WW) were maintained in well-watered, cool conditions until harvest. The remaining plants (D, D&H) were subject to a 6d drought treatment (soil water potential = - 0.6 MPa, gravimetric soil water content = 12 % (g/g)) starting 3d after anthesis on the main spike of each individual; this was followed, in half of these plants (D&H), by a 3d heat treatment at 37 °C/ 27 °C day/ night (n=7 for each accession in each treatment). Heat treatment was imposed in an adjacent glasshouse where plants were watered to weight manually. Drought was maintained until harvest in the D and D&H groups. LED lights (400 $\mu E/m^2/s$) were installed above plants to minimize variations due to light intensity. A graphical representation of the experimental design is shown in Supp. Fig. S1. Environmental data are shown in Supp. Fig. 2.

Water use and transpiration

The gravimetric platform was configured to weigh each pot at regular time intervals. All weight and water values were automatically logged and water usage estimated hourly for each pot throughout the experiment. During the heat treatment in an adjacent glasshouse, plants were watered to weight manually at similar times as the drought treatment and weights recorded. Pots were watered at least six times daily (6am, 10am, 12pm, 2pm, 4pm and 10pm). Pots containing soil only were weighed to estimate non-transpirational water loss under WW, D and D&H treatments. The water usage is a combination of plant transpiration and evaporation from the soil surface, which was negligible in all treatments as estimated from pots containing soil only.

Carbohydrates quantification

The main stem and spike of three plants per genotype per treatment were sampled 12d after anthesis (DAA), i.e. one day after heat treatment in drought and heat stressed plants, and stored at -80°C for further analysis. Measurements were conducted separately on the stem, flag leaf sheath, covered peduncle, exposed peduncle, rachis, grains, palea, lemma, awns and glumes. Dry weight was obtained by weighing the samples after freeze-drying. Total WSC in each tissue were determined using the anthrone method (Yemm and Willis, 1954) with some modifications: soluble sugars were extracted with 80 % ethanol at 80 °C for 1h, then extracted with distilled water at 60 °C for 1h. The extraction was repeated as many times as needed until no coloration was observed. Supernatants were combined in the same tube for colorimetric assay. Starch content in grains was measured using the Megazyme Total Starch HK (K-TSHK 08/18, Megazyme, Bray, Ireland) according to the manufacturer's instructions.

Individual WSC measurements in grains were performed in four genotypes (Frame, Odessa, Synthetic and Young). Glucose, fructose and sucrose were analyzed in the same samples used for total WSC analysis using high performance anion exchange chromatography with pulsed amperometric detection HPAEC-PAD (Dionex ICS-5000; Thermo Fisher Scientific, Sunnyvale, USA). Separations were performed at 30 °C and the flow rate was 0.5 mL/min. A 25 µL sample was injected on a Guard CarboPac PA20 (3 × 30mm) in series with an analytical CarboPac PA20 (3 × 150mm). The elution program consisted of 0.1M NaOH from 0 to 2 min, followed by increasing 1M sodium acetate concentration up to 20 % from 2 min to 35 min, followed by increasing 1M sodium acetate concentration up to 100 % from 35 min to 36.5 min, a steady concentration from 36.5 min to 37.5 min, followed by a 0.1M NaOH wash until return to equilibrium. Glucose, fructose and sucrose were identified based on glucose, fructose and sucrose standards. Fructans were identified by acid hydrolysis. Two WSC samples from the stem and awns were incubated with 0.2M trifluoroacetic acid (TFA) at 80 °C for 30 min together with untreated samples. Treated and untreated samples were analyzed using HPAEC-PAD as described above. Glucose, fructose, sucrose were quantified using external standards and peak areas determined using the instrument's Chromeleon software. Fructans were quantified using peak areas. As plant morphology and grain number varied greatly between the genotypes, WSC and starch contents were expressed as g/g DW to allow for comparison between genotypes.

Harvest data at maturity

Four plants per genotype per treatment were harvested at maturity to measure grain yield components. Total grain weight was determined for the main spike and for the whole plant. Seed number was counted using an automatic seed counter (Contador, Pfueffer

GmbH, Germany). Biomass weight included tillers, leaves and spikes but excluded grains. Plant height was measured from the base of the main stem to the top of the highest spike excluding awns. Biomass water use efficiency (bWUE) was calculated as the ratio of total aboveground biomass to total water use per plant. Grain water use efficiency (gWUE) was calculated as the ratio of total grain weight to total water use per plant.

Statistical analyses (1): Yield components

The data were analysed by two-way ANOVA with genotype and treatment as fixed factors for all measured yield component and biomass traits and for the analysis of carbohydrates within each tissue. Treatment means within genotypes were compared using Tukey’s HSD (honestly significant difference) test at $p = <0.1$. Statistical analyses (ANOVA, Tukey’s tests, correlation analyses) and graphical representation were performed using R software (version 3.4.4, R Core Team, 2019) and ASReml-R (Butler et al., 2009).

Statistical analyses (2): Water use and transpiration

The recorded water use data were used to identify genotype and treatment effects on hourly transpiration rate (TR, mL/hr) and specific transpiration rate (STR, mL/hr/g biomass), with the proviso that soil evaporation and plant transpiration water losses were indistinguishable.

VPD was computed hourly from Vapour Capacity (VC, kPa) using the following formula:

$$VC = 0.611 \exp(17.62\,T/(T + 243))$$

$$VPD = (1 - RH/100)\,VC$$

where T is temperature and RH is relative humidity. It was further decided to model TR or STR (henceforth denoted y) as a simple linear function of VPD with (a) genotype × treatment interaction incorporated into the VPD slope and intercept parameters, and (b) error variance modelled as a function of treatment (but not genotype). The resulting model comprises 2 x 8 x 3 = 48 fixed effects of interest (i.e. slope and intercept parameters) and 3 variance estimates, represented symbolically as

$$y_{ij} = \mathrm{Pot}_i + \mathrm{spatial}_i + \mathrm{Gen}_i \times \mathrm{Tr}_i + \mathrm{VPD}_j \times \mathrm{Gen}_i \times \mathrm{Tr}_i + \sigma^2_{\mathrm{Tr}}$$

where (a) y_{ij} is the TR or STR value for Pot i on hour j, (b) Pot_i is a random-effects term for variation between pots, and (c) *spatial* comprises fixed-effects terms for spatial variation within the greenhouse. The model was fitted separately to each of TR and STR using the R package ASReml-R4 (Butler et al., 2017). Analysis of the effect of 3d heat stress on transpiration was narrowed to 30d (0 to 30 days after treatment – DAT) after heat stress, while all genotypes were still using water in the well-watered treatment, to limit the effects of intrinsic differences of grain-filling duration on transpiration and distinguish the effects of treatments.

2017 experiment

In order to test the reproducibility of water use data, the 2016 experiment described above was replicated in an independent experiment with four genotypes (Currawa, Synthetic W7984, Mendos and Young) in 2017 with the same settings used for plant growth and treatments, except that plants were sown one month earlier. Plant water use was recorded using the gravimetric platform and three plants per genotype and per treatment were harvested at maturity and measured as before.

Results

Combined drought and heat stress differentially reduced total grain weight at harvest

The effect of drought and combined drought and heat stress (D&H) on total grain weight per plant at harvest (per plant yield) depended on genotype (Table 1). Drought reduced grain weight in Currawa, Odessa, Frame, Young and Gladius. Interaction of drought with high temperature further reduced plant yield in Odessa, Mendos and Young. In contrast, heat stress did not exacerbate the effect of drought in Currawa, Frame and Gladius. Plant yield in Koda was not sensitive to either drought or D&H stress. Overall, the combination of drought and high temperature was more detrimental to plant yield in some genotypes, but not all (Fig. 1a).

In order to assess the impact of drought and combined D&H on grain filling, grain dry weight in the main spike was measured at 12 DAA and at harvest. At 12 days after anthesis (DAA), total grain weight in the main spike was different among genotypes (Fig. 1b) but there was no effect of the treatments or genotype x treatment interaction (Table 1). At harvest, there was an effect of treatment on total grain weight in the main spike which was reduced by drought in Currawa and Gladius, and reduced by combined D&H in Koda, Mendos and Frame (Fig. 1c). There was no effect of drought or combined D&H on main spike total grain weight at harvest in Odessa, Synthetic W7984 and Young.

The relationship between aboveground biomass, water use and plant yield increased with increasing stress intensity

Plant yield was associated with aboveground vegetative biomass and total water use to different degrees depending on treatments (Fig. 2). Total grain weight was increasingly linearly related with aboveground biomass and total water used by the plant throughout the experiment with increasing stress intensity, from $r^2 = 0.2$ and $r^2 = 0.31$ under WW, to $r^2 = 0.35$ and $r^2 = 0.56$ under drought, to $r^2 = 0.46$ and $r^2 = 0.67$ under combined D&H, respectively (Fig. 2a). During the 3d heat treatment, plants generally used similar amounts of water as compared to well-watered conditions (Supp. Fig. S3), although soil water potential was halved.

When comparing modern genotypes (Frame, Young and Gladius) to older genotypes (Currawa, Odessa, Koda, Mendos and Synthetic W7984), the relationship between per plant yield and aboveground biomass was similar in both groups ($r^2=0.48$ and $r^2=0.46$, $p < 0.001$, respectively). Despite this, the slope of the regression for modern varieties was higher compared to older genotypes ($a=0.8$ and $a=0.3$, respectively, Fig. 2). The higher slope for modern genotypes was explained by the lower biomass required to produce similar grain weight compared to older genotypes under well-watered conditions, and reflects the high harvest index of modern genotypes in favourable conditions. However, the dependence of plant yield on total water used was higher in modern genotypes compared to older genotypes ($r^2 = 0.80$ and $r^2 = 0.63$, $p < 0.001$, respectively).

Combined drought and heat stress differentially reduced transpiration response to vapour pressure deficit

As total water use was strongly dependent on plant biomass (Supp. Fig. S4), water use was normalised to the final aboveground biomass and expressed as unit of water per unit of biomass to allow for comparison between plants. Water use differed between genotypes following combined D&H when all treated plants (D and D&H replicates) were in the same droughted conditions (Fig. 3a). Interaction of drought and 3d high temperature reduced subsequent daily water use in Odessa, Koda, Mendos and Young for the subsequent 30d, whereas daily water use following combined D&H was similar to D alone in Currawa, Frame, Synthetic W7984 and Gladius.

Plant water use is the summation of transpiration, which is driven by changes in vapour pressure deficit (VPD). Transpiration response to VPD was differentially altered by three-day high temperature treatment depending on genotype over the same grain-filling period when D and D&H plants were in the same droughted conditions (Fig. 3b). For both TR and STR, statistical significance was confirmed ($p < 0.05$) for the genotype × treatment interaction component of the VPD slope parameter. That is, the VPD effect on transpiration exhibited genotype × treatment interaction, both before and after normalisation to final aboveground biomass.

Heat stress reduced subsequent transpiration rate at VPD > 0.5 kPa in Odessa, at VPD > 0.7 kPa in Young and at VPD > 1.0 kPa in Koda and Mendos (Fig. 3b). In contrast, transpiration response to VPD was not altered following combined D&H stress in Currawa, Frame, Synthetic W7984 and Gladius. Transpiration response to VPD was affected in the same genotypes where daily water use was reduced by combined D&H. During drought, Young had the highest transpiration rate at VPD > 1.5 kPa whereas Currawa and Frame had the lowest transpiration rates. Following combined D&H, Synthetic W7984 had the highest transpiration rate at VPD > 1.5 kPa whereas Currawa and Odessa had the lowest transpiration rates (Supp. Fig. S5b).

Combined drought and heat stress increased WSC partitioning to the spike in old genotypes, but not in modern varieties

WSC were quantified in the main stem and the spike tissues following all treatments 12 DAA, i.e. immediately following the heat stress for the D&H replicates. There was a clear contrast between older genotypes (Currawa, Odessa, Koda, Mendos, Synthetic W7984) and more modern varieties (Frame, Young, Gladius) for WSC partitioning in stem parts compared with the spike (Fig. 4). In well-watered conditions, stem parts contained 67-87 % of total WSC in older wheat genotypes compared to 28-50 % in modern varieties. The spike tissues (excluding grains) contained 49-71 % of total WSC in modern varieties, whereas WSC in the spike were 12-33% of total WSC in older genotypes.

Drought and combined D&H differentially affected WSC distribution in the main stem and spike depending on genotype (Fig. 4). Drought significantly increased WSC partitioning into the spike in Currawa and Odessa and reduced the WSC fraction in the stem in Frame (Supp. Table 2). Combined D&H significantly increased WSC partitioning into the spike in older genotypes, whereas there was no change in WSC partitioning in modern varieties in both treatments. Changes in WSC allocation to the spike following combined drought and heat stress did not affect WSC partitioning to grains, except in the Synthetic genotype where WSC partitioning to grains was significantly increased by combined D&H compared to drought only.

The relationships between WSC concentrations and plant yield depended on plant organ, individual carbohydrate and the date of release of the variety

The relationship between WSC concentration at 12 DAA and plant yield depended on tissue and date of genotype release. Total WSC concentration in the stem at 12 DAA was positively related with plant yield in modern genotypes ($r^2 = 0.53$) whereas there was no significant regression observed in older genotypes ($r^2 = 0.1$) (Table 2, Fig. 5a). In contrast, total WSC concentration in awns at 12 DAA was positively related with plant yield in the two awned older genotypes ($r^2 = 0.85$) whereas there was no relationship in modern genotypes ($r^2 = 0.06$, Fig. 5b). Similarly, in grains, total WSC concentration at 12 DAA was positively related with plant yield in older genotypes (r^2 =0.41) whereas there was no relationship in modern genotypes in modern genotypes (r^2 =0.13) (Table 2, Fig. 5c). To determine whether individual WSC varied similarly to total WSC, we quantified glucose, fructose and sucrose concentrations in the stem, awns and grains. In the stem, a similar contrast was observed for individual WSC between older and modern varieties as was observed for total WSC (Table 2, Fig. 5a). Glucose and sucrose concentrations in the stem at 12 DAA were positively related with plant yield in modern varieties (r^2 =0.34 and 0.56, respectively). Stem fructose concentrations at 12 DAA were negatively related to plant yield in older varieties (r^2 =0.31). In awns, glucose, fructose and sucrose concentrations at 12 DAA were each negatively related with plant yield in the two awned older genotypes (r^2 =0.84, 0.81 and 0.67 respectively) but there were no significant relationships between these sugars and plant yield in more modern types (Table 2, Fig. 5b). In grains, unlike other tissues, sucrose concentrations at 12 DAA were low compared to glucose and fructose concentrations (Fig. 5c). Glucose and fructose concentrations were positively related with total grain weight at harvest (r^2=0.43 and 0.40, respectively – Fig. 5c). Two (unknown) fructans also appeared important for plant yield in modern varieties: fructan 1 in awns (r^2 =0.47) and fructan 2 in the stem (r^2 =0.73). In contrast with other sugars in the awns, fructan 1 concentrations at 12 DAA and yield per plant were positively related.

Drought and combined drought and heat stress altered WSC and starch balance in grains

In order to quantify WSC availability for starch synthesis, WSC and starch concentrations were measured in grains 12 DAA, immediately following D&H treatments. There was a significant interaction between genotype and treatment for WSC and starch concentrations in grains (Table 1). Drought significantly reduced WSC concentration in grains in the Synthetic type and heat stress did not exacerbate the effect of drought (Fig. 6). WSC concentration in grains was reduced by combined D&H in Odessa and Koda compared to WW and was specifically reduced by combined D&H in Currawa, Young and Gladius. Starch concentrations offset the reduction in WSC concentration in Currawa, Odessa and Synthetic W7984, resulting in a similar total non-structural carbohydrates (NSC) concentration in grains in all conditions. The balance between WSC and starch concentrations was altered in Koda, Young and Gladius. Total NSC concentration was reduced under drought in Koda and Young, and combined D&H reduced total NSC in Gladius. There was no significant effect of drought and combined D&H on WSC and total NSC concentration in grains in Currawa, Odessa, Mendos and Frame. Overall, there was a significant interaction between genotypes and treatments for total carbohydrates concentration in grains that was mainly driven by interaction between genotypes and treatments for WSC concentration. There was no relationship between starch concentrations at 12 DAA and plant yield (Fig. 5d).

Discussion

High water use and responsiveness to evaporative demand are indicators of higher grain weight under combined drought and heat stress

In this study, the impact of drought and combined D&H on total grain weight at harvest was assessed in eight, diverse wheat genotypes released between 1912 and 2007. The detrimental effect of heat stress combined with drought depended on genotype, illustrating genetic variation in grain weight response to combined D&H in wheat in the selected genotypes.

Per plant yield was increasingly dependent on both aboveground biomass and total water use with increasing stress intensity (drought, then additional heat stress) (Fig. 2), highlighting the important relationships between biomass, water use and grain weight under stress previously reported (Reynolds et al., 2006; Blum, 2009; Blum, 2011; Reynolds and Langridge, 2016). Biomass and water use are linearly related (de Wit, 1958) and mutually dependent during the plant's lifecycle: during the vegetative stage, transpiration drives biomass accumulation, which in turn results in high water use during grain filling when water is available. The maintenance of water use ensures the favourable water status of plant tissues and assimilates transport to grains.

We observed a high association between total grain weight at harvest and total water used in two independent experiments (Fig. 2b, Supp. Fig. S6), regardless of transpiration sensitivity to heat stress: the higher the water use, the higher the total grain weight at harvest. This indicated that high transpiration following 3d heat stress was a desirable trait in all the tested conditions, confirming the strong relationship between plant transpiration and yield (de Wit, 1958; Fischer and Turner, 1978; Sinclair et al., 2005).

Water used in transpiration is driven by the evaporative demand in the atmosphere due to stomatal aperture sensitivity to VPD (Turner et al., 1985; Grantz, 1990; Monteith, 1995). Our work illustrated the genetic variation in transpiration response to VPD previously observed in diverse wheat genotypes grown under well-watered and water-limited conditions (Schoppach and Sadok, 2012; Schoppach et al., 2016; Medina et al., 2019). In addition, we identified genetic variation in transpiration response to combined D&H (Fig. 3b). A three-day heat treatment altered transpiration response to VPD in the subsequent drought-only treatment in some genotypes.

As transpiration depends on green leaf area, the dynamics of senescence in response to heat stress could potentially explain genotypic differences in transpiration response to heat stress; water use would be quickly reduced in genotypes with faster heat-induced senescence compared to genotypes with lower senescence rate following heat stress. However, no significant differences or genetic variation for drought and heat stress-induced chlorophyll content (greenness) in comparison with drought were found in these genotypes in repeated experiments (Schmidt et al., 2020). Differences in water use between plants in the drought only treatment and during the same drought following heat treatment could also be the result of hydraulic plant properties. Yang et al. (2012) proposed that sensitivity of water viscosity and activity of aquaporins in high temperatures might explain changes in transpiration response to VPD under heat stress. The combination of high evaporative demand and water scarcity might also lead to the disruption of the water column in the xylem, which can cause cavitation. Cavitation damage might explain the lack of recovery in water use following heat stress observed in some genotypes in our experiments.

Here, with both drought and combined D&H stress, grainWUE was more important than WUE *per se*. In our experiments, we measured the effects of stress during grain filling when vegetative biomass was already accumulated, rather than during vegetative biomass production. In the field, where increased rooting depth to access available water and early vigour to enhance canopy coverage and reduce soil evapotranspiration are important, this might not be the case. Nonetheless, the transpiration driven water use changes observed here following heat stress, when plants were all subjected to drought, influenced final grain weight and separated more from less tolerant types.

Although studied in a limited number of genotypes, we observed clear increased grain weight per unit of biomass and per unit of water in more modern compared with older genotypes. In addition to improved plant architecture for assimilation and partitioning (harvest index), we also observed striking differences in the partitioning of assimilates between vegetative and spike tissues before the imposition of stress.

The spike is the main storage tissue for WSC in the more modern wheat varieties

Excess assimilates that are not used for growth and defence may be stored for further use during reproductive stages. The stem is considered an important source of stored WSC for grain filling, and the ability to store and remobilise stem reserves is regarded as a beneficial trait for wheat productivity under stress (Bidinger et al., 1977; Blum et al., 1994; Palta et al., 1994; Rebetzke et al., 2008). At 12 DAA, WSC content in the stem is at its peak (Zhang et al., 2015; Zhang et al., 2016; Shirdelmoghanloo et al., 2016). Our results showed that the stem was the main storage organ for WSC in tall genotypes in which the stem was the largest organ by weight, but not in more modern varieties where stems are much shorter as a consequence of the introduction of semi-dwarfing *Rht* genes (Borrell et al., 1993). More recent varieties partitioned more of the biomass to spikes, and the reproductive organ was also the major store of WSC in modern varieties (Fig. 4). Interestingly, in our experiment, a positive relationship between WSC concentration in the stem and total grain weight in the main spike at harvest was only observed in modern genotypes, which suggests that the important contribution of WSC content stored in the stem to grain filling may be a consequence of the introduction of semi-dwarfing genes (Richards, 1992; Miralles et al., 1998). Alternatively, it might suggest that plant breeders have selected for varieties that partition more of their WSC to spike tissues in the hot and dry conditions of South Eastern Australia, the origin of the more modern varieties. Older genotypes had large reserves of WSC in the stem for a limited sink in the spike (lower grain number), which could explain the absence of a relationship between both traits as stored WSC in the stem may not have been used (Borrell et al., 1993). In contrast with older types, more modern varieties had relatively low WSC concentration in the stem, indicating an opportunity to increase stem capacity for WSC storage in modern varieties.

WSC availability in grains rather than grain capacity limited grain weight under stress

Starch is the main component of final grain mass. It is synthesised from stored WSC or produced from current photosynthesis. Drought and heat stress alter WSC supply to grains, either by limiting carbon assimilation through photosynthesis or by interrupting assimilate remobilisation, thus WSC availability in grains might be a limiting factor for starch synthesis and grain filling (Jurgens et al., 1978; De Souza et al., 1997). In a field study on wheat genotypes grown in well-watered conditions, Fahy et al. (2018) quantified WSC and starch content, and key starch biosynthesis enzyme activity in grains at different grain developmental stages. They did not find any correlation between carbohydrate content in grains and final grain yield, suggesting that assimilate availability for starch synthesis is not a limiting factor for grain filling in wheat in favourable growing conditions. These findings are in accordance with our results in WW conditions where there was no relationship between WSC and starch content and plant yield. However, WSC concentration was reduced with increased stress intensity, and grains with relatively higher WSC concentrations at 12 DAA had higher yield (Fig. 5c). Glucose and fructose are the first substrates in the starch biosynthesis pathway (Emes 2003). Genotypes with higher glucose and fructose concentrations in grains at 12 DAA had higher yield, implying that shortage in glucose and fructose might limit starch biosynthesis later during grain filling and consequently final grain weight. Accelerated starch biosynthesis under stress depleted glucose and fructose in grains without any increase in sucrose content, indicating that insufficient sucrose supply to grains probably limited starch biosynthesis under stress.

Many studies propose sink strength (grain capacity) is the limiting factor for starch accumulation and grain filling in favourable environments (Jenner et al., 1991; Savin and Nicolas, 1996; Borrás et al., 2004; Borrill et al., 2015; Fahy et al., 2018). In our study, genotypes with higher grain capacity, represented by total grain dry weight at 12 DAA, had a higher yield in the well-watered treatment (Fig. 1a-b) suggesting that grain sink strength was a major determinant of grain weight at harvest when conditions were favourable. With D&H stress, however, genotypes with higher grain capacity did not have higher yield, indicating that high grain capacity was not sufficient to determine grain weight at harvest under stress, as has also been observed in barley (Savin and Nicolas, 1996). In our experiments, drought and combined D&H did not immediately reduce grain weight at 12 DAA. Reduction in grain filling occurred after 12 DAA, which corresponds to the end of cell enlargement and beginning of carbohydrate accumulation (Emes et al., 2003). Reduced grain weight under stress was due to altered grain filling, probably as a consequence of limited WSC supply to grains.

Conclusion

Drought and heat stress have rarely been studied together, despite their co-occurrence being a common scenario in wheat-growing regions. This work illustrated the effect of morphological changes introduced in wheat over a century on plant water use and carbohydrates partitioning. Results showed that heat stress occurring during grain filling, while plants were suffering from water stress, changed subsequent water use immediately so that some genotypes were unable to recover. Sensitivity to increased stress intensity was associated with low transpiration response to high VPD following heat stress and genetic variation in transpiration. Reduced availability of WSC in grains following combined D&H was also identified and important for final grain weight. This suggested that measurements of transpiration and WSC content in grains following heat stress might be used to identify genetic variation for tolerance of combined drought and heat stress.

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Zhang J, Chen W, Dell B, Vergauwen R, Zhang X, Mayer JE, Van den Ende W (2015) Wheat genotypic variation in dynamic fluxes of WSC components in different stem segments under drought during grain filling. Frontiers in Plant Science **6:** 624

Zhang J, Dell B, Ma W, Vergauwen R, Zhang X, Oteri T, Foreman A, Laird D, Van den Ende W (2016) Contributions of Root WSC during Grain Filling in Wheat under Drought. Frontiers in Plant Science **7:** 904

Table 1. Analysis of variance (ANOVA) showing the statistical significance of the traits measured for genotype, treatment and interaction between genotype and treatment.

Traits	Genotype	Treatment	Interaction
Total grain weight per plant	***	***	**
Total grain weight in the main spike at 12 DAA	***	ns	ns
Total grain weight in the main spike at harvest	***	***	ns
Aboveground biomass (excluding grains)	***	***	ns
Plant height	***	ns	ns
Total water use	***	***	ns
Water use after treatment	**	**	ns
biomass WUE	***	***	*
grain WUE	***	***	***
Harvest index	***	***	***
WSC concentration in grains	***	***	**
Starch concentration in grains	***	***	***
Total carbohydrates concentration in grains	***	**	*

* $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; ns not significant

Table 2. Correlation between carbohydrates concentrations at 12 DAA and final grain weight in different plant organs under all treatments r2 and p-values of linear regressions between carbohydrate concentrations on final grain weight in modern and older genotypes (Fig. 6). In bold: p < 0.1.

		Modern		Old	
Carbohydrate	Plant tissue	r^2	p	r^2	p
WSC	Stem	0.53	**0.03**	0.1	0.26
	Awns	0.06	0.54	0.85	**0.01**
	Grains	0.13	0.33	0.41	**0.01**
Glucose	Stem	0.34	**0.1**	0.01	0.73
	Awns	0.14	0.33	0.84	**0.1**
	Grains	0.47	0.13	0.00	0.9
Fructose	Stem	0.08	0.45	0.31	**0.03**
	Awns	0.11	0.39	0.81	**0.01**
	Grains	0.54	**0.09**	0.06	0.63
Sucrose	Stem	0.56	**0.02**	0.13	0.18
	Awns	0.07	0.49	0.67	**0.05**
	Grains	0.44	0.15	0.05	0.66
Fructan1	Stem	0.17	0.27	0.1	0.25
	Awns	0.47	**0.04**	0.35	0.22
	Grains	0.04	0.7	0.08	0.6
Fructan2	Stem	0.73	**0.003**	0.23	**0.07**
	Awns	0.05	0.56	0.44	0.15
	Grains	0.47	0.13	0.24	0.32
Starch	Grains	0.08	0.46	0.14	0.18

Figure legends

Figure 1. Combined drought and heat stress differentially reduced final grain weight. (a) Mean total grain weight per plant at harvest (n=4). (b) Mean total grain weight in the main spike at 12 DAA (n=3). (c) Mean total grain weight in the main spike at harvest (n=4). Error bars are standard error. Letters indicate the results of Tukey's test comparing treatment effect within each genotype ($p < 0.1$). Plants were grown under well-watered conditions (black), drought (dark grey) or combined drought and heat (light grey).

Figure 2. Aboveground biomass and water use explain more of the variation in grain weight under increasing stress intensity than in well-watered conditions. Relationships between aboveground biomass excluding grains (a), total water use (b) and final grain weight per plant. Each point represents one plant, grown under well-watered conditions (blue), drought (orange) or combined drought and heat (red). Confidence ellipses circle modern genotypes (in green) and older genotypes (in grey). (c) r^2 and p-value of linear regressions ('*' $p < 0.1$, '**' $p < 0.01$, '***' $p < 0.001$) are indicated for each treatment (well-watered, drought, drought and heat) and genotype group (modern, older).

Figure 3. Interaction of high temperature and drought differentially reduces daily water use and transpiration response to vapour pressure deficit. (a) Daily water use per plant estimated as total irrigation per day, normalised to aboveground biomass. Plants grown in well-watered conditions (black), drought (dark grey) or in drought following three-day heat stress (light grey). 0 DAT is the first day post heat treatment (12 days after

anthesis). Trend lines are loess regressions. Values are means of four replicates (n=4). The confidence interval (0.95) is displayed around smoothed regressions in grey. (b) Hourly transpiration rate response to VPD normalised to aboveground biomass. Plants grown in drought (orange) or in drought following three-day heat stress (red). Graphs include data from 0 DAT to 30 DAT. Trend lines are smooth regression lines.

Figure 4. Combined drought and heat stress increased WSC partitioning to the spike in old genotypes, not in modern varieties. Genotypes are shown in order of date of release (top – bottom, oldest = Currawa to newest = Gladius). Total water-soluble carbohydrates (WSC) as in different organs of plants as a percentage of total WSC (n=3). Plant organs are colour-coded as shown in the legend.

Figure 5. Relationship between total WSC, glucose, fructose, sucrose and starch concentrations in the stem (a), awns (b), grains (c-d) at 12 DAA, and final grain weight. Each point represents an average of carbohydrate concentration (n=3) in the main tiller for one genotype and one treatment. Ellipses represent old genotypes (grey) and modern genotypes (green). Plants grown under well-watered conditions (blue), drought (orange) or combined drought and heat (red). Regression lines include all data. r^2 and p-value of linear regressions (‘ns’ not significant, ‘*’ $p < 0.05$) are indicated.

Figure 6. Drought and combined drought and heat stress alter WSC and starch balance in grains at 12 DAA. Water-soluble carbohydrates (solid) and starch (transparent) concentration in grains at 12 DAA from plants grown under well-watered conditions (black), drought (dark grey) or combined drought and heat stress (light grey).

The sum of WSC and starch concentrations constitutes the non-structural carbohydrate (NSC). Values are means of three replicates (+/- SE). Letters (top: NSC, bottom: WSC) indicate the results of Tukey's test comparing treatment effects within each genotype ($p < 0.1$).

Supplementary Figure S1. (a) Schematic of the treatment design. Plants were well watered (soil water potential (SWP) = -0.3 MPa) until anthesis. Drought (SWP = -0.6 MPa) was applied 3 days after anthesis (DAA) and maintained until harvest. Three-day heat treatment was applied 9 DAA. Three replicates per genotype and per treatment were sampled 12 DAA, and four replicates were harvested. (b) Images of plants of different genotypes following combined D&H stress at 12 DAA.

Supplementary Figure S2. Daily maximum and minimum temperature (a), maximum daily light intensity (b), and daily maximum and minimum VPD (c) in the glasshouse experiment in 2016 (in grey) and 2017 (in black). Maximum values are shown in filled points; minimum values are shown in empty points.

Supplementary Figure S3. Water use per plant during three-day heat treatment, normalised to aboveground biomass. Plants grown in well-watered conditions (black), drought (dark grey) or combined drought and heat stress (light grey). Values are means and standard error of four replicates (n=4). Data were subjected to two-way ANOVA. Letters indicate the results of Tukey's test comparing treatment effect within each genotype ($p < 0.1$).

Supplementary Figure S4. Relationship between aboveground biomass (excluding grains) and total water used. Each point represents one plant. r^2 and p-value of the linear regression (‘***’ $p < 0.001$) are indicated.

Supplementary Figure S5. Significance of genotypic differences in final grain weight (a), transpiration rate at VPD = 2 kPa (b), plant height (c), harvest index (d), WUE for grain weight (e) and WSC concentration in the stem (f). Plants grown in well-watered conditions (WW), drought (D) or combined drought and heat stress (D&H). Values are means and standard error of four replicates (n=4). Values represent average per plant. Data were subjected to two-way ANOVA. Letters indicate the results of Tukey’s test comparing genotype effect within each treatment ($p < 0.1$).

Supplementary Figure S6. Relationship between total water used and final grain weight in 2017 experiment (n=3). Each point represents one plant. r^2 and p-value of the linear regression (‘***’ $p < 0.001$) are indicated. Plants grown under well-watered conditions (blue), drought (orange) or combined drought and heat (red).

Table 1. Analysis of variance (ANOVA) showing the statistical significance of the traits measured for genotype, treatment and interaction between genotype and treatment.

Table 2. Correlation between carbohydrates’ concentrations at 12 DAA and final grain weight in different plant organs under all treatments r^2 and p-values of linear regressions between carbohydrate concentrations on final grain weight in modern and older genotypes (Fig. 6).

Supplementary Table S1. Origins and pedigrees of the eight wheat genotypes used in the study.

Supplementary Table S2. Statistical significance of differences in percentages of water-soluble carbohydrates in different parts of wheat plants: stem, grains and spike, between WW and D, D and D&H, or WW and D&H. In bold: $p < 0.1$.

Figure 1

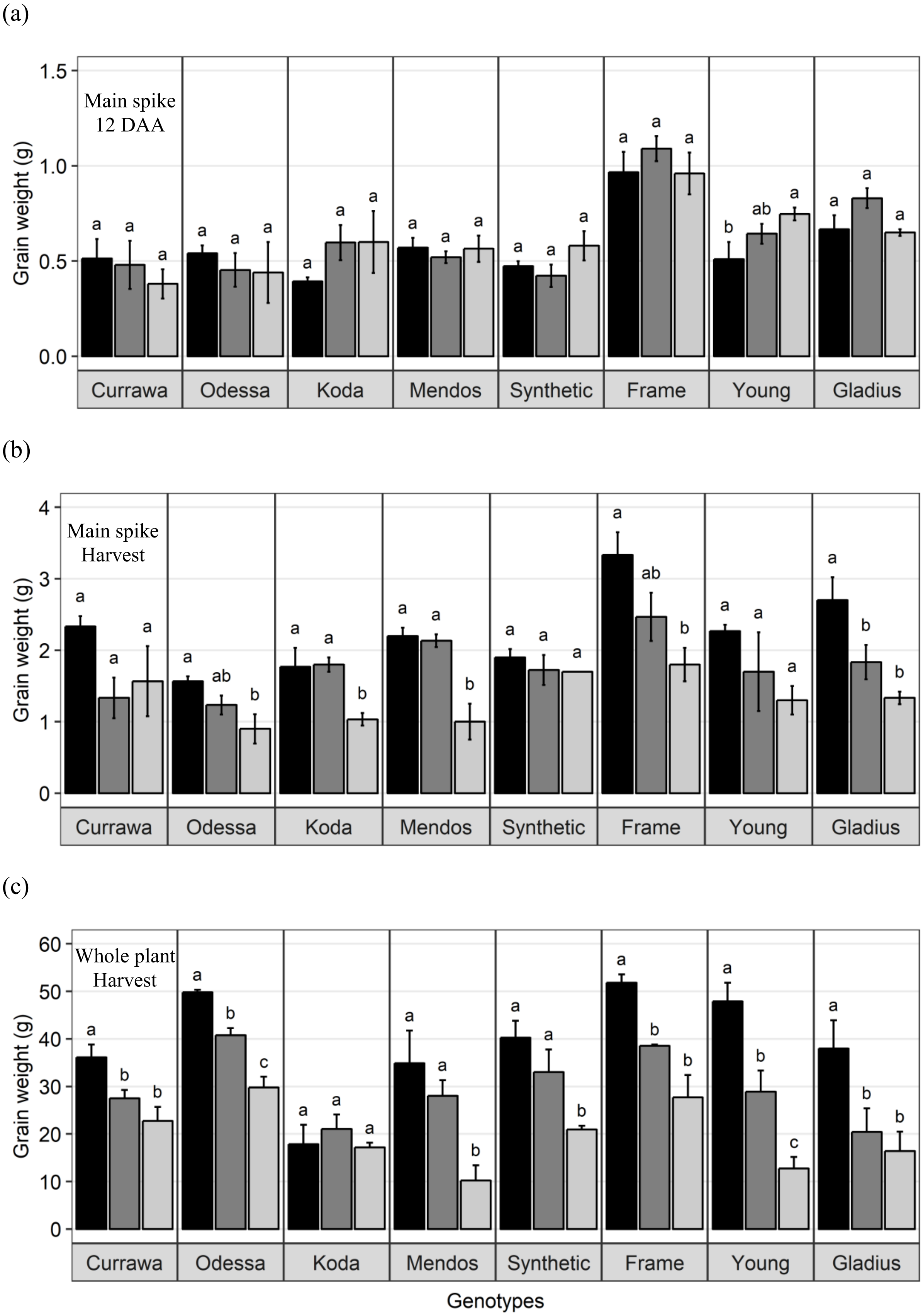
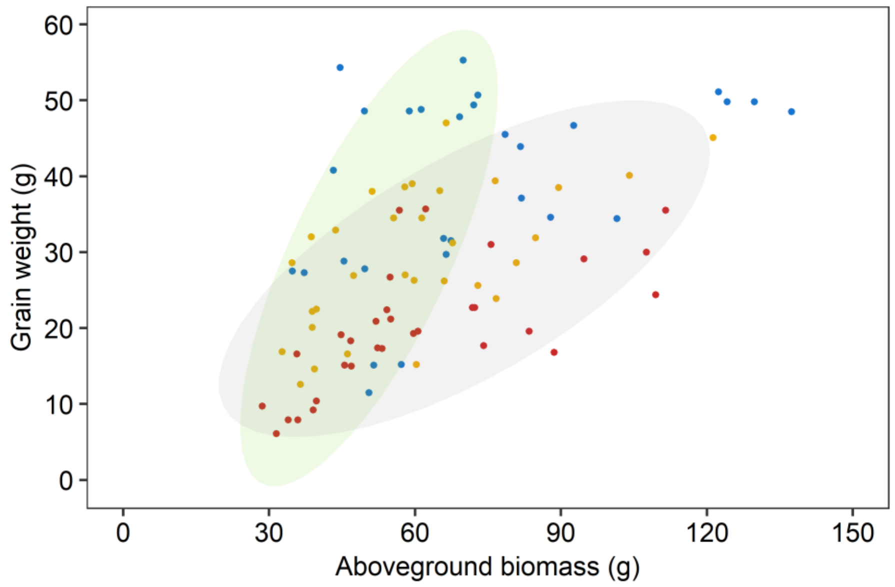
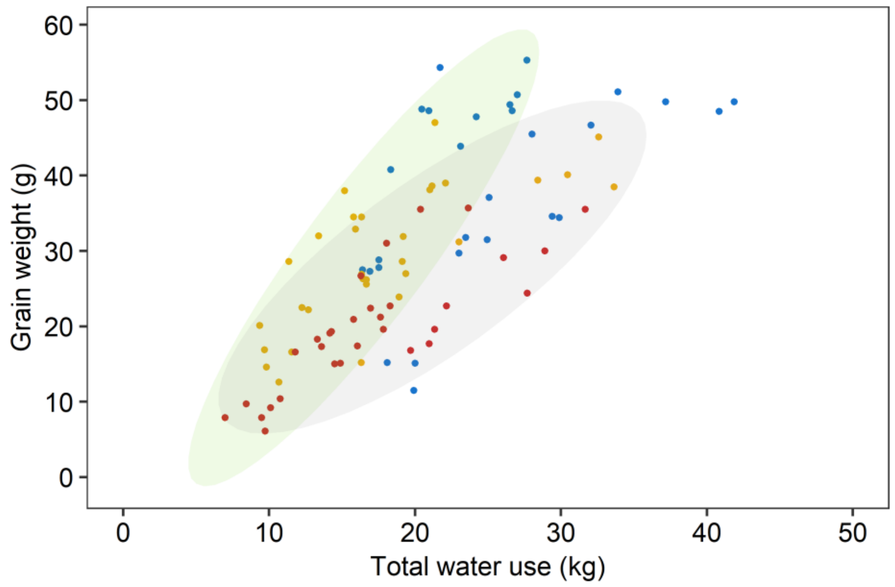

(a)
Main spike
12 DAA
Grain weight (g)
1.5
1.0
0.5
0.0
Currawa a a a
Odessa a a a
Koda a a a
Mendos a a a
Synthetic a a a
Frame a a a
Young b ab a
Gladius a a a
(b)
Main spike
Harvest
Grain weight (g)
4
3
2
1
0
Currawa a a a
Odessa a ab b
Koda a a b
Mendos a a b
Synthetic a a a
Frame a ab b
Young a a a
Gladius a b b
(c)
Whole plant
Harvest
Grain weight (g)
60
50
40
30
20
10
0
Currawa a b b
Odessa a b c
Koda a a a
Mendos a a b
Synthetic a a b
Frame a b b
Young a b c
Gladius a b b
Genotypes

Figure 2

(a)



(b)



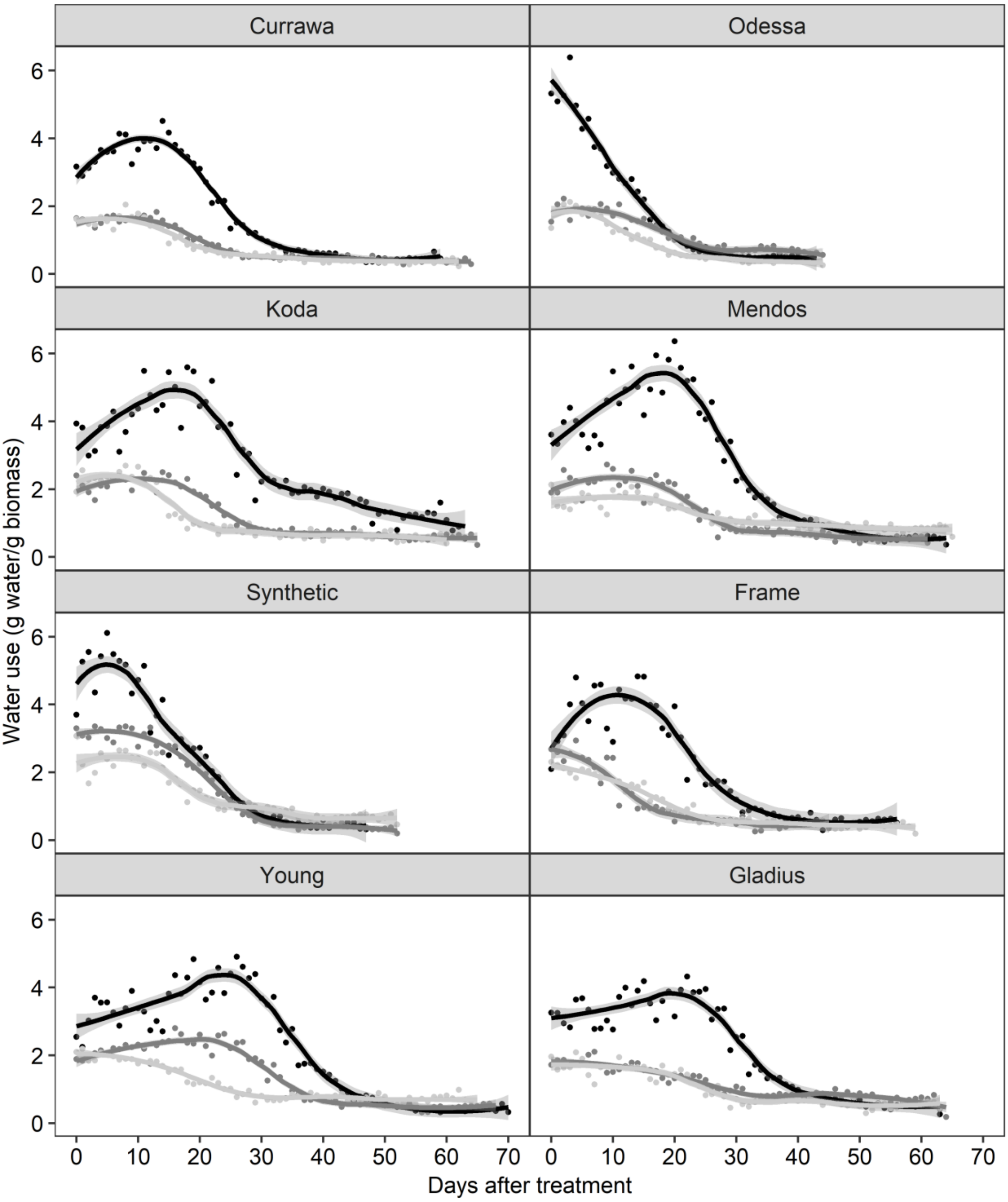
(c)

	Aboveground biomass		Total water use	
	r^2	p	r^2	p
Well-watered	0.2	*	0.31	**
Drought	0.35	*	0.56	***
Drought and heat	0.46	*	0.67	***
Modern genotypes	0.48	***	0.8	***
Older genotypes	0.46	***	0.63	***

* $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; ns not significant

Figure 3

(a)


Currawa
Odessa
Koda
Mendos
Synthetic
Frame
Young
Gladius
0
2
4
6
Water use (g water/g biomass)
0
10
20
30
40
50
60
70
Days after treatment

(b)

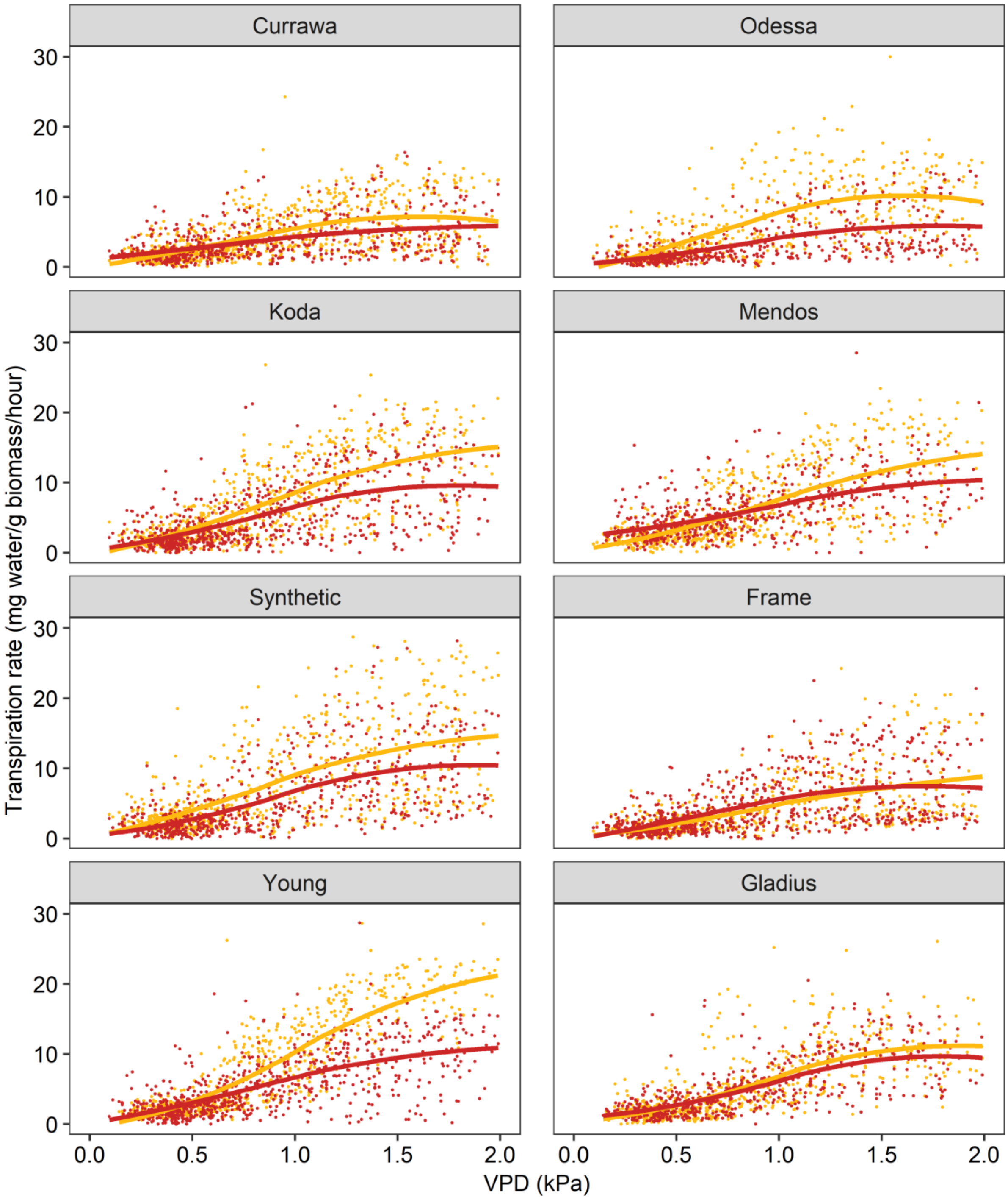
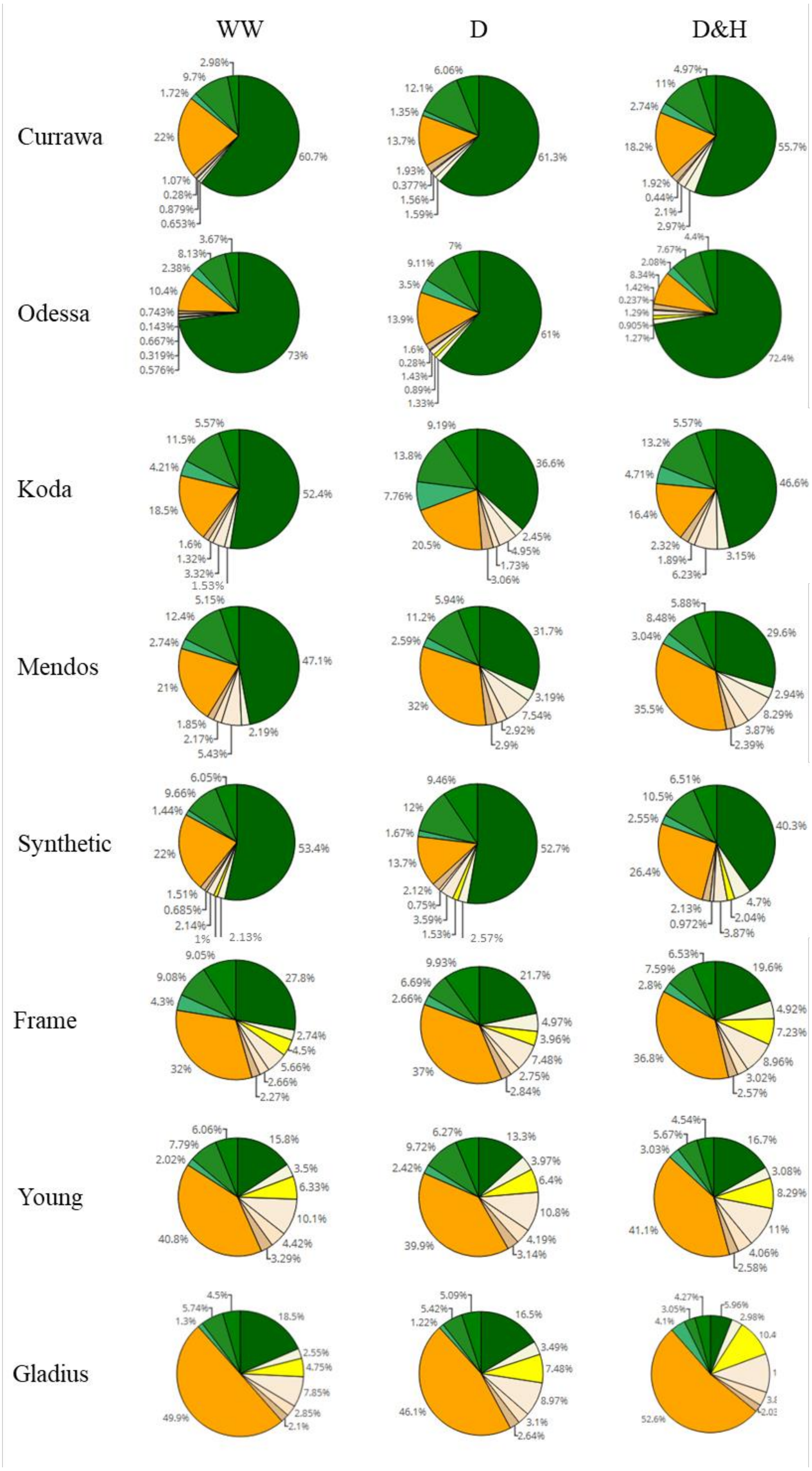

Currawa
Odessa
Koda
Mendos
Synthetic
Frame
Young
Gladius
Transpiration rate (mg water/g biomass/hour)
30
20
10
0
0.0
0.5
1.0
1.5
2.0
VPD (kPa)

Figure 4


WW
D
D&H
Currawa
Odessa
Koda
Mendos
Synthetic
Frame
Young
Gladius
2.98%
9.7%
1.72%
22%
60.7%
1.07%
0.28%
0.879%
0.653%
6.06%
12.1%
1.35%
13.7%
61.3%
1.93%
0.377%
1.56%
1.59%
4.97%
11%
2.74%
18.2%
55.7%
1.92%
0.44%
2.1%
2.97%
3.67%
8.13%
2.38%
10.4%
0.743%
0.143%
0.667%
0.319%
0.576%
73%
7%
9.11%
3.5%
13.9%
61%
1.6%
0.28%
1.43%
0.89%
1.33%
4.4%
7.67%
2.08%
8.34%
1.42%
0.237%
1.29%
0.905%
1.27%
72.4%
5.57%
11.5%
4.21%
18.5%
52.4%
1.6%
1.32%
3.32%
1.53%
9.19%
13.8%
36.6%
7.76%
20.5%
2.45%
4.95%
1.73%
3.06%
5.57%
13.2%
4.71%
46.6%
16.4%
2.32%
1.89%
6.23%
3.15%
5.15%
12.4%
2.74%
47.1%
21%
1.85%
2.17%
5.43%
2.19%
5.94%
11.2%
2.59%
31.7%
32%
3.19%
7.54%
2.92%
2.9%
5.88%
8.48%
3.04%
29.6%
35.5%
2.94%
8.29%
3.87%
2.39%
6.05%
9.66%
1.44%
22%
53.4%
1.51%
0.685%
2.14%
1%
2.13%
9.46%
12%
1.67%
13.7%
52.7%
2.12%
0.75%
3.59%
1.53%
2.57%
6.51%
10.5%
2.55%
40.3%
26.4%
2.13%
0.972%
4.7%
2.04%
3.87%
9.05%
9.08%
4.3%
27.8%
32%
2.74%
4.5%
5.66%
2.66%
2.27%
9.93%
6.69%
2.66%
21.7%
37%
4.97%
3.96%
7.48%
2.75%
2.84%
6.53%
7.59%
2.8%
19.6%
36.8%
4.92%
7.23%
8.96%
3.02%
2.57%
6.06%
7.79%
2.02%
15.8%
3.5%
6.33%
40.8%
10.1%
4.42%
3.29%
6.27%
9.72%
2.42%
13.3%
3.97%
6.4%
39.9%
10.8%
4.19%
3.14%
4.54%
5.67%
3.03%
16.7%
3.08%
8.29%
41.1%
11%
4.06%
2.58%
4.5%
5.74%
1.3%
18.5%
2.55%
4.75%
7.85%
49.9%
2.85%
2.1%
5.09%
5.42%
1.22%
16.5%
3.49%
7.48%
8.97%
46.1%
3.1%
2.64%
4.27%
3.05%
4.1%
5.96%
2.98%
10.4
1
52.6%
3.5
2.03

🟩 Stem 🟩 Leaf sheath 🟩 Peduncle (covered) 🟩 Peduncle (exposed)
🟧 Grains 🟫 Rachis ⬜ Palea ⬜ Lemma 🟨 Awns ⬜ Glumes

Figure 5

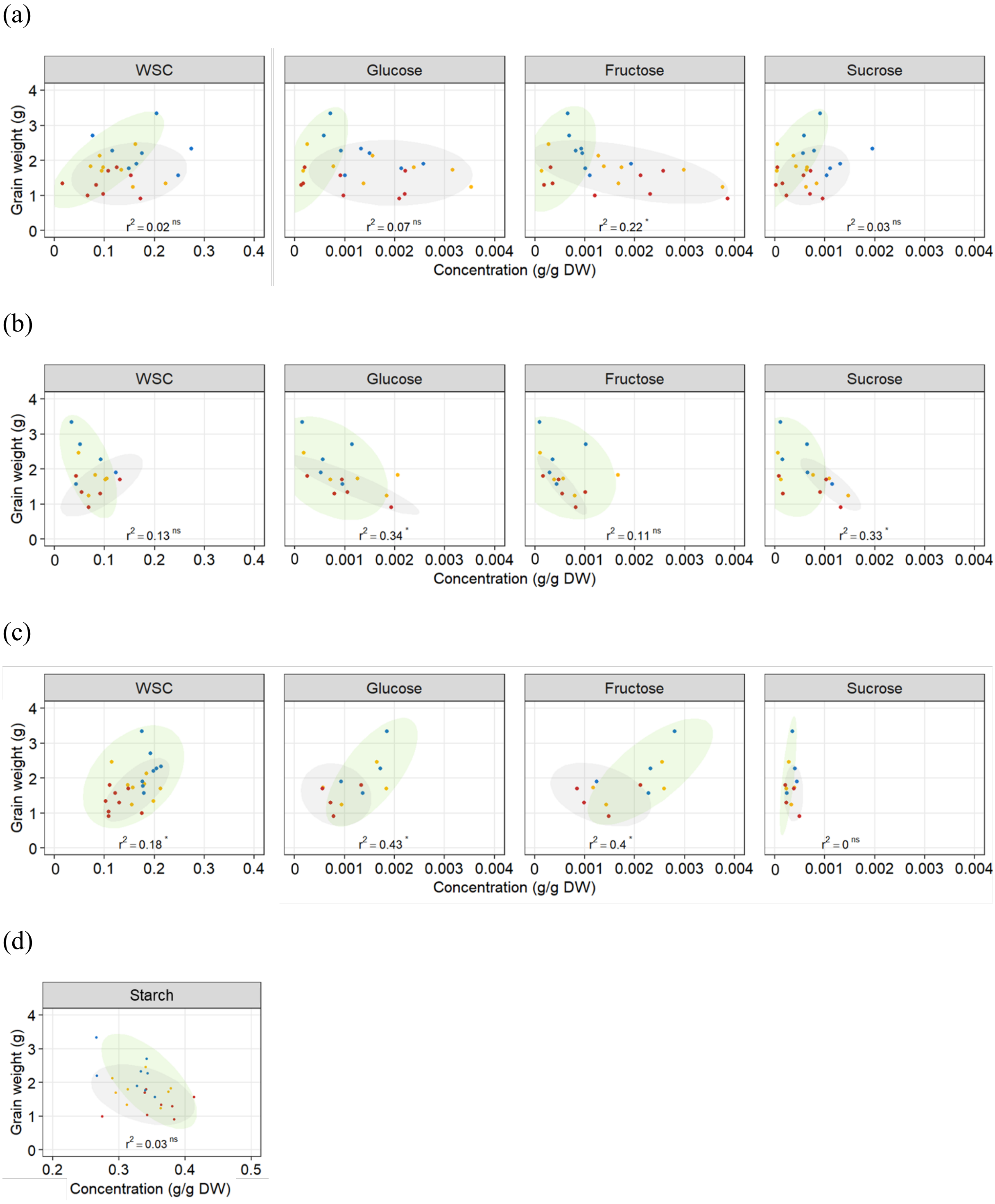
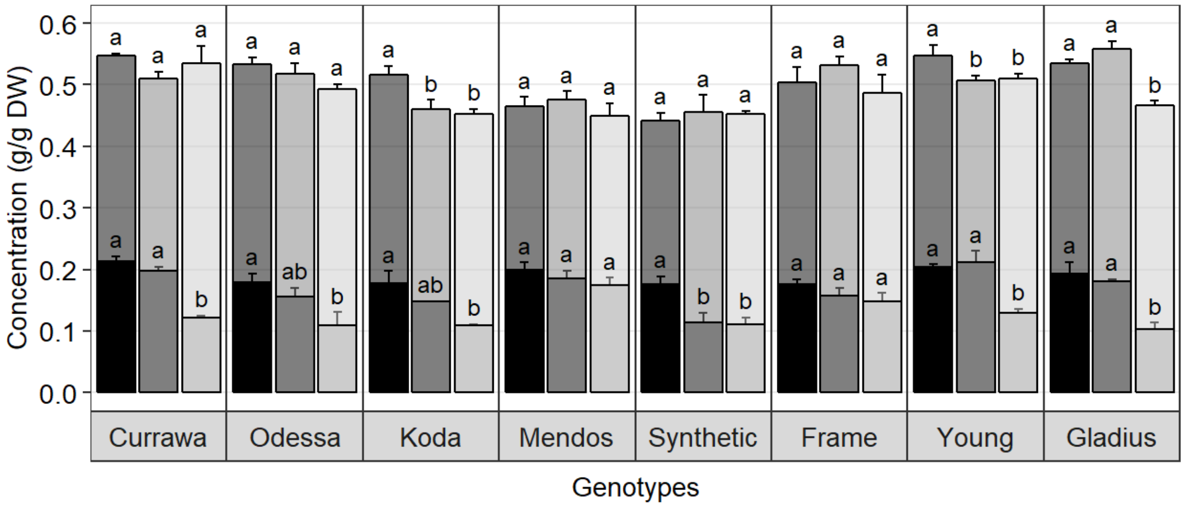
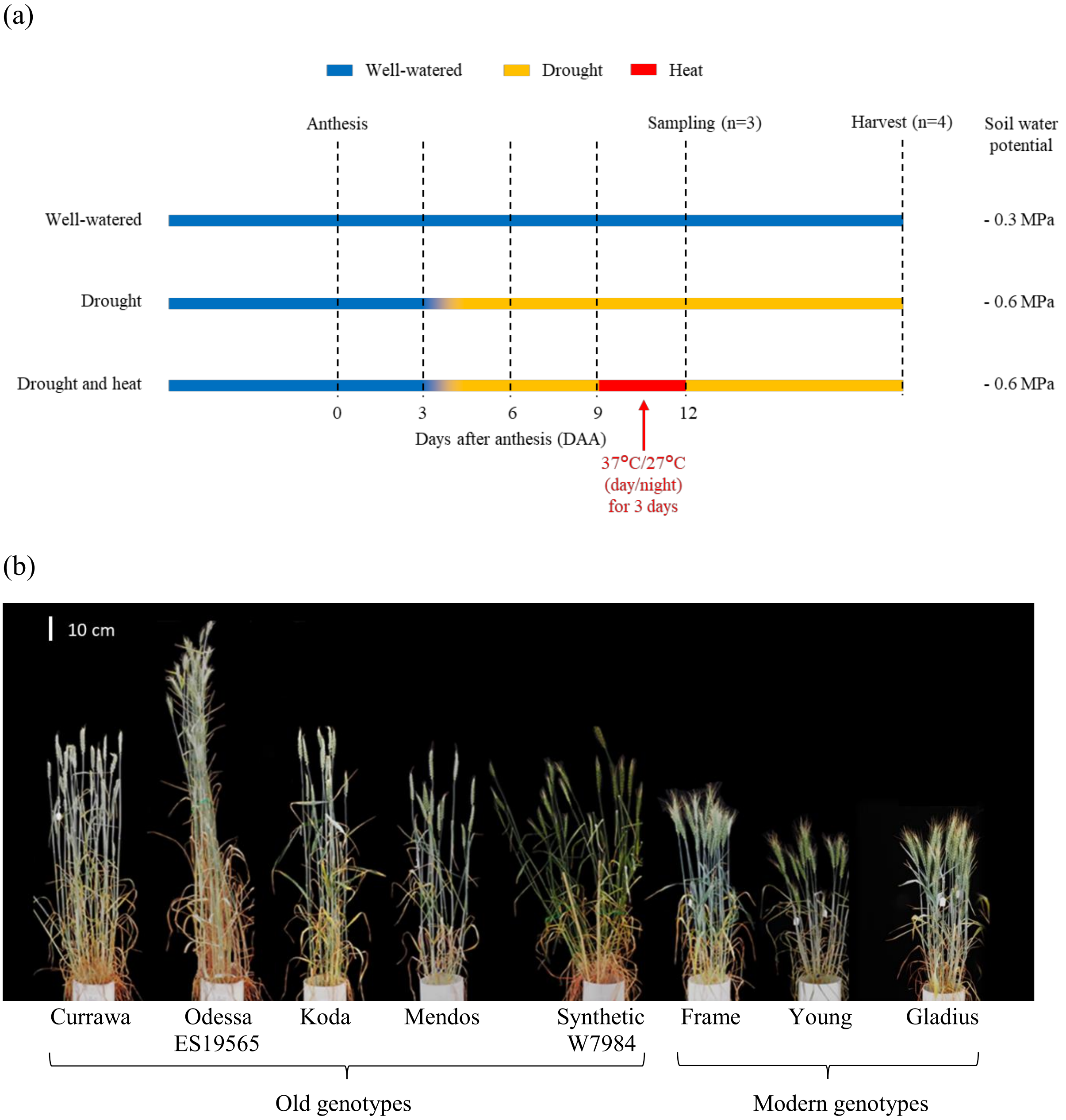

(a)
WSC
Glucose
Fructose
Sucrose
Grain weight (g)
0
1
2
3
4
$r^2 = 0.02^{ns}$
$r^2 = 0.07^{ns}$
$r^2 = 0.22^{*}$
$r^2 = 0.03^{ns}$
0
0.1
0.2
0.3
0.4
0
0.001
0.002
0.003
0.004
Concentration (g/g DW)
(b)
WSC
Glucose
Fructose
Sucrose
Grain weight (g)
$r^2 = 0.13^{ns}$
$r^2 = 0.34^{*}$
$r^2 = 0.11^{ns}$
$r^2 = 0.33^{*}$
Concentration (g/g DW)
(c)
WSC
Glucose
Fructose
Sucrose
Grain weight (g)
$r^2 = 0.18^{*}$
$r^2 = 0.43^{*}$
$r^2 = 0.4^{*}$
$r^2 = 0^{ns}$
Concentration (g/g DW)
(d)
Starch
Grain weight (g)
$r^2 = 0.03^{ns}$
0.2
0.3
0.4
0.5
Concentration (g/g DW)

Figure 6

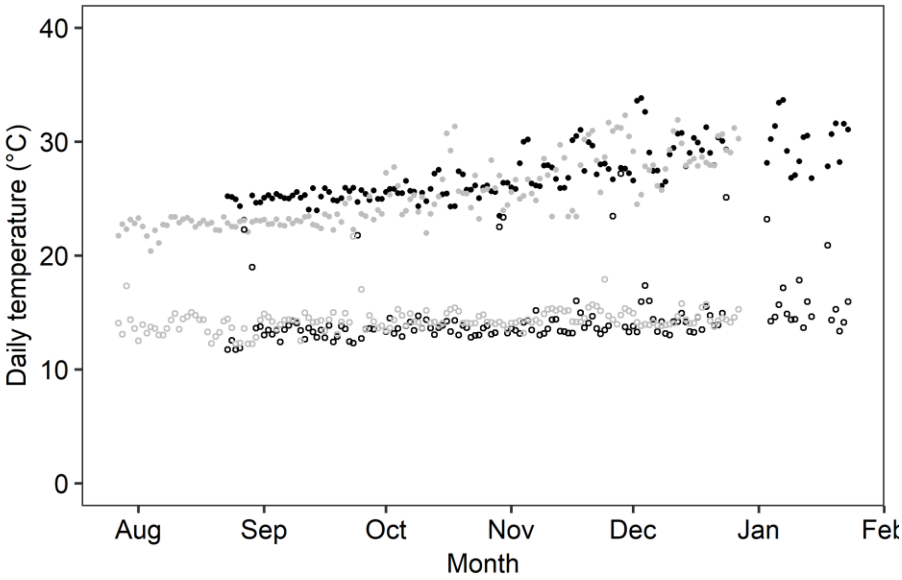

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Concentration (g/g DW)
Currawa
Odessa
Koda
Mendos
Synthetic
Frame
Young
Gladius
Genotypes
a
b
ab

Supplementary figure S1

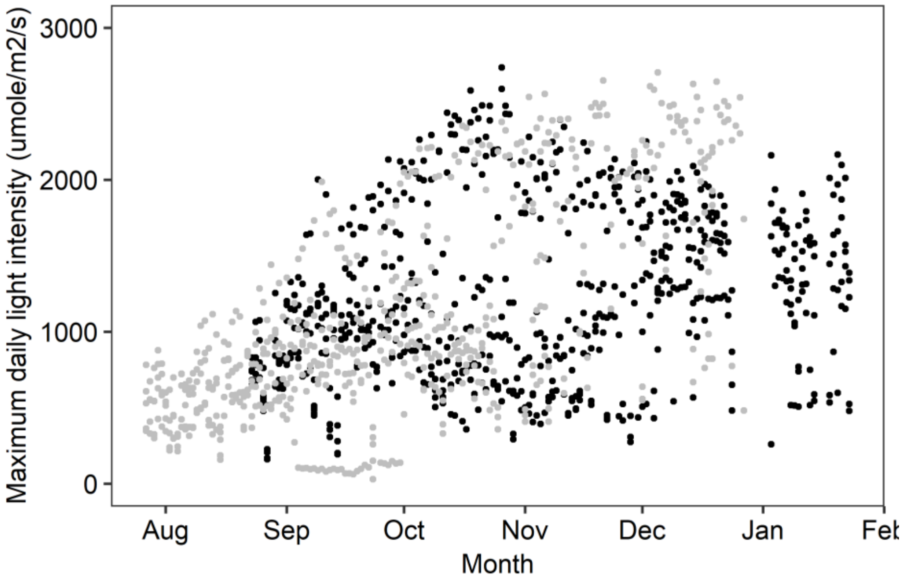


Supplementary figures S2

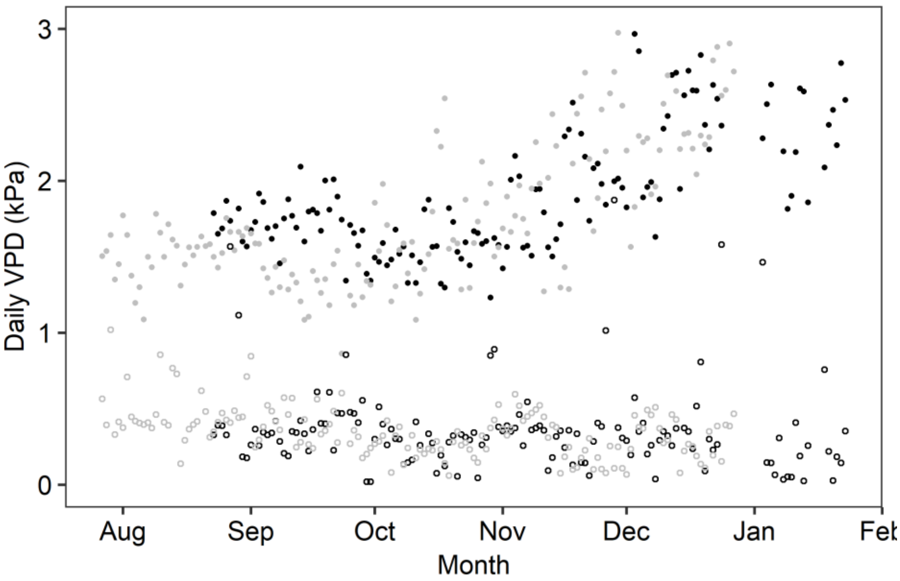
a)



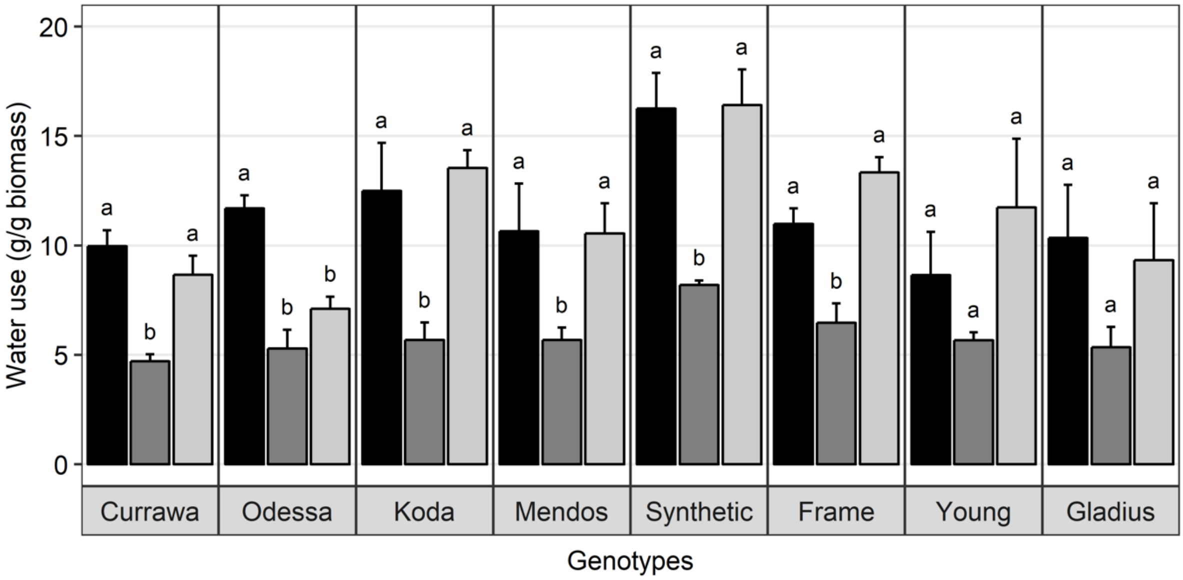
b)



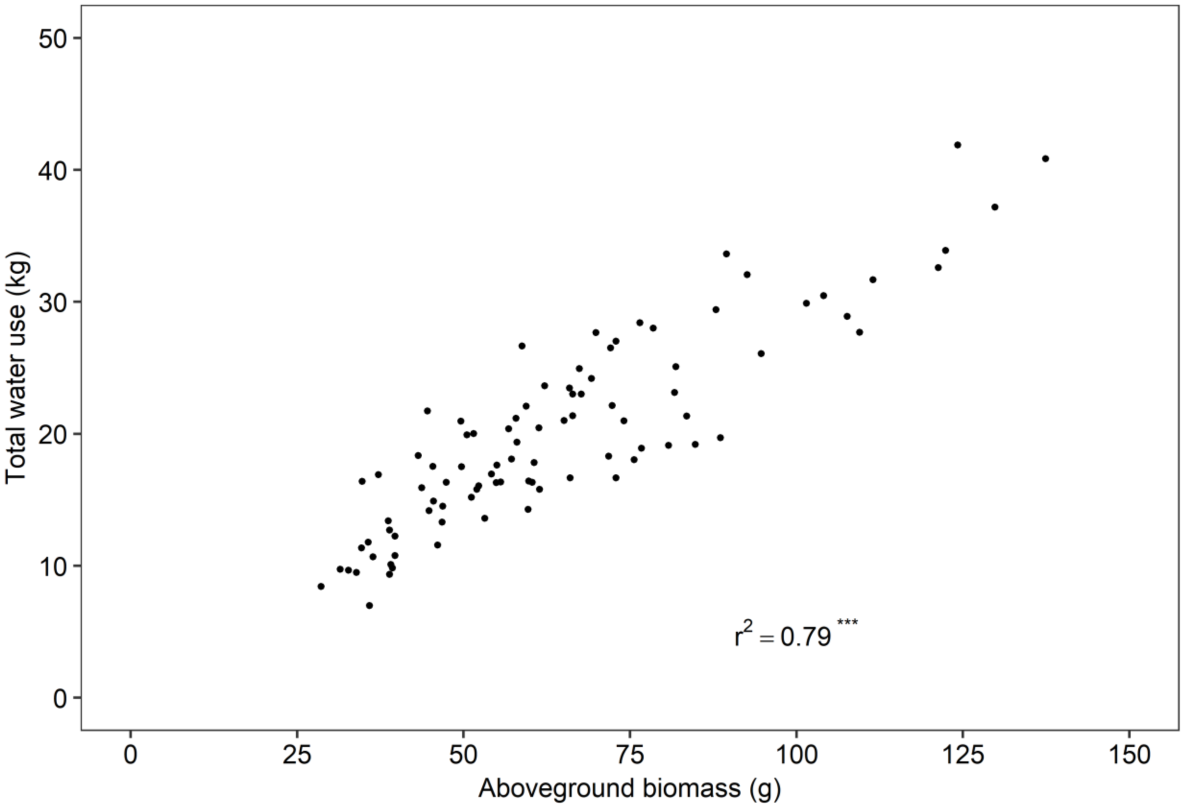
c)



Supplementary figures S3

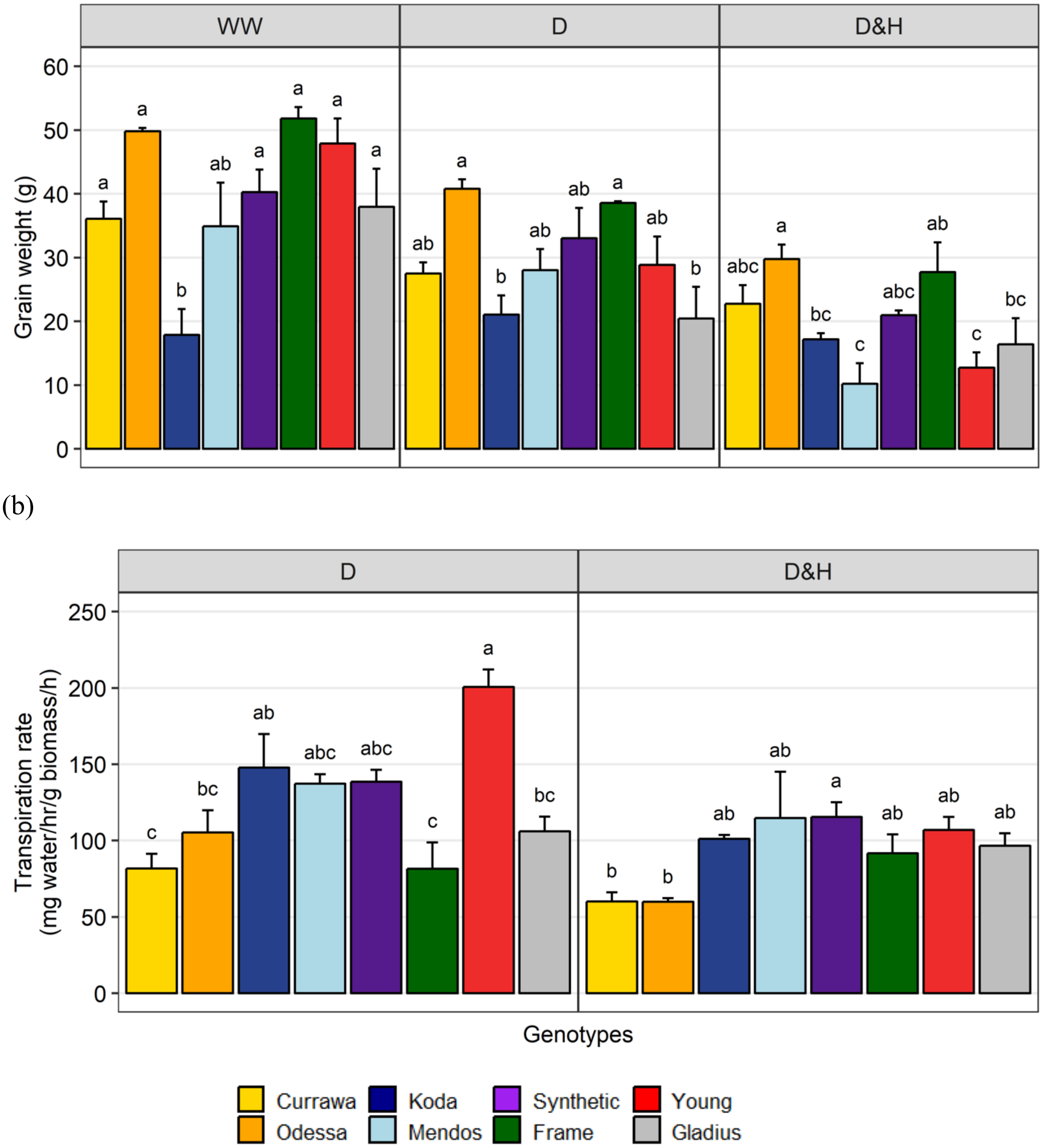


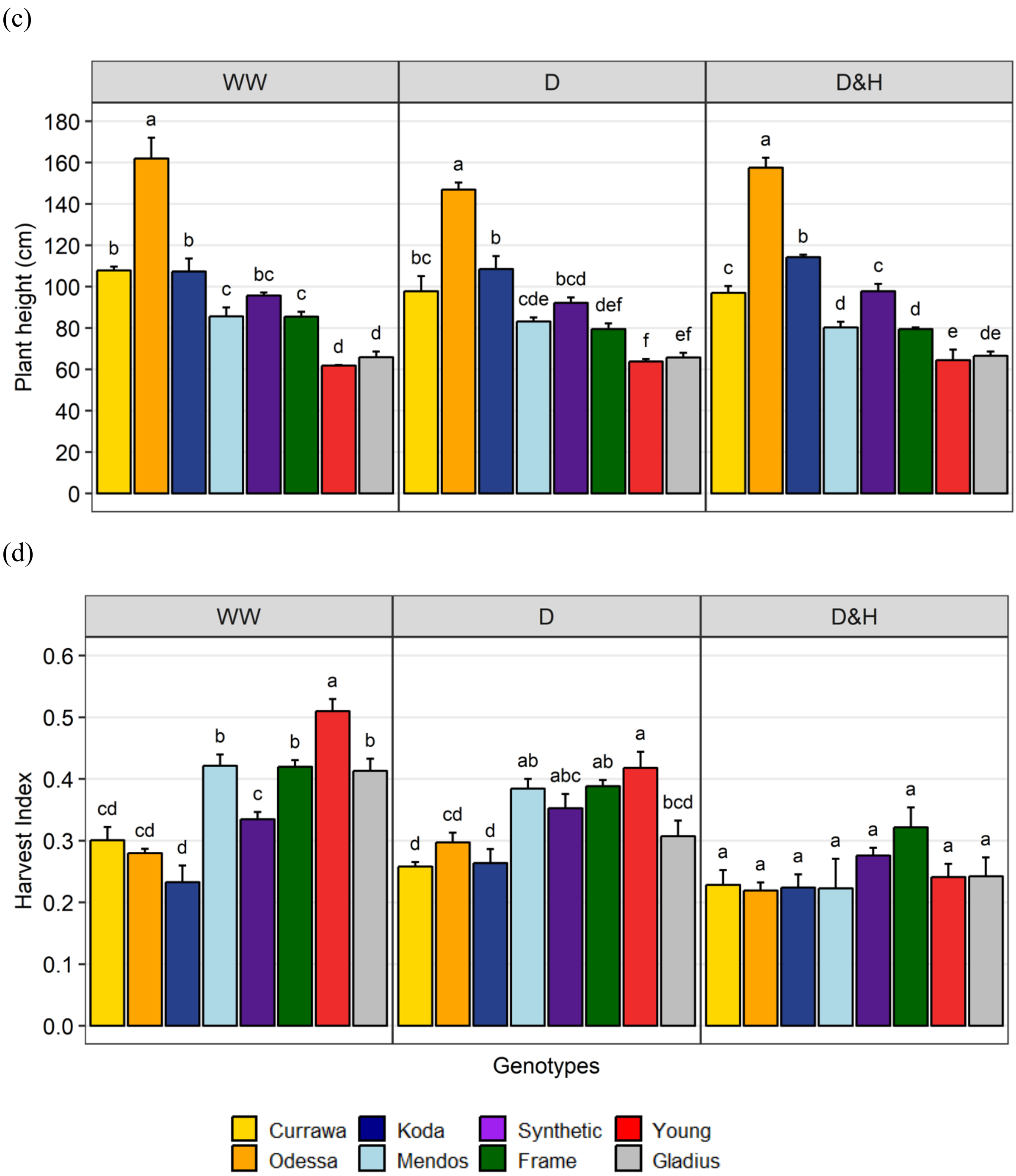
Supplementary Figure S4



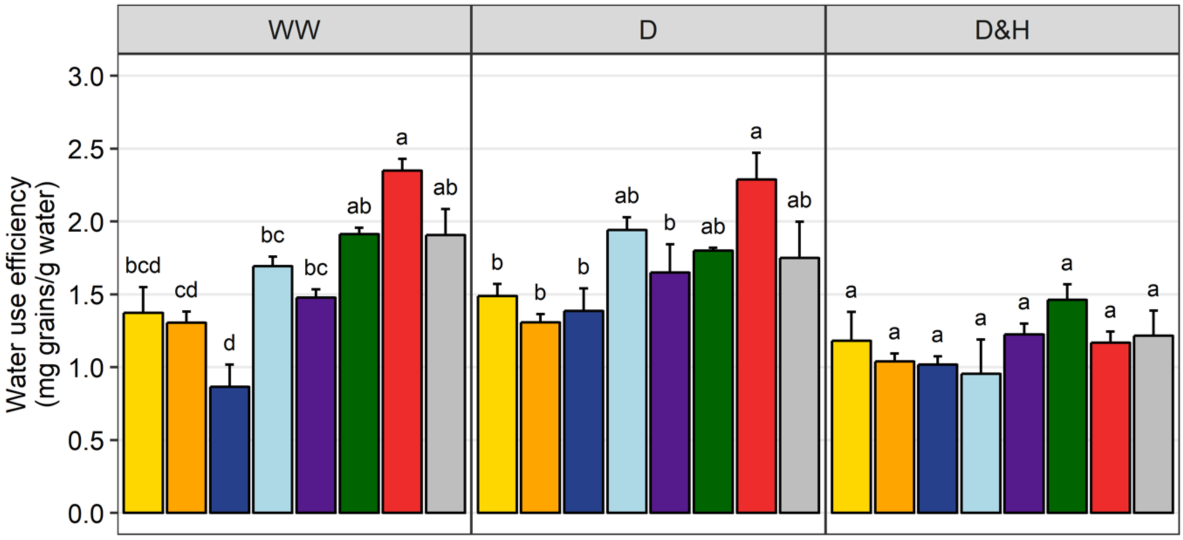
Supplementary Figure S5

(a)

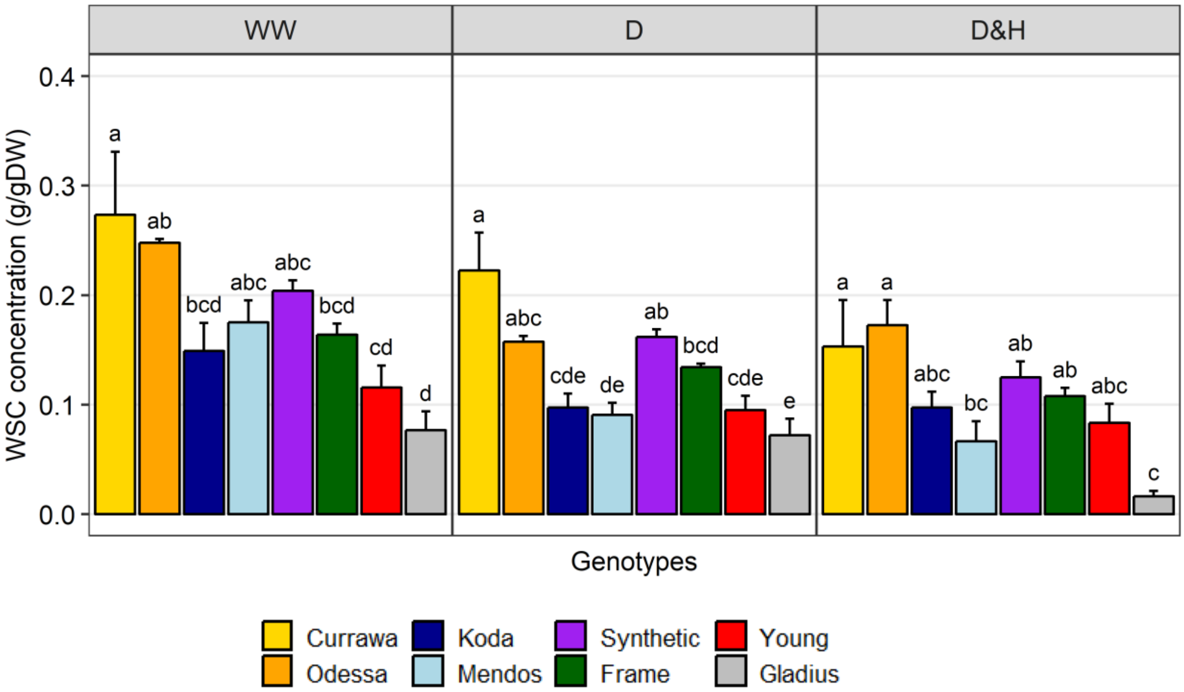



(c)
WW
D
D&H
Plant height (cm)
0
20
40
60
80
100
120
140
160
180
b
a
b
c
bc
c
d
d
bc
a
b
cde
bcd
def
f
ef
c
a
b
d
c
d
e
de
(d)
WW
D
D&H
Harvest Index
0.0
0.1
0.2
0.3
0.4
0.5
0.6
cd
cd
d
b
c
b
a
b
d
cd
d
ab
abc
ab
a
bcd
a
a
a
a
a
a
a
a
Genotypes
Currawa
Odessa
Koda
Mendos
Synthetic
Frame
Young
Gladius

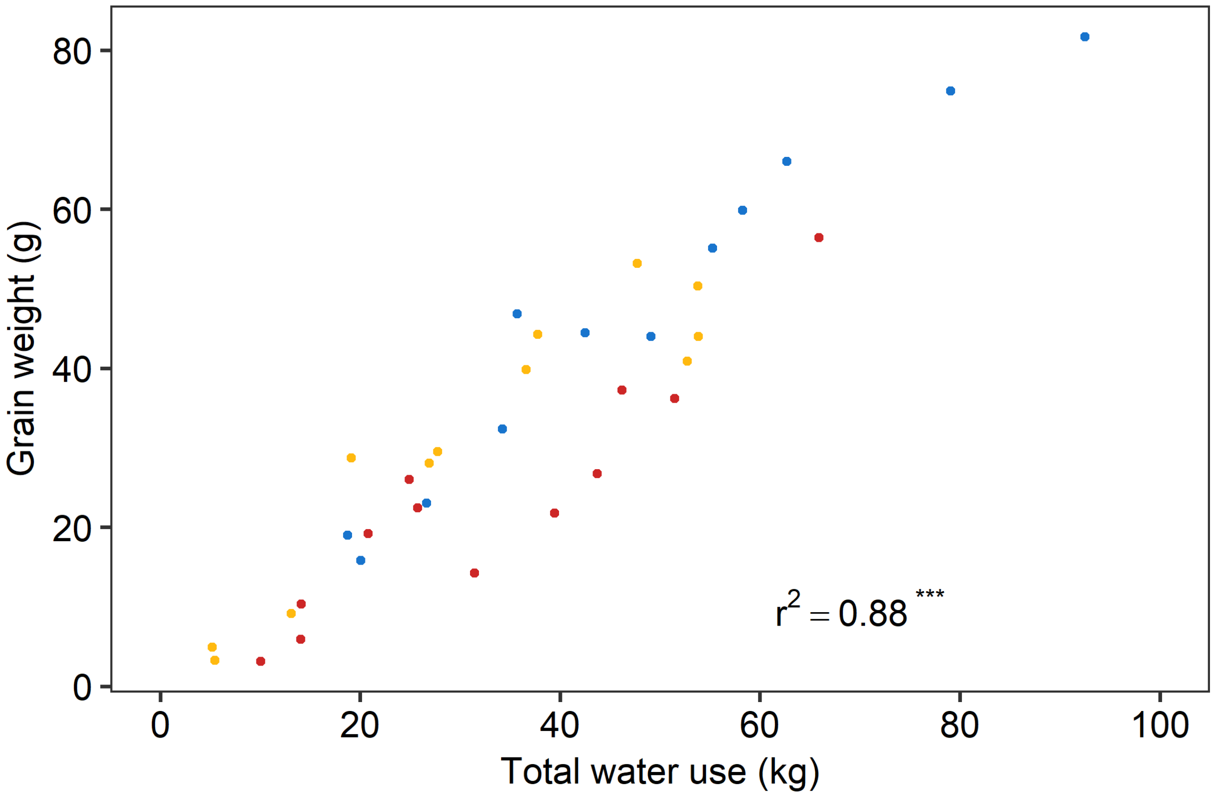
(e)


WW
D
D&H
Water use efficiency
(mg grains/g water)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
bcd
cd
d
bc
bc
ab
a
ab
b
b
b
ab
b
ab
a
ab
a
a
a
a
a
a
a
a

(f)


WW
D
D&H
WSC concentration (g/gDW)
0.4
0.3
0.2
0.1
0.0
a
ab
bcd
abc
abc
bcd
cd
d
a
abc
cde
de
ab
bcd
cde
e
a
a
abc
bc
ab
ab
abc
c
Genotypes
Currawa
Odessa
Koda
Mendos
Synthetic
Frame
Young
Gladius

Supplementary Figure S6



Supplementary Table S1

Name	Pedigree	Year of release	Origin
Currawa	Northern-Champion/Cretan//Little-Club[56][37][39][1138][1451]	1912	Australia
Odessa ES19565	Not available	1930	Ethiopia
Koda	Dundee/Kenya-745-C-6042//Bobin*2/Gaza[39][1138]; Dundee/Gabo[56]	1955	Australia
Mendos	Spica/Koda//Gabo/3/(Sib)Mengavi[39][117][1138]	1964	Australia
Synthetic W7984	Altar-84(TR.DR)/(TR.TA)CI-18[1540]		Mexico
Frame	Molineux/3*Dagger[1866][2854]	1997	Australia
Young	VPM-1/3*Beulah//Silverstar[3810]	2005	Australia
Gladius	(DH)RAC-875/Krichauff//Excalibur/Kukri/3/RAC-875/Krichauff/4/RAC-75//Excalibur/Kukri[3794]	2007	Australia

Supplementary Table S2

Genotype	Part	WW/D	D/D&H	WW/D&H
Currawa	Stem	0.42	0.35	0.99
	Spike	**0.05**	0.31	**0.01**
	Grains	0.18	0.47	0.71
Odessa	Stem	0.44	0.19	0.78
	Spike	**0.03**	0.98	**0.06**
	Grains	0.55	0.38	0.89
Koda	Stem	0.11	0.59	0.41
	Spike	0.15	0.78	**0.06**
	Grains	0.73	0.32	0.7
Mendos	Stem	0.25	0.86	**0.10**
	Spike	0.13	0.89	**0.06**
	Grains	0.34	0.87	0.15
Synthetic	Stem	0.64	**0.05**	0.15
	Spike	0.19	0.27	**0.02**
	Grains	0.14	**0.03**	0.47
Frame	Stem	**0.08**	0.45	**0.02**
	Spike	0.55	0.50	0.13
	Grains	0.52	1.00	0.55
Young	Stem	0.91	0.97	0.98
	Spike	0.99	0.99	1.00
	Grains	0.86	0.97	0.95
Gladius	Stem	0.96	0.32	0.23
	Spike	0.50	0.65	0.17
	Grains	0.75	0.45	0.85

Chapter 3:

Dissimilarity between leaf and spike transpiration following combined drought and heat stress

Introduction

Hydration is essential for normal cellular function in plants, and water use through transpiration is a fundamental component of plant growth and productivity (Blum 2009, Brodribb 2015). Plant water status is the result of the balance between water uptake by roots and water loss in aerial tissues via transpiration. When water resources in the soil are scarce, transpiration rate is reduced and biological processes are slowed, resulting in reduced growth and limiting grain yield (Brodribb 2015). In the experiment described in chapter 2, plant water use explained a high proportion of the variation in final grain weight. This proportion increased with increasing stress intensity and was highest following combined drought and heat stress (D&H), confirming the importance of plant water use in determining plant productivity in unfavourable environments and highlighting the need to advance our knowledge of the physiological basis of plant water use.

Water movement in plants is governed by environmental factors as well as the hydraulic properties of plants (Boyer 1985). Evaporative demand in the atmosphere drives transpiration through the stomata and causes water loss in transpiring organs, resulting in a decreasing gradient of water potential along the plant and a passive movement of water along the soil-plant-atmosphere continuum (Dixon, 1896). Water is conducted through xylem vessels that form a resistance to water flow dependent on conduit geometry (Tyree & Ewers 1991). Xylem and phloem conduits are adjacent to each other and their functions are interconnected (Hölttä et al., 2009). Water movement in the xylem and assimilate transport in the phloem rely on gradients of water and osmotic potential along the vessels, resulting in a dynamic exchange of water between both conduits. Disrupted function of either conduit can impair the function of the other conduit (Sevanto 2018). The combination of the water potential and hydraulic conductance of plant organs and water-conducting tissues determine water flow from the soil through the plant to the atmosphere.

The wheat spike is an important site for assimilate production and transpiration during grain filling, especially when water supply is limited (Teare 1972, Blum 1985, Araus 1993, Maydup 2010, Sanchez-Bragado 2014). This is mainly due to its proximity to grains and the more favourable water status compared to the rest of the plant under drought (Barlow 1980, Dorion 1996, Wardlaw 2002, Abbad 2004, Neghliz 2016). Studies on the effect of drought and heat stress on the spike function are scarce, mainly due to complex morphology that makes measurements difficult compared to leaves. Results in the previous chapter showed that 3d heat stress combined with drought altered transpiration response to environment in some wheat genotypes. Given the strong relationship between transpiration and final grain weight, I hypothesised that combined D&H reduced transpiration and therefore water flow to the spike. This could alter the spike water status and consequently the process of grain filling. In this experiment, plant water use, water flow to the spike and two determinants of water flow, hydraulic conductance and water potential, were monitored in two genotypes contrasting for water use response to combined D&H.

Material and methods

Plant material and growth

Two bread wheat genotypes contrasting for water use response to combined D&H were used in this study (*Triticum aestivum* L. cultivars Frame and Young). Single plants were sown in in 40 cm x 15 cm round pots containing 8 kg of a mixture of 1/3 clay/loam, 1/3 UC mix and 1/3 cocopeat and grown in a growth room (Phoenix Research, Australia) at 22°C/ 15°C day/ night with a 12h photoperiod (250 µmol m^{-2} s^{-1} photon flux density). Plants were well-watered (soil water potential = - 0.3 MPa, soil water content = 20 %) until anthesis of the main spike. One third of the plants (n=16 for each genotype and in each treatment;) were maintained in well-watered, cool conditions until harvest. A 7d drought treatment (soil water potential = - 0.6 MPa, soil water content = 12 %) was applied 3d after anthesis for the remaining plants, followed, in half of these plants, by a 3d heat treatment at 37 °C/ 27 °C day/ night (n=16 for each genotype and in each treatment) applied in another growth room. After heat treatment, drought was maintained until harvest. Pots were weighed daily to the target pot weight corresponding to the soil water potential of the treatment. A graphical representation is shown in Figure 1.

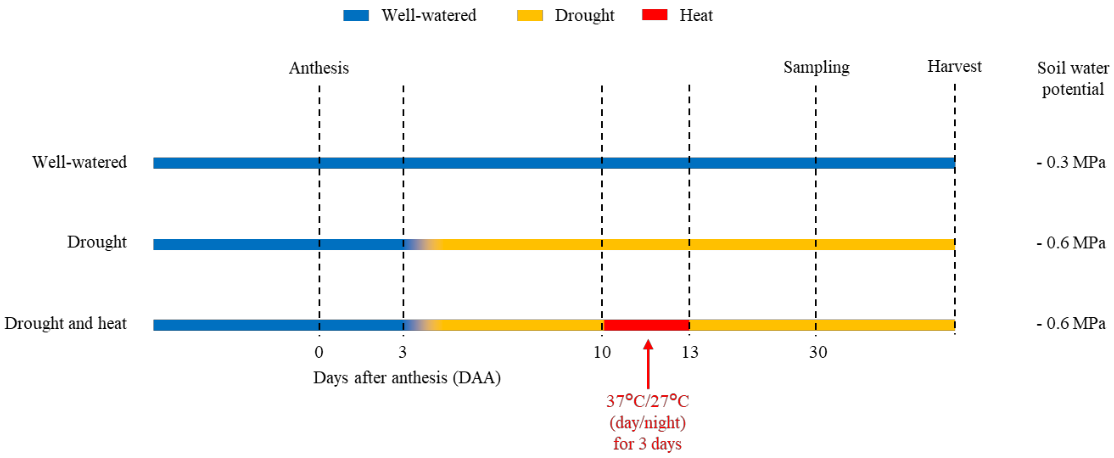


Figure 1. The experimental design for applying drought and combined drought and heat stress. Plants were well watered (soil water potential (SWP) = -0.3 MPa) until anthesis. Drought (SWP = -0.6 MPa) was applied 3 DAA and maintained until harvest. Three-day heat treatment was applied 10 DAA.

Sampling and measurements of water potential and hydraulic conductance

Sampling and measurements were performed 30 days after anthesis (DAA) at midday. Thirteen spikes (seven replicates for water potential measurements, six replicates for hydraulic conductance measurement) per genotype and per treatment were cut 5 cm from the base of the spike, immediately put vertically in a bottle with the peduncle immersed in deionised water and transported in a humidified sealed box. Measurements of water potential and hydraulic conductance were performed separately within 10 minutes after sampling. For water potential measurements, spikes were separated in three parts with similar numbers of spikelets: basal, central and apical. Water potential was measured using a dewpoint hygrometer (WP4C, Decagon Devices Inc. München, Germany). Measurements were recorded when values became stable. Spike hydraulic conductance was measured using a High Pressure Flow Meter (HPFM-Gen3, Dynamax, Houston, TX). The HPFM was connected to the peduncle recut under water at 1 cm below the base of the spike. Increasing pressure from 0 to 600 kPa applied to the spike and flow rate were recorded every 2 seconds and all measurements were corrected to values at 25°C. A second measurement was done on the same spike after removing all spikelets to measure the hydraulic conductance of the rachis. Hydraulic conductance was calculated from the slope of the plot of water flow versus applied pressure.

Sap flow and water use measurements

Sap flow was measured using SF-4 sap flow sensors (Edaphic Scientific, Moorabbin, Australia) connected to a CR1000 data logger (Campbell Scientific, Logan, USA). Sap flow sensors were installed on the peduncle, 1 cm below the base of the spike. For Frame, sensors were placed on the leaf sheath around the peduncle as the peduncle was not fully emerged. Measurements were performed every 15 minutes on three plants per genotype and per treatment from 10 to 30 days after treatment (DAT).

Difference between pot weights at two irrigation events was considered as daily water use (between 10 DAT to 30 DAT), after correcting for soil evaporation using pots with soil only.

Gas exchange measurements

Leaf transpiration was measured at ambient light in the flag leaf after anthesis, continuously for one day, using a Ciras 3 Portable Photosynthesis System connected to the PLC3 Universal Leaf Cuvette (PP Systems, Amesbury, USA).

Harvest data

Three plants per genotype and per treatment were harvested when plants were fully dry. Grain weight and grain number were measured separately in different spikes, and aboveground biomass included all tillers and spikes.

Data analysis and statistical analyses

Sap flow data baselines were different depending on days and on the sensor. Day values (Mv) were normalised to the sap flow baseline of 5 h of the preceding night. Repeated measures ANOVA was performed on daily water use and sap flow rate, with genotype and/or treatment as fixed factors and days after treatment as the error term using the aov function in R software. Two-way ANOVA was performed on grain weight and water potential, with genotype and treatment as fixed factors for grain weight, and with genotype, treatment and position in the spike as fixed factors for water potential. Treatment means within genotypes were compared using Tukey’s HSD (honestly significant difference) test ($p =< 0.05$). When the number of replicates was not sufficient, a Student’s t-test was used to compare means of two treatment within the same genotype.

Statistical analyses (ANOVA, Tukey's test, Student's t-test) and graphical representation were performed using R software version 3.4.4 (R Core Team, 2018) and ASReml-R (Butler et al., 2009).

Results

Environmental conditions

Mean temperatures in the growth room were 25.5 ± 1.0 °C during the day and 15.4 ± 0.8 °C at night (Fig. 2A), except during the applied heat treatment. Relative humidity in the growth room was very high, 89.4 ± 10.0% during the day and 54.5 ± 12.7% at night (Fig. 2B). VPD was 1.4 ± 0.5 kPa during the day and 0.2 ± 0.2 kPa at night (2C). During the last month of the experiment, a 2°C increase in temperature and significantly reduced relative humidity resulted in increased VPD.

Anthesis in cv. Young plants was about three weeks earlier than in cv. Frame. For logistical reasons, heat treatments were applied in two different growth rooms, one per genotype. Environmental conditions were similar in both growth rooms. During heat treatment, temperature was 39.5 ± 0.3 °C/ 26.3 ± 0.2 °C day/ night for Young, 40.3 ± 1.2 °C/ 25.8 ± 0.4 °C for Frame. Relative humidity was 77.4 ± 4.8%/ 36.6 ± 10.1% for Young, 40.3 ± 1.2 °C/ 25.8 ± 0.4 °C for Frame. VPD was 4.2 ± 0.4 kPa/ 0.8 ± 0.2 kPa for Young, 4.7 ± 0.7 kPa/ 0.6 ± 0.2 kPa for Frame.

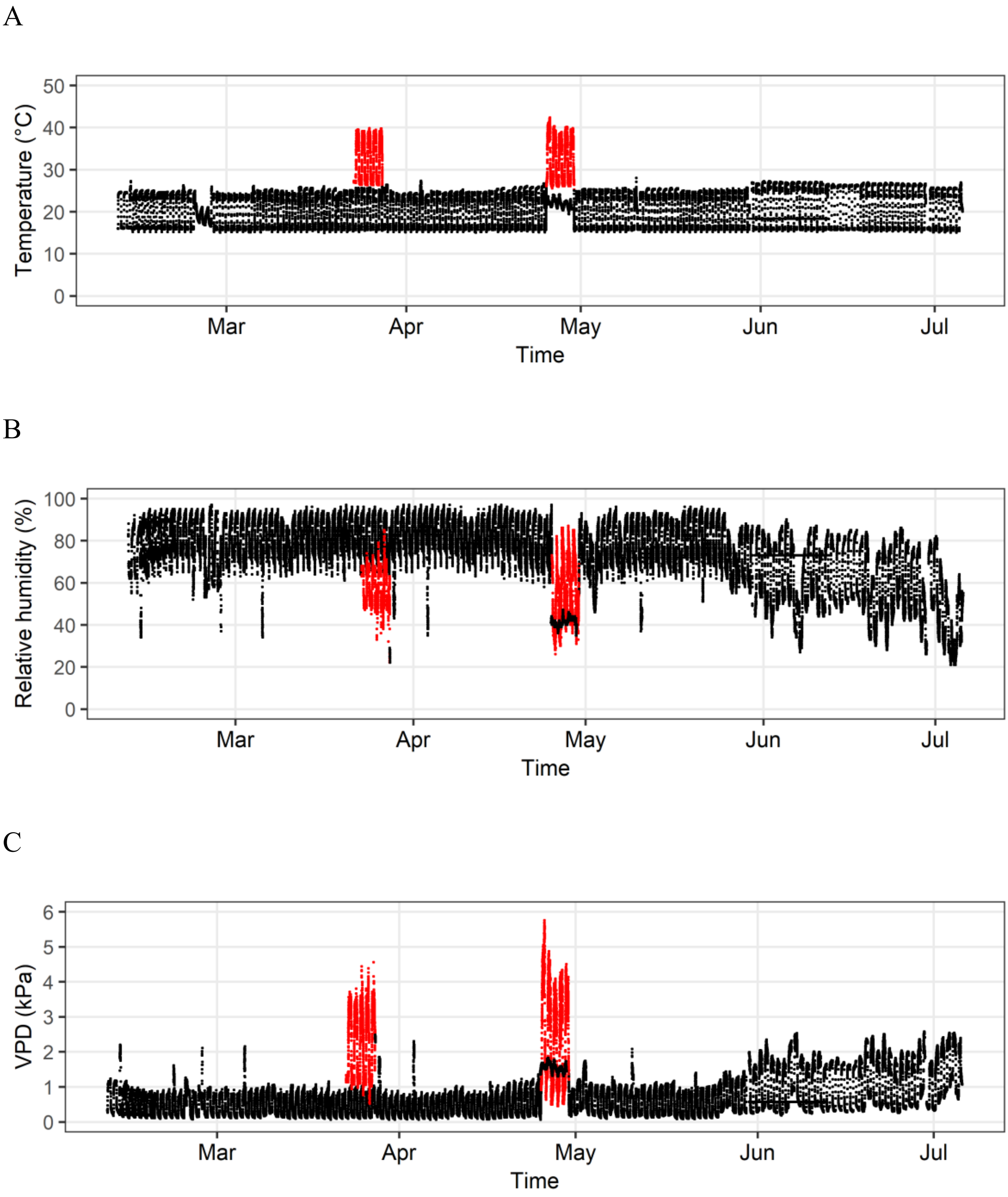


Figure 2. Temperature (A), relative humidity (B) and vapour pressure deficit (C) during plant growth. Data points in red refer to heat treatment for Young in March and Frame in April.

Daily water use, sap flow rate and leaf transpiration

As water use strongly depends on biomass and Frame and Young genotypes differ in aboveground biomass, plant water use was normalised to biomass and expressed as unit of water per unit of biomass.

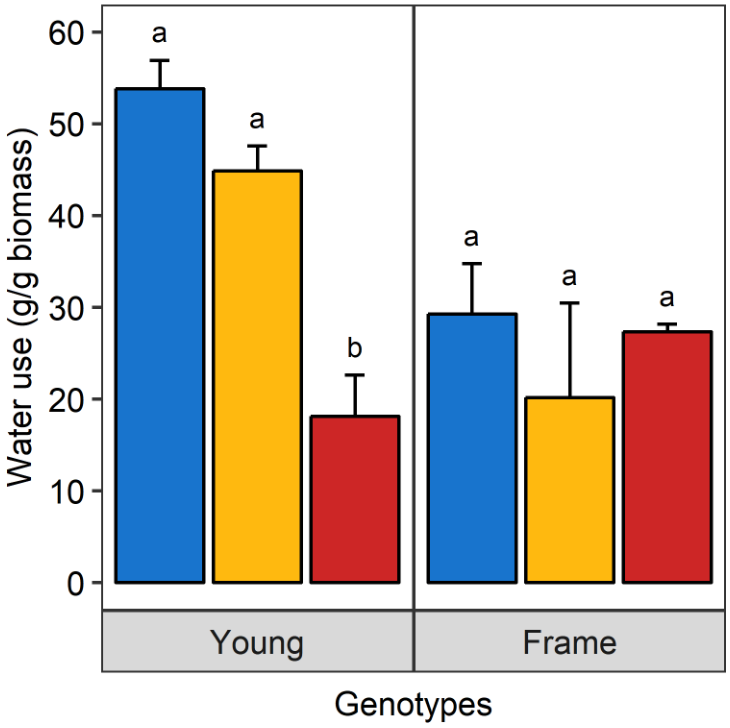
There was a significant interaction between genotype and treatment for total water use per plant between 10 and 30 DAT (Table 1). Drought did not have a significant effect on total water use during this period in either genotype, whereas combined drought and heat stress (D&H) significantly reduced water use only in Young (Fig. 3A).

				Interactions			
Traits	Genotype	Treatment	Position	GxT	GxP	TxP	GxTxP
Total water use normalised to biomass (10-30DAT)	**	***		**			
Daily water use (10-30DAT)	***	***		***			
Water potential	***	***	***	**	ns	ns	*
Grain number in the main spike	***	ns		ns			
Grain weight in the main spike	ns	***		*			
Grain weight per spike	ns	***		ns			

* $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; ns not significant

Table 1. Analysis of variance (ANOVA) showing the statistical significance of the traits measured for genotype, treatment and interaction between genotype and treatment. Position in the spike was also included in the ANOVA analysis for water potential.

A



B

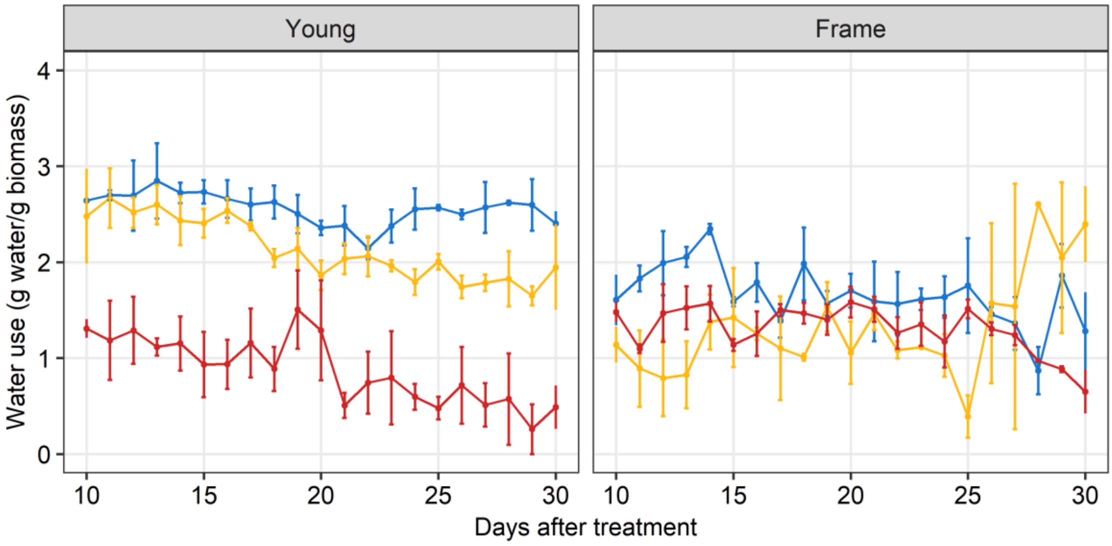
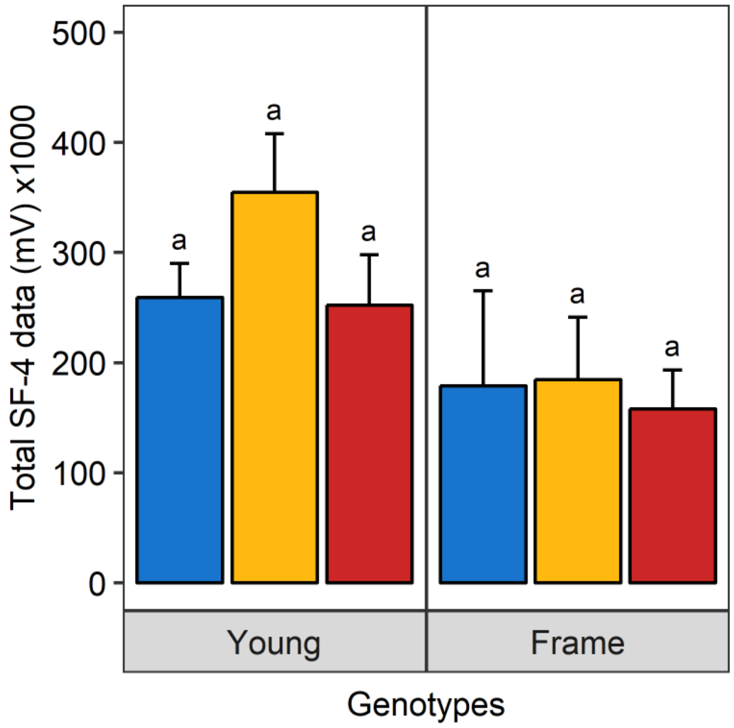


Figure 3. Plant water use between 10 and 30 days after treatment. (A) Total water use per plant normalised to biomass (B). Daily water use per plant normalised to biomass. Plants grown in well-watered conditions (in blue), drought (in orange) or following a three-day heat stress (in red). 0 DAT is the first day post-treatment (= 12 DAA). Values are means of three replicates. Error bars are standard errors.

There was a significant interaction between genotype and treatment for daily normalised water use per plant between 10 and 30 DAT (Table 1). The daily amount of water used per unit of biomass (g water/g biomass) during the drought treatment from 10 to 30 DAT was similar than in well-watered (WW) conditions in Young up to 22 DAT, after which daily water use was lower than in WW conditions ($p = 0.2$, Fig. 3B). In Frame, daily water use was reduced under drought compared to WW conditions up to 25 DAT, after which daily water used suddenly increased ($p < 0.001$, Fig. 3B). Increase in water use after 25 DAT in Frame plants under drought results from increased VPD during the last month of experiment (Fig. 2), affecting Frame plants that were the latest to reach anthesis, under drought. In contrast with drought, combined D&H reduced daily water use in Young, whereas daily water use was similar under drought and following combined D&H in Frame up to 24 DAT ($p < 0.001$). Daily water use was higher in Young compared to Frame when grown in WW conditions and under drought only up to 25 DAT ($p = 0.008$ and $p < 0.001$, respectively). Following combined D&H, water used was similar in both genotypes ($p = 0.9$).

Variation in sap flow sensor values, which reflected sap flow rate in the peduncle and correspond to water flow to the spike, between 10 and 30 DAT differed depending on the genotype and the treatment (Fig. 4A). In Young, sap flow rate was similar in WW plants and following combined D&H and decreased gradually ($p = 0.5$). Under drought, sap flow rate was constant between 10 and 18 DAT, then decreased gradually, resulting in higher water flow under drought compared to WW and combined D&H treatment between 15 and 22 DAT ($p = 0.08$ and $p = 0.3$, respectively). In Frame, water flow to the spike was highly variable between 10 and 19 DAT but was constant between 20 and 30 DAT. After 20 DAT, water flow was higher under drought compared to WW conditions. Following combined D&H, water flow was low compared to drought until 15 DAT ($p = 0.004$), gradually increased to reach WW levels at 18 DAT and surpassed WW levels after 20 DAT, then gradually decreased after 27 DAT ($p = 0.002$).

A



B

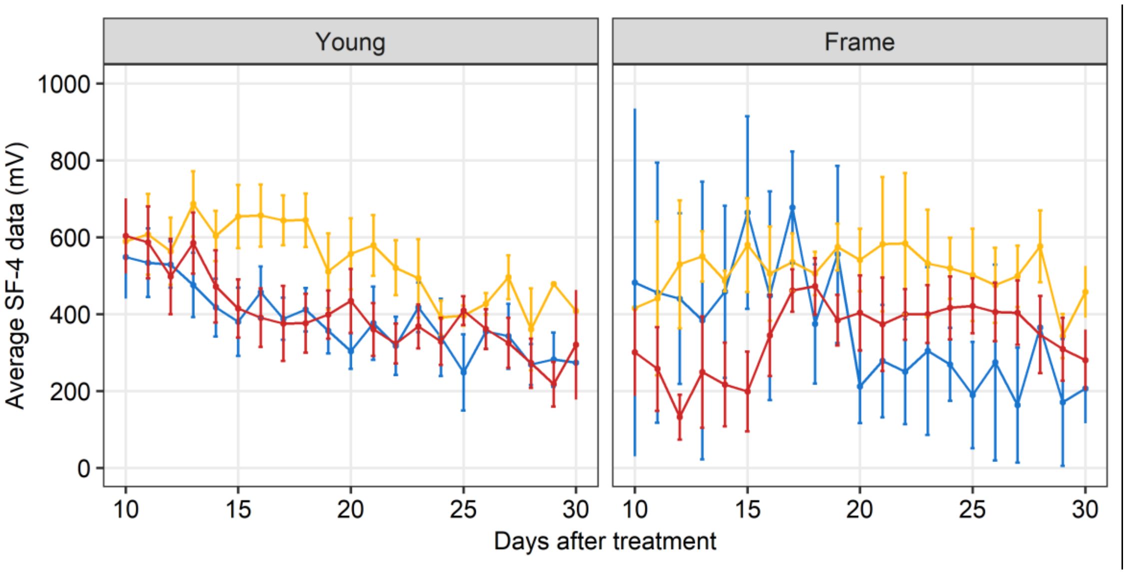


Figure 4. Normalised data from sap flow sensors in the peduncle between 10 and 30 days after treatment. (A) Total sap flow. (B) Average sap flow rate per day. Plants grown in well-watered conditions (in blue), drought (in orange) or following a three-day heat stress (in red). 0 DAT is the first day post-treatment (= 12 DAA). Data are means of two to eight replicates. Error bars are standard errors.

In flag leaves, combined D&H significantly reduced transpiration compared to drought in Young and Frame ($p = 0.03$ and $p = 0.02$, respectively) (Fig. 5). Leaf transpiration was not significantly different between both genotypes under drought and combined D&H ($p = 0.5$ and $p = 0.3$, respectively).

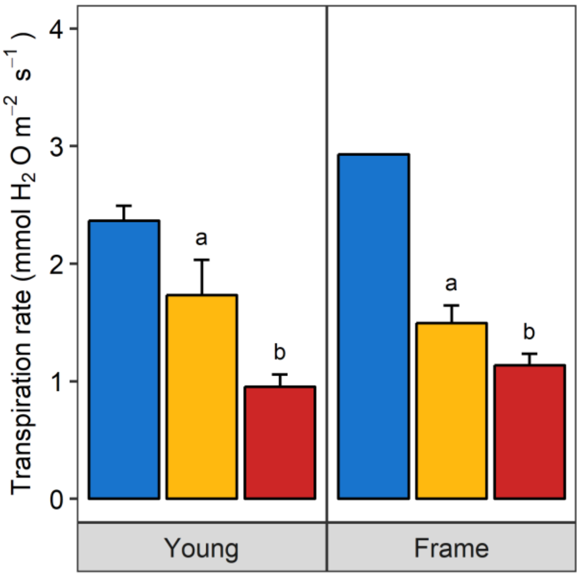


Figure 5. Average transpiration rate in the flag leaf measured after anthesis during light period (12h). Plants grown in well-watered conditions (in blue), drought (in orange) or following a three-day heat stress (in red). Values are means of 6 replicates for Young, 3 replicates for Frame under D or combined D&H. Error bars are standard errors. Plants grown in well-watered conditions (in blue) are shown as check lines because of the unique replicate for Frame but were not included in analyses. Letters indicate the results of a Student's t-test comparing mean under drought and mean following combined D&H within each genotype ($p < 0.1$).

Hydraulic conductance and water potential

Hydraulic conductance was measured in the peduncle immediately proximal to the spike. Hydraulic conductance of the peduncle in Young was double the hydraulic conductance in Frame (0.031 and 0.014 mmol/s/MPa, respectively) (Fig. 6A). There was no effect of drought on hydraulic conductance in Young, and there were not enough data to conclude on the effect of drought on hydraulic conductance in Frame. 3d heat treatment did not affect hydraulic conductance under drought in either genotype. Removal of spikelets increased hydraulic conductance in both genotypes (Fig. 6B). Differences in hydraulic conductance between Frame and Young following spikelet removal were reduced. In the rachis, hydraulic conductance averaged 0.06 mmol/s/MPa in Frame and 0.07 mmol/s/MPa in Young. The only spike replicate under drought had higher hydraulic conductance compared to WW and combined D&H, whereas two spike replicates out of four had a lower hydraulic conductance compared to WW and drought following combined D&H, resulting in high variation following combined D&H in Young.

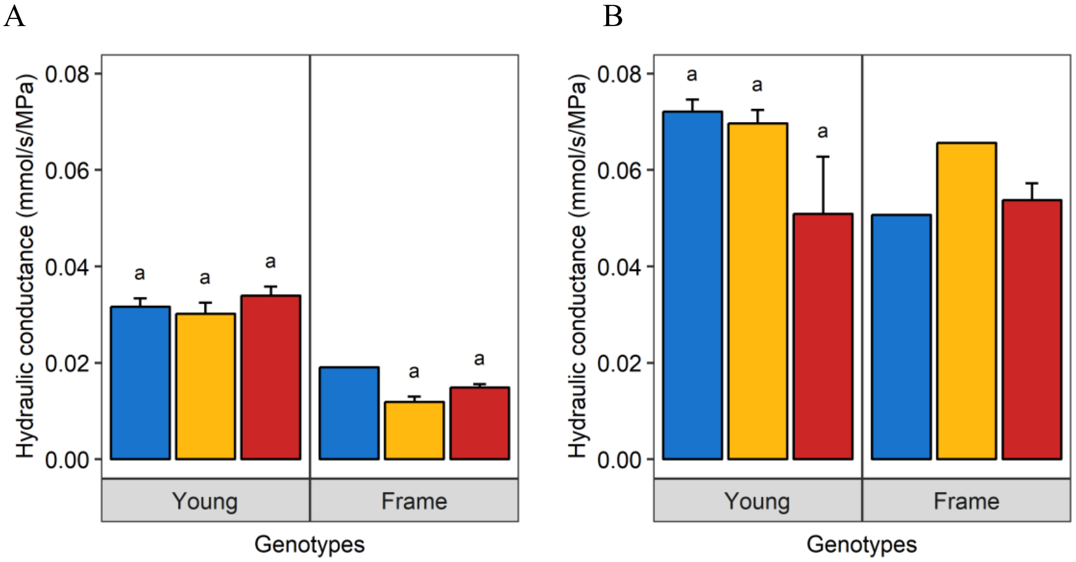


Figure 6. Hydraulic conductance of the peduncle with whole spike (A), or spike without spikelets (B), measured 30 DAA. Plants grown in well-watered conditions (in blue), drought (in orange) or following a three-day heat stress (in red). Values are means of 2 to 5 replicates. Error bars are standard errors. Letters indicate the results of a Student's t-test comparing two treatments within each genotype ($p < 0.1$).

There was a significant interaction between genotype and treatment for spike water potential (Table 1). The effect of treatment on water potential depended on the genotype and on the position in the spike. In Frame, only drought decreased water potential, and only in the central part of the spike (Fig. 7A). In Young, water potential was more negative in the basal part of the spike. Heat stress further decreased water potential in the central part in Young, and significantly decreased water potential in the apical part. When comparing genotypes, water potential was similar under WW and drought in both genotypes, but was significantly lower in the central and apical parts of the spike in Young.

A gradient in water potential was observed along the spike in both Frame and Young in WW conditions (Fig. 7B), where water potential was lower in the apical part of the spike. However, no other significant gradient was observed in Frame under drought and following combined D&H. In Young, a stronger gradient along the spike was measured following combined D&H. No gradient in water potential was observed under drought in Young. There was no genotypic difference in water potential in WW conditions and drought. Water potential was significantly higher in the central and apical parts of Young spikes compared to Frame following combined D&H

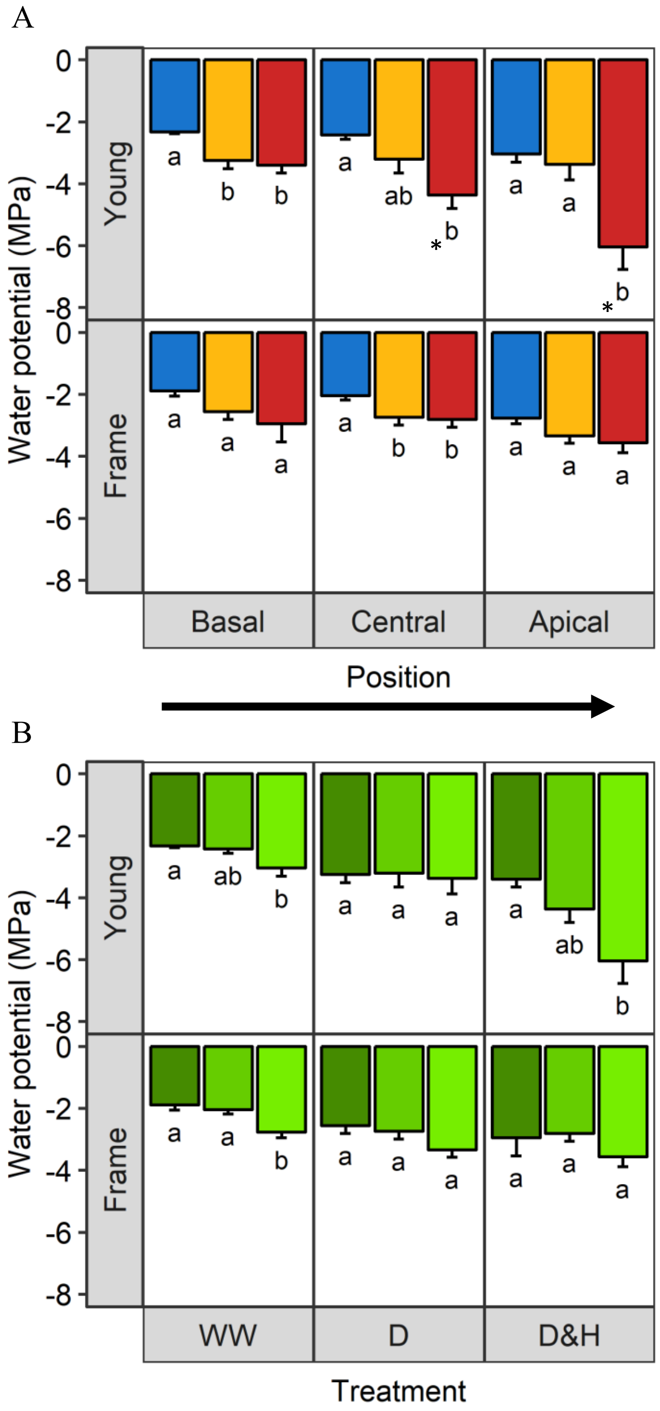


Figure 7. Water potential of the basal, central and apical part of the main spike. (A) Plants grown in well-watered conditions (in blue), drought (in orange) or following a three-day heat stress (in red). (B) Colours indicate different parts of the spike, from basal (dark green) to apical (light green) part of the spike. Values are means of 5 replicates. Error bars are standard errors. Letters indicate the results of a Tukey's test comparing treatments within each genotype ($p < 0.1$).

Grain number and final grain weight

There was a significant genotypic difference for grain number in the main spike (Table 1). Grain number was higher in Young compared to Frame, averaging 66.6 grains in Young and 40.2 grains in Frame (Fig. 8). Grain number per spike was not affected by drought or combined D&H (Table 1).

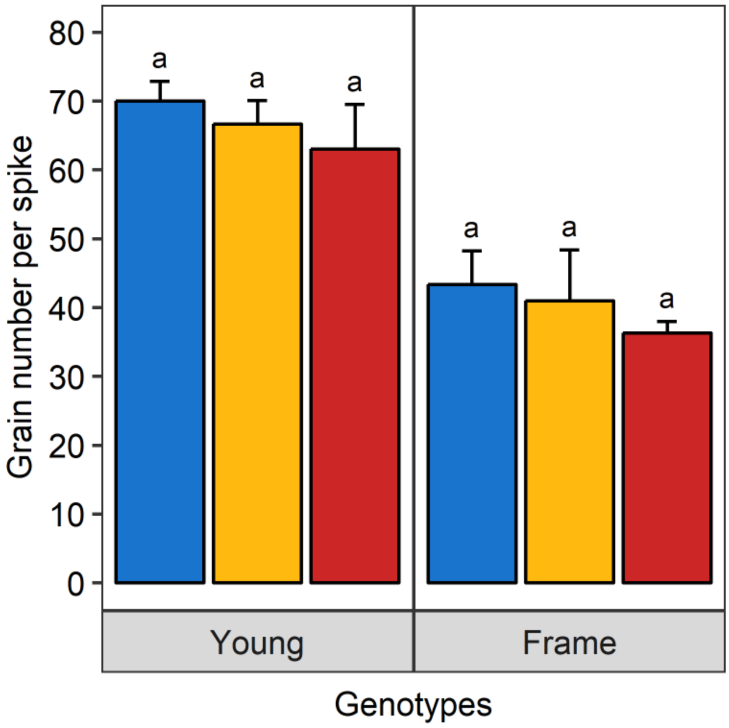


Figure 8. Number of grains in the main spike of Young and Frame plants. Plants grown in well-watered conditions (in blue), drought (in orange) or following a three-day heat stress (in red). Values are means of 3 replicates. Error bars are standard errors. Letters indicate the results of Tukey's test comparing treatment effect within each genotype ($p < 0.1$).

There was a significant interaction between genotype and treatment for final grain weight in the main spike (Table 1). Grain weight in the main spike was similar in WW conditions to the drought treatment in both genotypes (Fig. 9A). Combined D&H significantly reduced grain weight in Young but not in Frame. There was no significant interaction between genotype and treatment for final grain weight per plant (Table 1). Grain weight per plant was similar in WW and drought treatments in Young. Grain weight per plant in Frame was more variable under drought. Combined D&H reduced total grain weight per plant in Frame and Young (Fig. 9B). Grain weight per plant was higher in Frame compared to Young following combined D&H ($p = 0.07$).

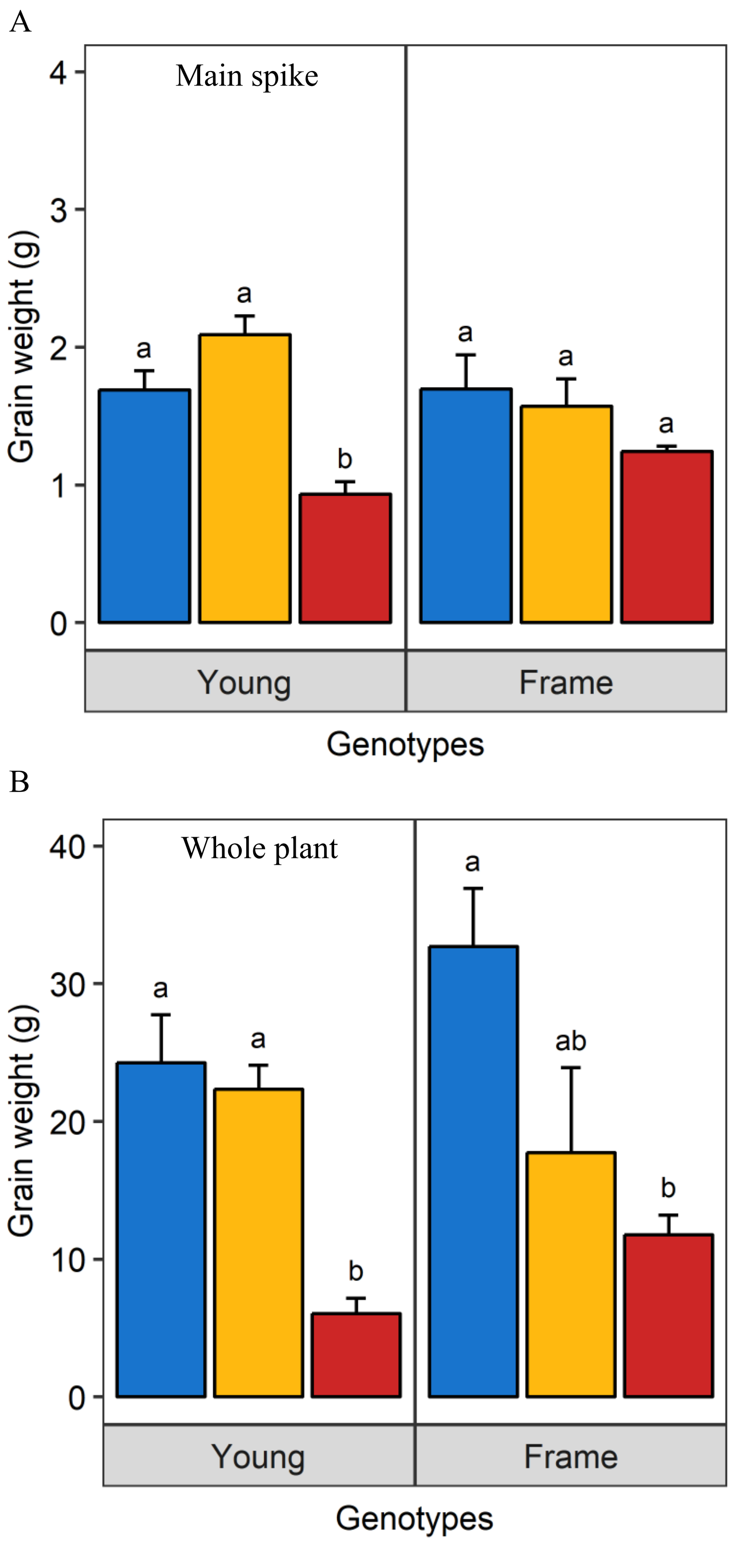


Figure 9. Final grain yield in the main spike (A) and whole plant (B). Plants grown in well-watered conditions (in blue), drought (in orange) or following a three-day heat stress (in red). Values are means of 3 replicates. Error bars are standard errors. Letters indicate the results of Tukey's test comparing treatment effect within each genotype ($p < 0.1$).

Discussion

This work combined, for the first time in wheat, measurements of hydraulic conductance of the peduncle, water potential of the spike and water flow to the spike measured on the peduncle in proximity to the spike. There were, however, limitations to the experiment that influence the interpretation of the results. Technical difficulties incurred from measurements conducted for the first time resulted in a low number of replicates and may explain variability observed. In addition, phenology in Frame was different in this experiment compared to the previous experiment in chapter 2. Frame plants reached anthesis 84.4 ± 3.8 days after germination in this experiment, 22 days later than anthesis in Frame plants in the previous experiment (62.4 ± 3.1 days after germination). This was not the case for Young plants that reached anthesis at a similar time, 51.8 ± 1.0 days after germination in this experiment, 53.1 ± 2.6 days after germination in the experiment in chapter 2. This was probably due to higher sensitivity of this genotype to the lower light intensity in the growth room (250 µmol m^{-2} s^{-1} photon flux density) compared to the glasshouse experiment (>1000 µmol m^{-2} s^{-1} photon flux density), as Frame plants did not grow as in the previous experiment; the peduncle did not emerge after heading. Consequently, sap flow sensors were placed on the leaf sheath covering the peduncle instead of the peduncle only as was the case for Young.

We observed a gradient of water potential along the spike of both genotypes in well-watered conditions (Fig. 7B). Lower water potential in more apical than basal parts of the spike creates a water potential gradient, and grain weight at the apical part of wheat spikes is usually lower than grain weight in the rest of the spike (Herzog 1983, Xie 2015, Feng 2018, Baillot 2019). Xie et al. (2015) and Baillot et al. (2019) explained spatial variation of individual grain weight within the spike as a lower grain filling rate and lower initial carpel size. Lower water potential at the apical part of the spike may also explain lower grain weight due to less favourable water status compared to other parts of the spike. Reduced water potential of the spike and grain weight following combined D&H only, in Young only, suggests that the relationship between spike water potential and grain weight also applies to stress conditions. Accelerated senescence of Young spikes after additional heat stress may explain reduced water potential in these spikes.

We did not observe a significant effect of drought and combined D&H on hydraulic conductance of the peduncle in both genotypes, which might imply that the hydraulic pathway in the peduncle was not damaged by drought or combined D&H. However, there was a significant difference in the hydraulic conductance of the peduncle in Frame compared with Young. Removal of the spikelets eliminated the genotypic difference in hydraulic conductance, suggesting that hydraulic conductance of the peduncle was mainly determined by spikelets rather than the rachis. In grapevine, hydraulic conductance of the bunch showed a linear relationship with the number of berries; bunches with a higher number of berries had a higher hydraulic conductance (Tyerman et al. 2004). This relationship was also apparent in this experiment, where Frame spikes that had fewer grains had a lower hydraulic conductance compared to Young spikes that had more grains (Fig. 8).

Water flow to the spike indicated by sap flow sensor values was not constant during grain filling but gradually decreased in Young under WW conditions, similar to the reported decline in water flow to grapes in grapevine (Tyerman et al. 2004, Tilbrook and Tyerman 2009). Water flow in Frame appeared constant but included water flow to the spike and to the flag leaf, which probably explains the higher variability in water flow in this genotype. The relative amount of water flow to the spike compared to water flow to leaves is still to be determined. Water flow to the spike can be used as a surrogate of spike transpiration. Interestingly, in this experiment, water flow was higher under drought, unlike in leaves where stomatal closure following drought represents a major resistance to water flow and reduces leaf transpiration as observed in this experiment (Fig. 5). This observation implies that stomata in the spike tissues may remain open even when the whole plant experiences stress, which is in accordance with the higher water status observed in the spike compared to leaves when water supply is limited (Barlow 1980, Dorion 1996, Wardlaw 2002, Abbad 2004). This suggests that transpiration in leaves and in the spike may be regulated differently. Differential regulation of water flow between the flag leaf and the spike may be a mechanism by which the plants favour water movement to reproductive organs when water supply is not sufficient to meet the water need of all plant organs. An interaction between drought and heat stress was observed for water flow to the spike. 3-day heat stress prevented increase in water flow to the spike observed under drought only, implying sensitivity of this hypothetical mechanism to high temperature. I hypothesised that high hydraulic conductance of the peduncle and low water potential of the spike would favour water flow to the spike. At 30 DAA, despite the significantly higher spike hydraulic conductance in Young compared to Frame in all conditions, and higher water potential in the central and apical parts of Young spikes compared to Frame following combind D&H, water flow to the spike was not significantly different in both genotypes under the same treatment (Fig. 4), suggesting that the rate of water flow to the spike may not be driven by hydraulic conductance at the peduncle or water potential of the spike, and that water flow could be determined by other factors. Despite the higher hydraulic conductance in Young, water flow to the spike was similar to water flow in Frame that had a significantly higher hydraulic resistance, implying that water flow to the spike in Young was subject to higher resistance. Water flow from roots to transpiring organs is driven by root pressure and transpiration at the stomata (Lopez 2017). It is unlikely that observed differences were caused by different root pressures in Frame and Young; first because measurements of hydraulic conductance were conducted at midday, and root pressure is relatively small during the day compared to transpiration (Lopez 2017), and second because changes in root pressure would affect leaves and spikes similarly, which was not the case in this experiment, in which leaf transpiration, whole plant transpiration and water flow to the spike varied differently depending on the genotype and the treatment. This observation strengthens the hypothesis of differential stomatal regulation in the spike compared to leaves, possibly due to a better water status in the spike. In this case, water flow to the spike in Young would be limited by a higher stomatal resistance in the spike, despite the higher hydraulic conductance of the peduncle.

Reduced plant water use following combined D&H did not negatively affect water flow to the spike, although the difference in whole plant water use following heat stress in Young still negatively influenced final grain weight more in this genotype, as observed in the previous experiment (Chapter 2). Transpiration in the spike was not reduced under drought unlike leaf transpiration, suggesting differential regulation of transpiration under stress depending on the transpiring tissue, and the hydraulic conductance of the spike including spikelets was relatively insensitive to stress. Further work is needed to confirm these results and to explore the potential implications of differential transpiration in vegetative and reproductive organs.

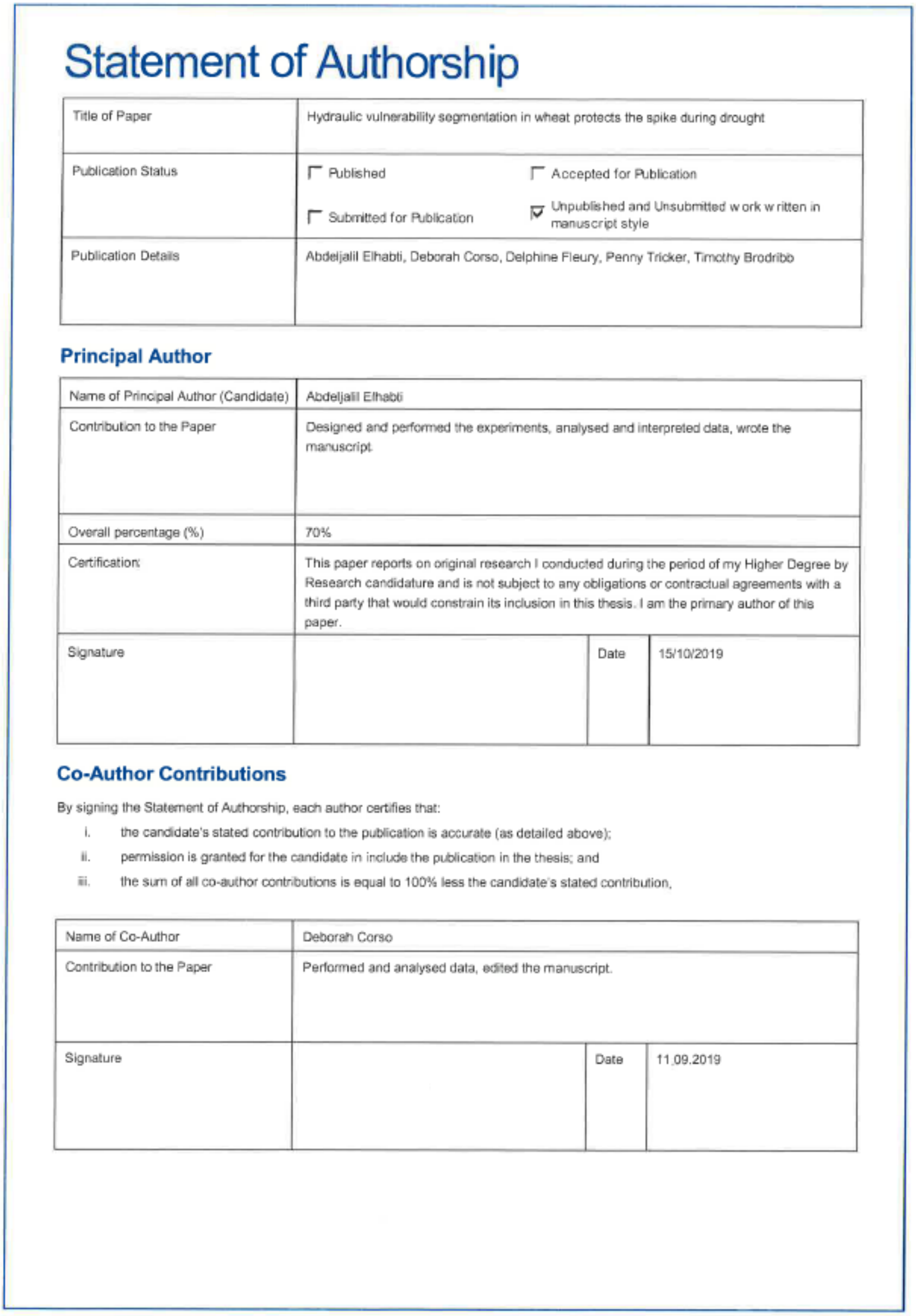
Chapter 4:

Hydraulic vulnerability segmentation in wheat protects the spike during drought

Supplementary introduction

Additional heat stress on drought altered transpiration response to vapour pressure deficit and reduced daily water use in some wheat genotypes (chapter 2). Reduction in water use did not occur in the spike as evidenced by water flow data (chapter 3), which implies that reduction in transpiration occurred at the leaf level. This apparent hydraulic protection of the spike is in accordance with the higher water status of the spike compared to leaves in wheat (Barlow 1980, Dorion 1996, Wardlaw 2002, Abbad 2004). In this chapter, we explored embolism as a potential limiting factor for water use under drought, and resistance to embolism as a mechanism that allows the peduncle to maintain water flow to the spike when water is less available.

Statement of authorship



Statement of Authorship

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iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Hydraulic vulnerability segmentation in wheat protects the spike during drought

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Summary

- Drought increasingly challenges crop production in many areas of the world. Plant water use is a key aspect of crop performance under drought. Knowledge of new traits associated with plant water use can be used to identify and breed for wheat varieties with high performance in dry environments.
- Hydraulic and vulnerability segmentation have been associated with a reduced risk of hydraulic failure in woody plants. In this work, we explored the vulnerability segmentation hypothesis in wheat using an optical method to visualise embolism in the flag leaf and the peduncle.
- We found that cavitation events in the stem peduncle consistently occurred later than in the flag leaf. This delay was associated with hydraulic and vulnerability segmentation in the plant. The peduncle had larger xylem conduits and higher wall thickness to breadth ratio, a proxy for wall collapse resistance, compared to the flag leaf.
- Hydraulic vulnerability segmentation in wheat seems to protect the spike from fluctuations in water availability by delaying embolism in the peduncle.

Key words: Cavitation, monocot, *Triticum aestivum*, embolism, optical vulnerability, conduit dimensions

Introduction

Water scarcity is increasingly straining agricultural production. Climate projections predict that droughts will be more common and more intense in the future (Sheffield & Wood, 2008), raising concerns about the damaging social and economic consequences of drought for food security. One of the response measures to protect crop yields is to develop new crop varieties adapted to the changing climate (IPCC, 2013). Improving our knowledge of the physiological mechanisms triggered by drought is essential for discovering novel traits to identify wheat varieties with improved performance in dry environments.

The ability of plants to use water effectively in dry environments is crucial for plant growth and sustained yields (Blum, 2011). Plant water uptake is driven by evaporative demand of the atmosphere creating a gradient of decreasing water potential in the soil-plant-atmosphere continuum, allowing water to flow from soil to roots through xylem vessels to transpiring organs such as leaves (Dixon, 1896). Continuity of water flow ensures maintained transpiration and photosynthesis, biomass accumulation and assimilate transport to sink tissues (Brodribb *et al.*, 2015). When soil water supply is not sufficient to sustain transpiration, increasing tension in xylem vessels ultimately results in the formation of air bubbles, or cavities, in the water column. This process, called cavitation, blocks water flow in xylem conduits, which disrupts plant water relations and impairs many physiological processes such as photosynthesis, assimilate transport and grain filling (Brodribb *et al.*, 2015). Embolism repair, also called xylem refilling, has been proposed as a mechanism allowing plants to avoid the long-term damage of xylem cavitation. However, this process may not occur in all plant species, suggesting that cavitation damage can be irreversible (Knipfer *et al.*, 2015). Another way to limit the impact of embolism on whole plant physiology is through hydraulic and vulnerability segmentation within a plant (Zimmermann, 1983; Tyree & Ewers, 1991). In woody angiosperms, differential resistance to water flow and vulnerability to cavitation cause drought-induced embolism to occur primarily in distal parts (e.g. leaves) showing higher water tension and higher vulnerability to cavitation, in contrast with high-energy parts of the plant (e.g. trunk) showing lower water tension and higher resistance to cavitation. This segmentation limits cavitation damage to distal structures and reduces the risk of hydraulic failure in the critical organs. While this segmentation of vulnerability is well-studied in perennial plants, less is known in annual plants in which reproductive structures are the most important organ.

Despite the significant implications for resistance to drought, there is no report of hydraulic or vulnerability segmentation in monocots, which is likely due to technical limitations for measuring embolism. In this work, we quantified cavitation events in the wheat flag leaf and the peduncle, the closest stem segment to the spike, using the optical vulnerability method (Brodribb *et al.*, 2016; Brodribb *et al.*, 2017). This technique has been used successfully to measure the temporal and spatial propagation of embolism in leaves and stems separately in several species, including wheat (Johnson *et al.*, 2018). As resistance to cavitation has been related to conduit dimension in some studies (Hargrave *et al.*, 1994; Brodribb *et al.*, 2016; Scoffoni *et al.*, 2017), imaging data were complemented with quantification of bundle sheath vessel dimensions. We hypothesised that hydraulic or vulnerability segmentation would favour water status in the spike to protect it from fluctuation in water availability in the rest of the plant.

Material and methods

Plant material

Single plants of bread wheat (*Triticum aestivum* L. cultivar Young) were sown in 118mm x 125mm (diameter/height) standard pots containing a mixture of 1/3 clay/loam, 1/3 UC mix and 1/3 coco peat. Six plants were grown in a glasshouse with a photoperiod of 14h, temperatures of 23°C/ 15°C (day/ night) and relative humidity of 60%-70%. Plants were well-watered until anthesis, after which plants were moved to the laboratory to perform the optical vulnerability assays.

Optical vulnerability measurements

Plants were removed from pots and most of the soil was removed to enhance drying during measurements. Roots were placed in wet paper towel to prevent initial rapid loss of moisture and paper towel was left to dry. The optical method was performed as described by Lucani et al 2019. Briefly, flag leaf and stem were secured on a flatbed scanner (Epson Perfection V700 Scanner) using microscope slides and tapes. Plants were scanned horizontally, continuously, one at a time in laboratory conditions. Scanning was done in reflective mode every 5 min at a resolution of 1200 DPI until no cavitation events were observed. The whole scanning period usually lasted for less than 3 days. Water potential was measured in the flag leaf of other tillers using a PSY1 leaf psychrometer (ICT International, Armidale, NSW, Australia) connected to the PSYPRO eight-channel water potential datalogger (Wescor, Utah, USA). If plants had additional tillers, water potential of leaf and peduncle in additional tillers was measured using a WP4 Dewpoint Potential Meter (Decagon Device, Pullman, Washington, USA). Image analysis was performed as described by Lucani et al 2019.

Microscopy images

Prior to scanning, fragments of 1 cm were sampled on the second tiller, half-way along the length of the flag leaf and 1 cm below the spike base. Tissue samples were fixed in FAA solution and stored at 4 °C until use. Stored tissue samples were washed with 50 mM sodium phosphate buffer (pH 7.0) for 5 min and embedded in 5% low-melting agarose (Bioline Reagents, London).). Transverse cross-sections 100 ~ 150 µm thick) were made using a vibratome (VT1200, Leica Microsystems, Germany). Sections were stained with 5% toluidine blue followed by three-time rinses with ddH_2O, before mounting onto glass slides with 75% glycerol. Bundle sheath anatomy was photographed at different magnifications (x40, x100, x200) using a digital camera (DS-Ri1, Nikon, Japan) attached to a light microscope (ECLIPSE NiE, Nikon, Japan).

Data analysis

Tissue means were compared using Tukey's HSD (honestly significant difference) test at p = <0.05. Statistical analyses and graphical representation were performed using R software version 3.4.4 (R Core Team, 2019).

Results

Vulnerability to cavitation

As plant biomass and the amount of soil removed from each plant varied, desiccation period differed slightly between plants, ranging from 2 to 4 days. Despite this variation, embolism consistently occurred later in the peduncle compared to the leaf (Fig. 1, 2). Time differences between first embolism events in leaves and peduncles varied depending on drying period, ranging from 8 to 26 hours. P50, the water potential inducing a 50% of total embolism, averaged -1.95 MPa in the flag leaf and -3.44 MPa in the peduncle (Fig. 3). Water potential in the peduncle was on average 0.7 MPa higher than in the flag leaf (Supp. Fig. 1).

Vessel distribution and anatomy

Vessels in the flag leaf consisted of major veins separated by three minor veins structured in one layer (Fig. 4a). In the peduncle, veins were structured in two rings, the outer layer containing minor veins and the inner layer major veins. Despite the different morphology and structure, both organs had the same number of veins, comprising 23% of major veins in leaf and 41% of major veins in peduncle (Fig. 4b). In the peduncle, both minor and major veins had clear xylem conduits, whereas in the leaf only major veins had distinct xylem conduits (Supp. Fig. 2). Compared to the leaf, the peduncle had a higher major and minor vein density, higher vein area, and higher wall thickness to breadth ratio (Fig. 4c-f).

Discussion

Vulnerability segmentation has been widely observed in various woody plants (Cochard *et al.*, 1992; Tyree *et al.*, 1993; Tsuda & Tyree, 1997; Johnson *et al.*, 2011; Charrier *et al.*, 2016), but no information is available for monocots. In this work, we report hydraulic vulnerability segmentation in monocots for the first time, in the important crop, wheat. Unlike perennial plants in which most of the energy investment lies in the trunk and branches, in wheat, an annual monocot, seeds are the final sink for assimilates captured during growth. Assimilation, biomass production, translocation of stored assimilates and sustained grain filling require a continued supply of water and are slowed when water supply is limited (Brodribb *et al.*, 2015). Our findings show that the peduncle, the closest stem segment to the spike, is less prone to embolism than the flag leaf, partly due to a more favourable water potential in the peduncle, and provide evidence of hydraulic and vulnerability segmentation in wheat. A few studies have reported a higher water potential in the spike compared to the leaf (Morgan, 1977; Saini & Aspinall, 1982; Wardlaw 2002; Neghliz *et al.*, 2016). Higher resistance to cavitation in peduncles could be a mechanism that protects the spike from fluctuation in water availability in the vegetative tissues, delaying the risk of embolism in reproductive organs and maintaining favourable water status in the spike under drought (Barlow *et al.*, 1980).

Many studies reported the correlation between vulnerability to cavitation and conduit diameter; with large vessels being more vulnerable to cavitation (Hargrave *et al.*, 1994; Brodribb *et al.*, 2016; Scoffoni *et al.*, 2017). Other studies suggested that vulnerability to cavitation was rather associated with the number and size of pit membrane pore diameters that are correlated with xylem size (Tyree & Sperry, 1989; Wheeler *et al.*, 2005; Christman *et al.*, 2009). Here we found that the wheat peduncle, which contains large vessels, was not more vulnerable to embolism compared to the leaf with smaller vessels, indicating that embolism formation was not related to conduit diameter.

Wall thickness to breadth ratio is a proxy for resistance to wall collapse (Hacke *et al.*, 2001). In woody angiosperms, high P50 - the water potential inducing a 50% loss in leaf hydraulic conductance - corresponded to high thickness to breadth ratio (Blackman *et al.*, 2010). As smaller veins were shown to be less vulnerable to cavitation than major veins (Brodribb *et al.*, 2016), the high thickness to breadth ratio of xylem conduits in minor veins, present in the peduncle but absent in leaves, suggests that resistance to wall collapse may contribute to the observed delayed embolism in minor veins.

It is noteworthy that cavitation in this study was observed in fast-desiccating plants. Such extreme conditions contrast with field conditions where the development of water stress is expected to be a longer process, and vulnerability thresholds are reached more slowly, implying that the interval between occurrence of cavitation in leaves and peduncle may be longer in the field. Limiting hydraulic failure in leaves and prolonging water flow to the spike during this interval could prove significant to maintain grain yields. The degree of hydraulic vulnerability segmentation in wheat is a potential trait for drought tolerance of wheat and other crops. Further work is needed to explore this trait on a larger sample size and on diverse genotypes for possible genetic variation.

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Author contributions

A.E., P.T., D.F. and T.B. conceived and designed the research; A.E. and D.C. performed the experiments and analysed the data; All authors contributed to writing the manuscript.

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Figure legends

Figure 1. Time course of percentage embolism in the flag leaf (light green) and the peduncle (dark green) of six individuals of *T. aestivum*.

Figure 2. Spread of embolism in the flag leaf (a,c) and the peduncle (b,d) recorded during dehydration of plant (3) (Fig. 1) using a transmission light scanner (a-b) and processed images of embolism (c-d). Time (in hours) since the beginning of dehydration is shown at the bottom left corner. See Movie for the full image sequence by clicking here.

Figure 3. Vulnerability curves of the flag leaf (light green) and the peduncle (dark green) in three individuals of *T. aestivum*, representing the relationship between percentage of embolism and the corresponding water potential. Each point represents one or several embolism events that occurred within the same period (5 min).

Figure 4. Conduit dimensions in the flag leaf and the peduncle of wheat. (a) Cross-section of the flag leaf (top) and the peduncle (bottom). (b-f) Number of vessels, vessel density, meta- and proto-xylem areas and xylem wall thickness to breadth ratio in flag leaf (light green) and peduncle (dark green). (b) Plain colour (bottom) = minor veins; light colour (top) = major veins. Values are means and standard error of four replicates. Letters indicate the results of Tukey's test comparing treatment effect within each genotype ($p < 0.05$).

Supplementary figure 1. Relationship between water potentials in the flag leaf and the peduncle.

Supplementary figure 2. Cross-section of the flag leaf (top) and the peduncle (bottom) showing one major vein and one minor vein.

Figures

Figure 1.

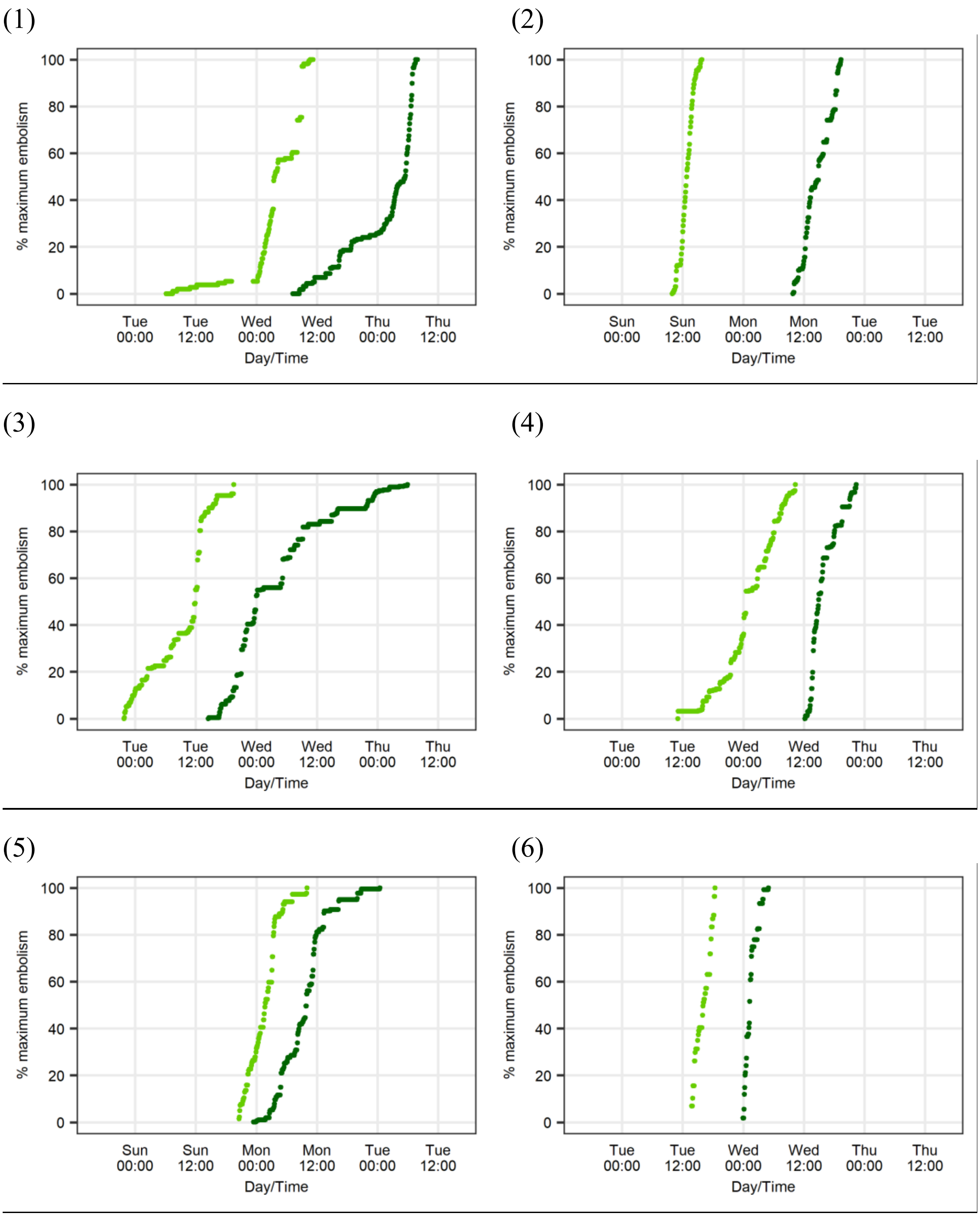


Figure 2.

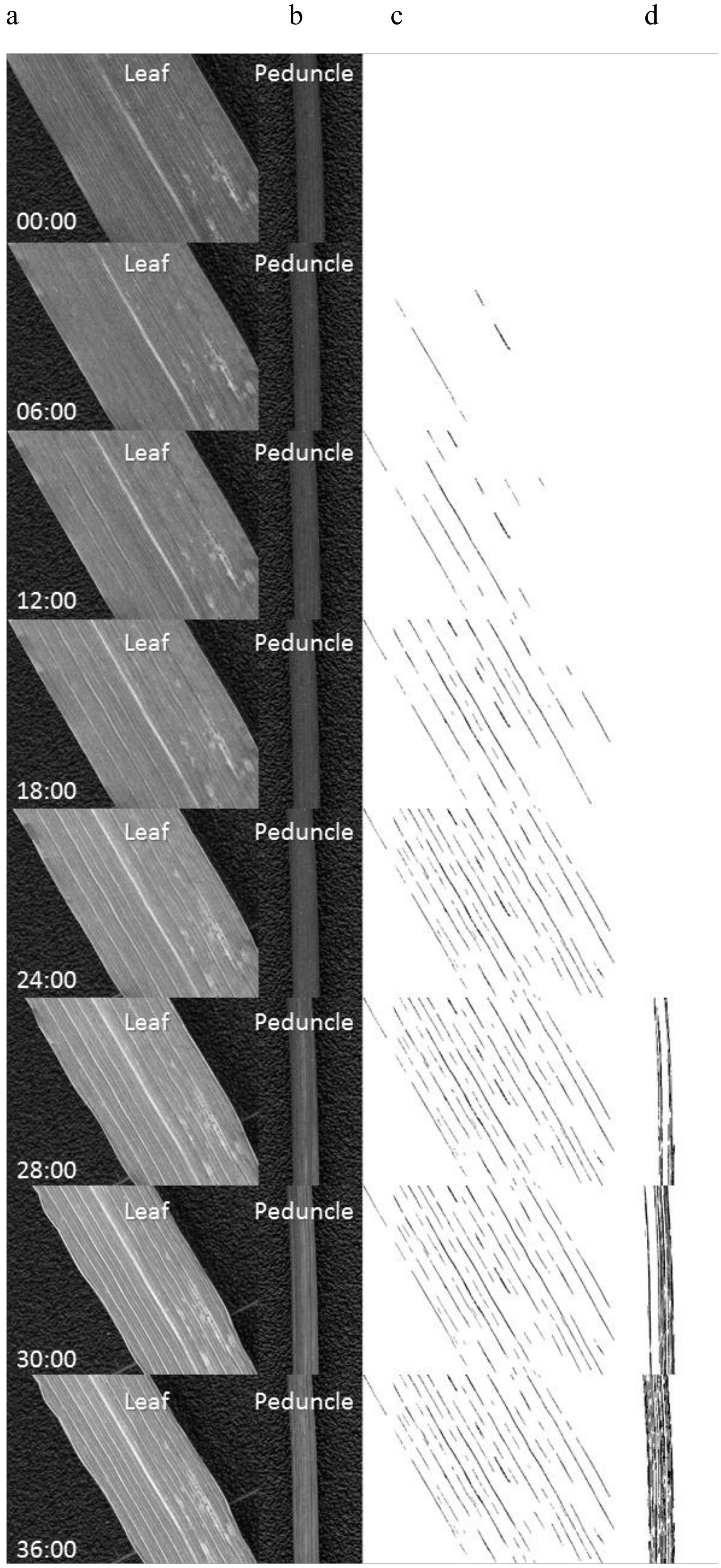


Figure 3.

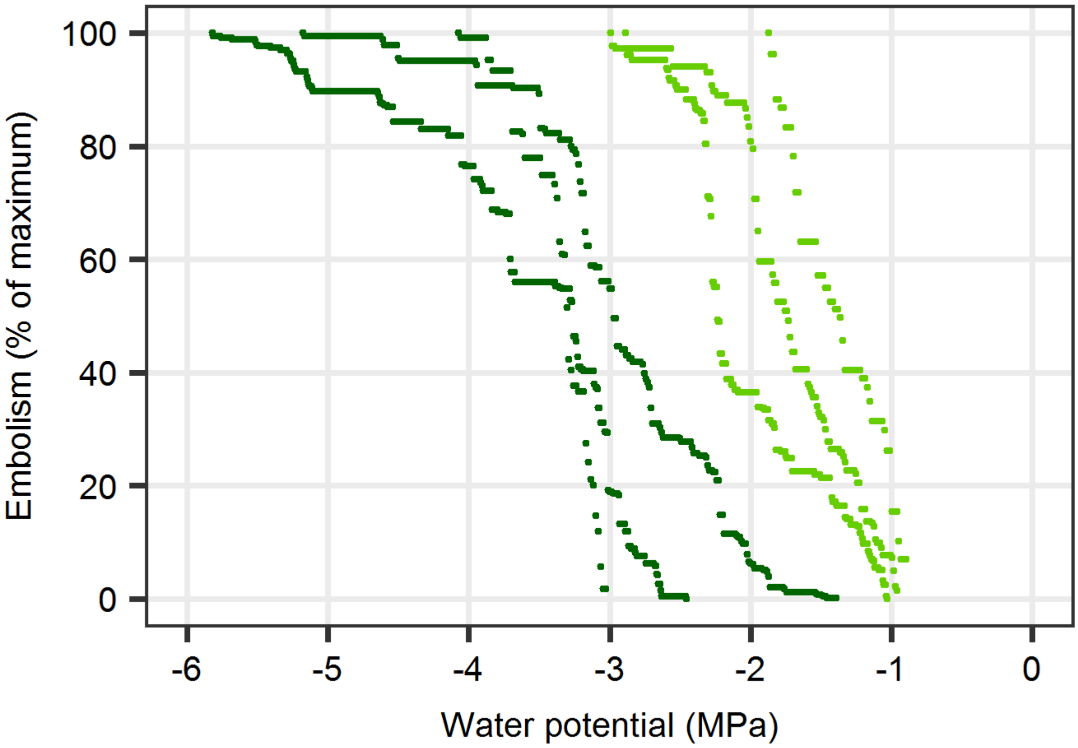
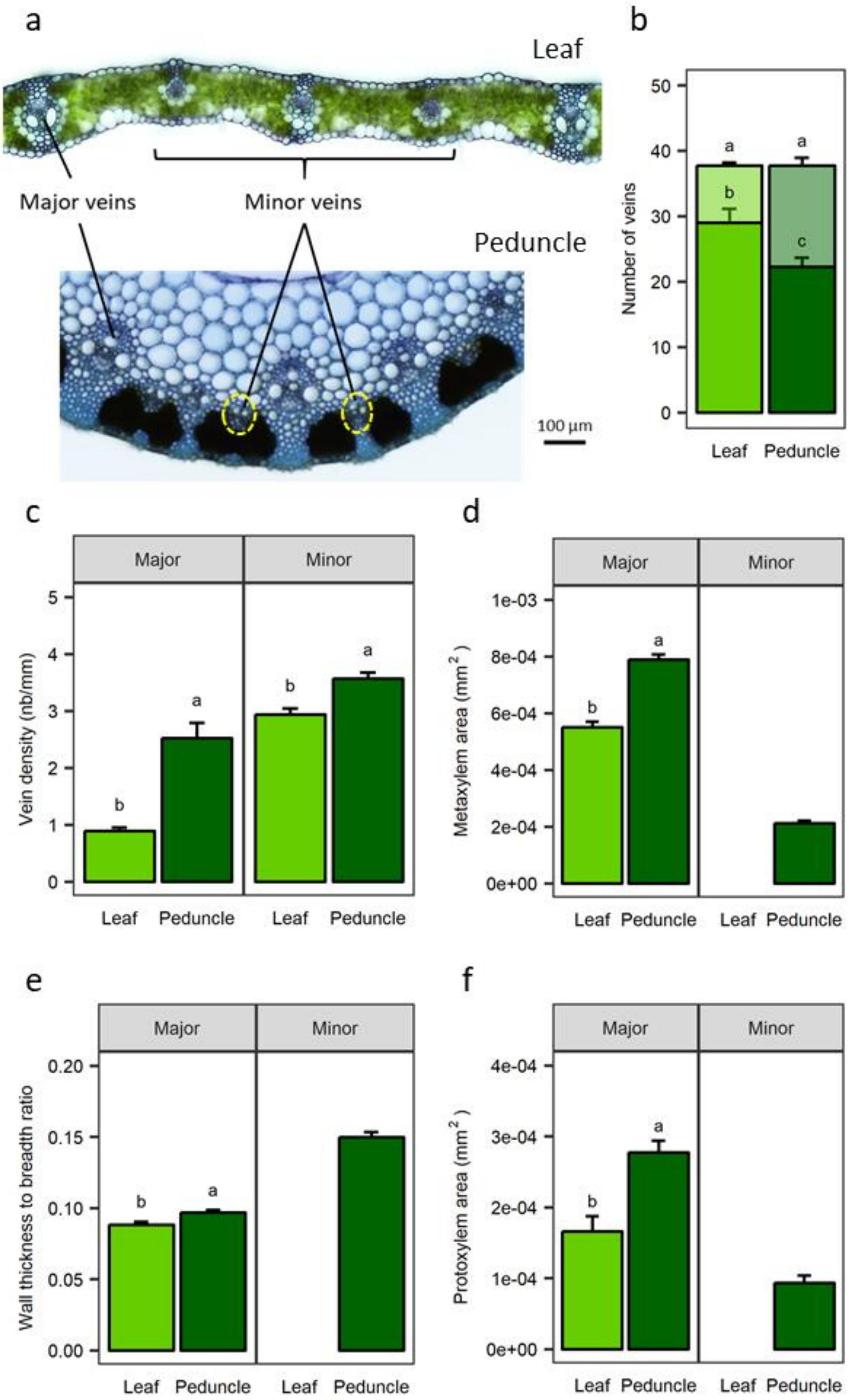
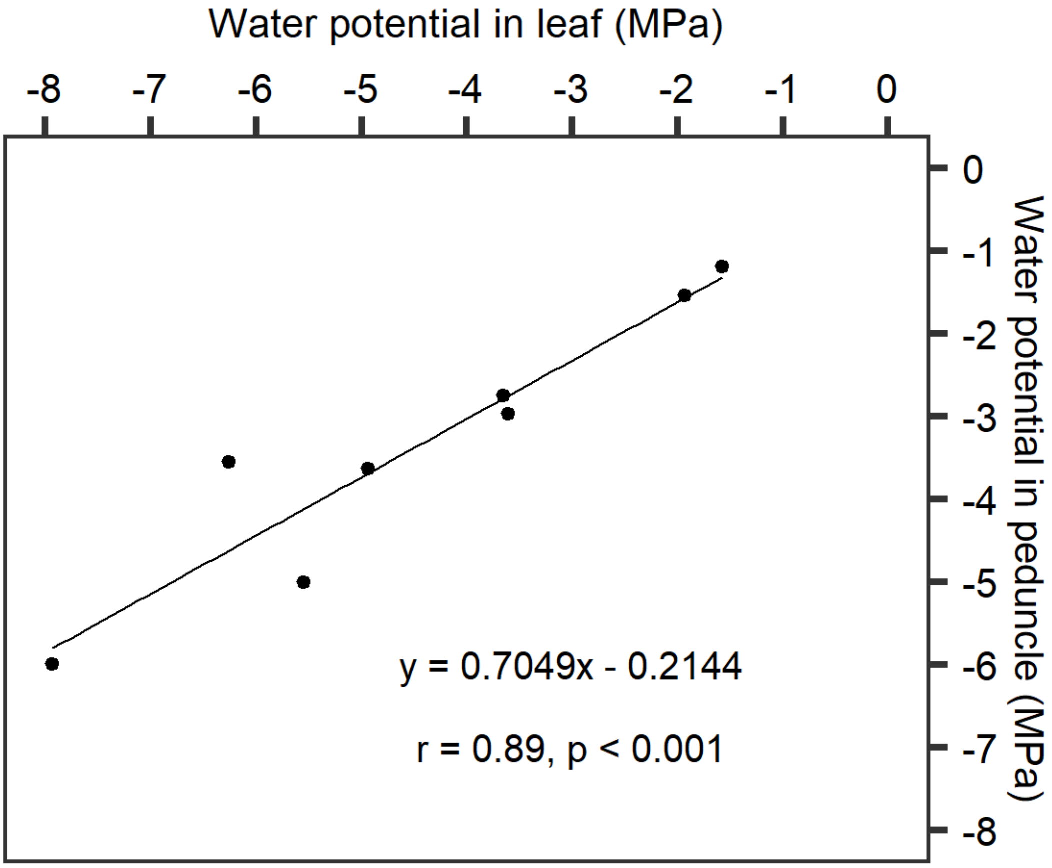

Embolism (% of maximum)
100
80
60
40
20
0
-6
-5
-4
-3
-2
-1
0
Water potential (MPa)

Figure 4.

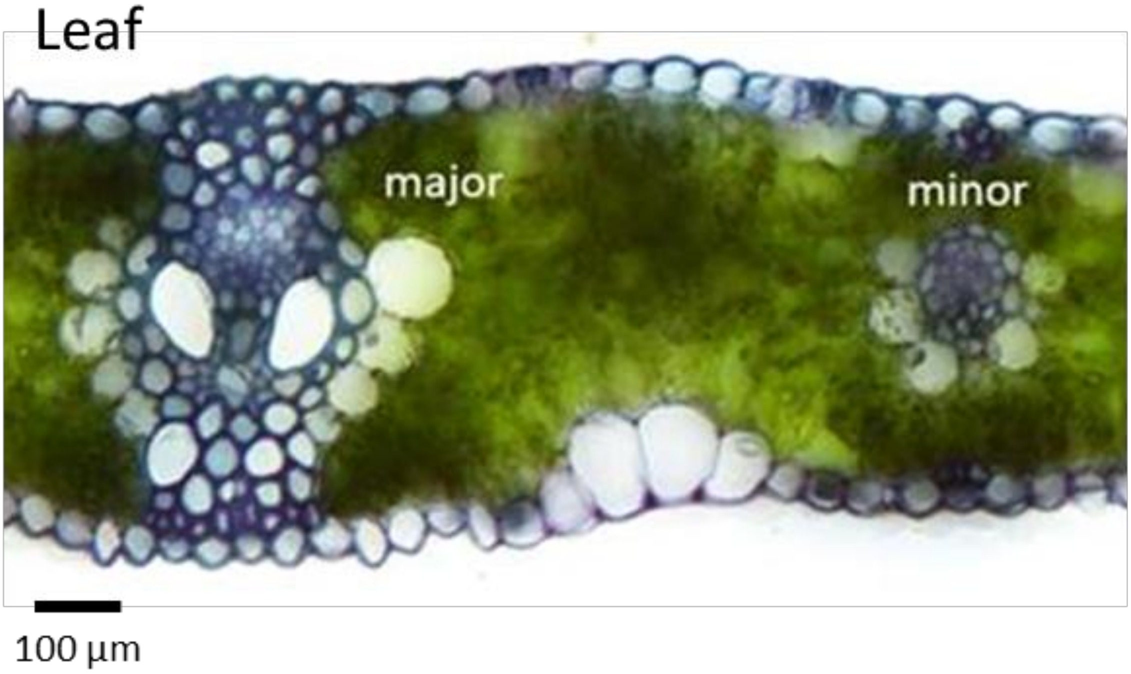

a
Leaf
Major veins
Minor veins
Peduncle
100 µm
b
Number of veins
0
10
20
30
40
50
a
a
b
c
Leaf
Peduncle
c
Major
Minor
Vein density (nb/mm)
0
1
2
3
4
5
b
a
b
a
Leaf
Peduncle
Leaf
Peduncle
d
Major
Minor
Metaxylem area (mm^2)
0e+00
2e-04
4e-04
6e-04
8e-04
1e-03
b
a
Leaf
Peduncle
Leaf
Peduncle
e
Major
Minor
Wall thickness to breadth ratio
0.00
0.05
0.10
0.15
0.20
b
a
Leaf
Peduncle
Leaf
Peduncle
f
Major
Minor
Protoxylem area (mm^2)
0e+00
1e-04
2e-04
3e-04
4e-04
b
a
Leaf
Peduncle
Leaf
Peduncle

Supplementary figure 1.

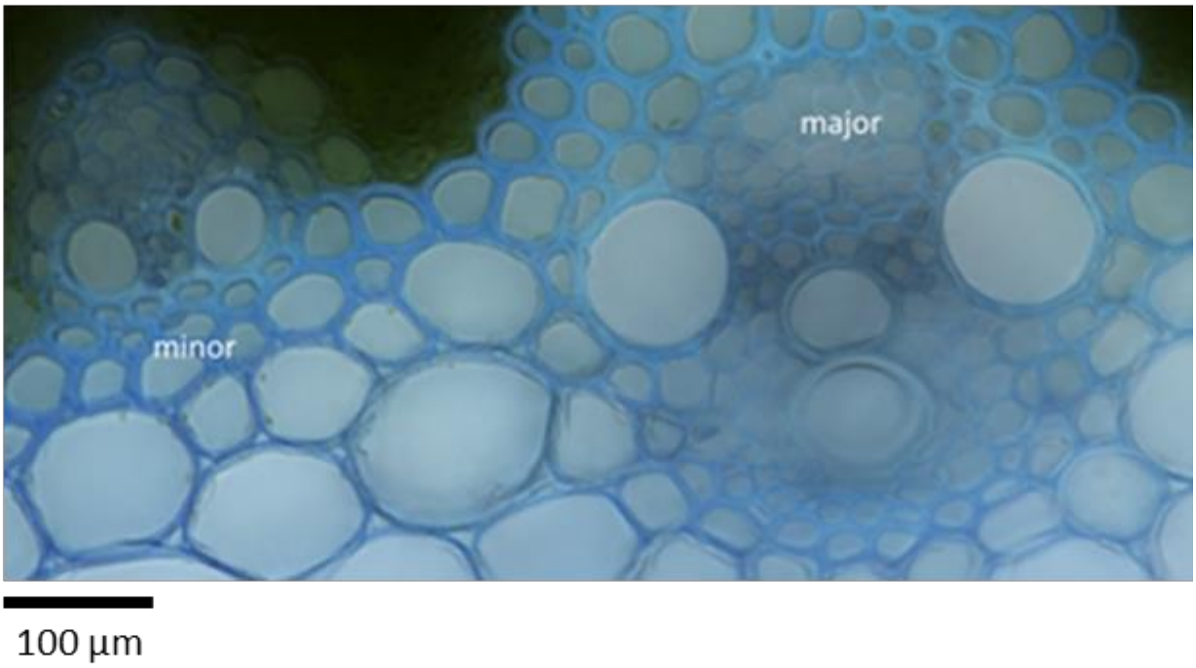


Supplementary figure 2.

Leaf



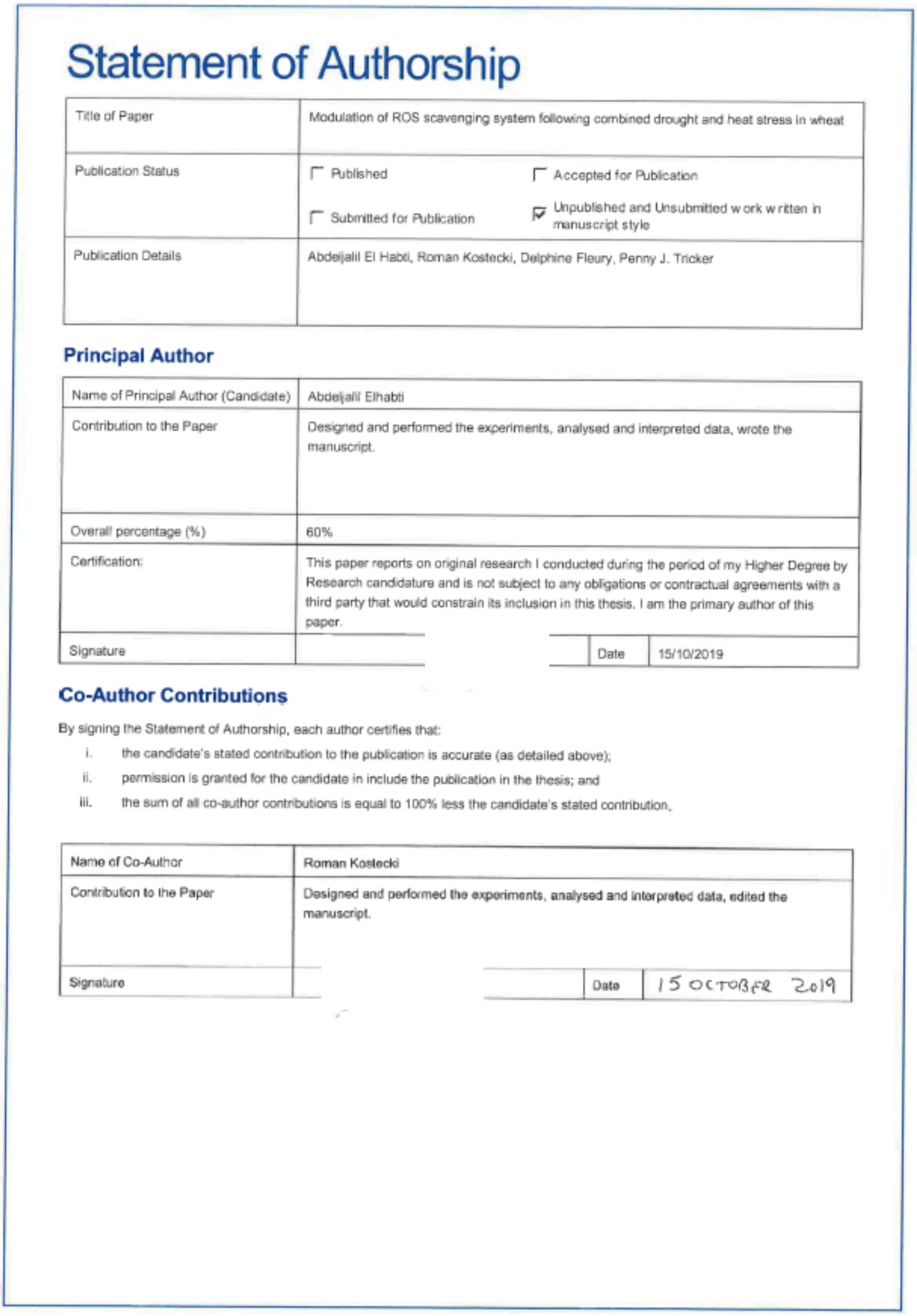
Peduncle



Chapter 5:

Modulation of the reactive oxygen species scavenging system following combined drought and heat stress in wheat

Statement of authorship



Statement of Authorship

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Contribution to the Paper	Designed and performed the experiments, analysed and interpreted data, wrote the manuscript.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	15/10/2019

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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Designed and supervised the experiments.		
Signature		Date	14/10/2019

Name of Co-Author	Penny J. Tricker		
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Modulation of the reactive oxygen species scavenging system following combined drought and heat stress in wheat

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Abstract

Reactive oxygen species (ROS) are important signals in plant response to abiotic stress. Cellular ROS concentration in leaves rises following drought and high temperatures and causes oxidative stress and damage. The ability of the ROS scavenging system to maintain redox homeostasis under stress is associated with tolerance to abiotic stress. In this work, we investigated the response of two antioxidant enzymes, superoxide dismutase (SOD) and glutathione reductase (GR), and non-enzymatic antioxidant capacity to drought and to combined drought and heat stress in diverse wheat genotypes contrasting for grain weight following stress. Unlike SOD that was not sensitive to stress, we observed a reduction in GR activity that was specific to combined drought and heat stress in some genotypes. Reduced GR activity was accompanied with increased antioxidant capacity. Induced non-enzymatic scavenging seems to compensate for reduced enzymatic scavenging under combined stress. Given the difficulty accurately measuring cellular concentrations of ROS with current assays, a novel method was developed to measure ROS concentration *in vivo*.

Introduction

Reactive oxygen species (ROS) are natural by-products of oxygen metabolism and an important component of redox homeostasis and cell signalling (Waszczak *et al.*, 2018). In plants, ROS are involved in various biological processes including development, metabolism, response to abiotic and biotic stresses and cell death (del Río, 2015). Physiological ROS concentrations are low, finely regulated by the elaborate ROS scavenging system comprised of enzymatic and non-enzymatic antioxidants that maintain a balance between ROS production and ROS scavenging. This balance can be altered when plants undergo stressful growth conditions such as drought or high temperatures. During abiotic stress, excessive oxygen free radicals lead to oxidative damage, disrupting many biological processes and potentially leading to cell death (Mittler, 2002).

Impaired redox homeostasis is one of the first signals of abiotic stress. In wheat, drought increases ROS production at different developmental stages in the leaf and the spike, induces oxidative stress and causes cellular damage (Loggini *et al.*, 1999; Sairam & Saxena, 2000; Selote & Khanna-Chopra, 2006; Cheng *et al.*, 2016; Abid *et al.*, 2018; Lou *et al.*, 2018; Nemati *et al.*, 2018; Fábián *et al.*, 2019). High ROS content is accompanied by an increase in antioxidants activity (Loggini *et al.*, 1999; Sairam & Saxena, 2000; Selote & Khanna-Chopra, 2006; Cheng *et al.*, 2016; Abid *et al.*, 2018; Lou *et al.*, 2018). At the transcript level, drought induces the increased expression of genes related to ROS detoxification (Sečenji *et al.*, 2010). Heat stress increases ROS content and membrane damage but, unlike drought, decreases enzymatic and non-enzymatic antioxidant activity in some wheat genotypes (Wang *et al.*, 2011; Narayanan *et al.*, 2015; Wang *et al.*, 2015). The degrees of cellular damage and changes in ROS scavenging activity under drought or heat stress are genotype-dependent: high antioxidant activity is generally associated with reduced oxidative stress and correlated with stress tolerance (Loggini *et al.*, 1999; Wang *et al.*, 2015; Cheng *et al.*, 2016; Abid *et al.*, 2018; Nemati *et al.*, 2018).

In several species, modulation of ROS metabolism was shown to be specifically affected by combined drought and heat stress (D&H) compared to individual stresses. Cytosolic ascorbate peroxidase 1, a ROS scavenging enzyme, specifically accumulated in *Arabidopsis* plants subjected to D&H (Koussevitzky *et al.*, 2008). In tobacco, many enzymes involved in ROS scavenging (e.g. glutathione reductase, glutathione peroxidase) were specifically induced under D&H (Rizhsky *et al.*, 2002). In citrange, limited oxidative damage following combined D&H in a tolerant genotype was associated with a higher activity of specific antioxidant enzymes (e.g., superoxide dismutase, ascorbate peroxidase) (Zandalinas *et al.*, 2017). The effect of combined drought and heat stress on ROS metabolism in wheat has not yet been explored, despite the prevalence of both stresses in wheat growing regions and the sensitivity of ROS metabolism to stress intensity. In this work, we assessed the impact of combined D&H on two antioxidant enzymes, superoxide dismutase (SOD) and glutathione reductase (GR), and on non-enzymatic antioxidant capacity in wheat flag leaves. We hypothesised that higher antioxidant enzyme activity would keep ROS concentrations low in tolerant plants. Given the difficulty measuring ROS contents in biological tissues due to their low concentration and short half-life (Queval *et al.*, 2008; Smirnoff & Arnaud, 2019), we explored the possibility of developing a method to measure oxidative stress *in vivo*. We developed optical microfibres coupled with probes for detecting H_2O_2, one of the ROS, and protein carbonyls that are generated by ROS and are widely used as markers of oxidative stress.

Material and methods

Plant material

The experimental design was the same as in chapter 2. Eight wheat genotypes (*T. aestivum* L.) were selected from a diversity panel representing 534 wheat accessions from 44 countries and all continents. The diversity panel was previously subjected to post-anthesis drought and combined drought and heat stress in a polytunnel and evaluated for grain weight. The selected genotypes contrasted for grain weight following drought and combined drought and heat stress.

Growth conditions and sampling

The growth conditions were as described in chapter 2: single plants were sown in 40 cm x 15 cm round pots containing 8.2 kg of a mixture of 1/3 clay/loam, 1/3 UC mix and 1/3 cocopeat. Plants were grown in a glasshouse (34°58'17.8"S, 138°38'23.4"E) on a gravimetric platform (Droughtspotter, Phenospex, Heerlen, The Netherlands) that automatically irrigated to the pre-defined pot weight six times daily and recorded weights hourly. Plants were well-watered (soil water potential = - 0.3 MPa, soil water content = 20 %) and grown in temperate conditions (22 °C/ 15 °C day/ night) until anthesis of the main spike. Anthesis date was the first day anthers were observed on the main spike. One third of the plants were maintained in well-watered, cool conditions until harvest. The remaining plants (D, D&H) were subject to a 6d drought treatment (soil water potential = - 0.6 MPa, gravimetric soil water content = 12 % (g/g)) starting 3d after anthesis on the main spike of each individual; this was followed, in half of these plants (D&H), by a 3d heat treatment at 37 °C/ 27 °C day/ night (n=7 for each accession in each treatment). Heat treatment was imposed in an adjacent glasshouse where plants were watered to weight manually. Drought was maintained until harvest in the D and D&H groups.

The flag leaves of three plants per genotype per treatment were sampled 12 days after anthesis (DAA) and stored at -80°C for further analysis.

Antioxidant enzyme activity

50 mg of flag leaf were ground using a Genogrinder (SPEXSample Prep, München, Germany). The obtained powder was suspended in 500 µl of extraction buffer containing 50 mM phosphate buffer (pH 7.5), 1% (w/v) polyvinylpyrrolidone (PVP), 0.5% (v/v) Triton X-100, 1 mM EDTA and a cocktail of protease inhibitors (P9599, Sigma-Aldrich). After centrifugation (15 min, 10,000 *g*), the supernatant was recovered and a second extraction was performed. The two supernatants were pooled and immediately used for enzyme activity measurements. Superoxide dismutase (SOD) activity (EC 1.15.1.1) was determined using the method of Beauchamp and Fridovich (1971). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5), 10 mM methionine, 2 µM riboflavin, 0.1 mM EDTA, 70 µM NBT and enzyme sample. Reactions were carried out at 25°C under a light intensity of ~120 µmol m^{-2} s^{-1} for 10 min. Sample absorbance was determined at 560 nm. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of the photochemical reduction of NBT. Glutathione reductase (GR) activity (EC 1.6.4.2) was determined using the method of Smith *et al.* (1988) by following the oxidation of $NADPH_2$ spectrophotometrically at 340 nm. The reaction mixture contained 50 mM Hepes-NaOH buffer (pH 7.5), 0.5 mM oxidized glutathione, 0.25 mM NADPH, 0.5 mM EDTA and enzyme sample.

Non-enzymatic antioxidant capacity

Non-enzymatic antioxidant capacity was measured according to Rice-Evans and Miller (1994). 50 mg of ground sample flag leaf was mixed in 1 mL of 80% ethanol then centrifuged at 2000 *g* for 10 minutes. The supernatant was retained. 0.1 mL of sample extract was added to 3.9 mL of DPPH 60 μM solution (in 80% ethanol). The mix was incubated in the dark at ambient temperature for 30 minutes, then the absorbance was measured at 517 nm. The percentage of inhibition was obtained using the following formula:

$$\% \text{ inhibition} = [(A0-A)/A0]*100$$

where A0 is the absorbance of the control, which is 0.1 mL of 80% ethanol in 3.9 mL of DPPH solution. A is the absorbance of the sample extract mixed with DPPH after 30 minutes.

Development of a method to measure oxidative stress using optical fibres *in vivo*

The functionalised fibre used in this experiment was developed at the Centre for Nanoscale BioPhotonics (University of Adelaide). The tip of a 600 nm fibre was coated with a fluorescent sensor (Purdey *et al.*, 2015) doped polymer that detects increases in endogenous H_2O_2 levels. Another sensor that detects increases and decreases in protein carbonyl derivative levels was made in a similar way and used later in the experiment. Sensitivity of the functionalised optical fibre was first tested in solutions of increasing concentrations of H_2O_2 or decreasing concentrations of β-hydroxybutyrate which contains a carbonyl group. The functionalised tip of the fibre was then inserted in the flag leaf, the stem and developing grains for *in vivo* measurements. All measurements were conducted on wheat plants (*Triticum aestivum* cv. Frame) at the grain filling stage. Fluorescence of the H_2O_2 sensor and protein carbonyls sensor were measured at 522 and 517nm, respectively.

Statistical analysis

Statistically significant differences were analysed by two-way ANOVA with genotype and treatment as fixed factors for enzyme activity and antioxidant capacity. Treatment means within genotypes were compared using Tukey's HSD (honestly significant difference) test at $p = <0.1$. Statistical analyses were performed using R software version 3.4.4 (R Core Team, 2019).

Results

Enzymatic and non-enzymatic activity

SOD and GR activity and non-enzymatic antioxidant capacity were measured 12 days after anthesis. SOD activity was not sensitive to stress and was similar in all genotypes, averaging 32 units/g FW (Fig. 1A, D). There was a significant interaction between genotypes and treatments for GR activity (Fig. 1B, D). GR activity was specifically decreased by combined D&H in Odessa, Synthetic W7984 and Young compared to drought only, whereas no significant effect of the treatments was observed on GR activity in the other genotypes. Non-enzymatic antioxidant capacity was increased by drought in Currawa and by combined D&H in Odessa, Synthetic and Young (Fig. 1C).

Method to measure oxidative stress using optical fibres *in vivo*

Prior to measuring H_2O_2 contents *in vivo*, the first step was to characterise the response of the functionalised fibre to increased H_2O_2 concentrations. The H_2O_2 sensor was sensitive to concentrations as low as 0.625 µM (Fig. 2A), but was not reversible i.e. did not detect decreases in ROS concentrations. A protein carbonyl sensor was chosen as an alternative to the H_2O_2 sensor for its reversible property. The sensor's response to varying β-hydroxybutyrate concentrations was first characterised (Fig. 2B). The intensity of the fluorescence peak at 517 nm was proportional to the concentration of β-hydroxybutyrate which contains a carbonyl group. The functionalised fibre was then inserted in the stem and in developing grains for *in vivo* measurements. Signals obtained from measurements in both tissues were different, a higher magnitude increase being observed in developing seeds compared to the stem (Fig. 2C).

Discussion

SOD and GR activities were measured as indicators of ROS scavenging status in the flag leaf following drought or combined D&H. SOD converts singlet oxygen to the less reactive H_2O_2 and is the first step of ROS detoxification (Supp. fig. 1). GR catalyses the reduction of glutathione using NADPH and is essential for glutathione and ascorbate recycling. Regeneration of glutathione and ascorbate, two antioxidant metabolites, is a major pathway of H_2O_2 detoxification (Apel & Hirt, 2004). Drought or combined D&H did not affect SOD activity in the flag leaf in any genotype, as has also been observed in wheat plants grown in field conditions (Kong *et al.*, 2015). Wang *et al.* (2011) reported unchanged SOD activity in the flag leaf after 6 days of a 34°C heat stress and decreased activity on the seventh day, suggesting that SOD activity may be less sensitive to stress compared to other enzymes. Alternatively, an increase in SOD activity was reported in wheat leaves subjected to drought during the vegetative stage (Selote & Khanna-Chopra, 2006; Cheng *et al.*, 2016; Abid *et al.*, 2018), suggesting that SOD may act differently depending on the developmental stage.

GR activity was not significantly affected by drought, but the additional three day-heat stress reduced GR activity, suggesting that the ascorbate-glutathione cycle might be altered by combined stress and highlighting a predominant heat stress effect on GR activity. Reduced GR activity following combined D&H was genotype-dependent, indicating genetic variation in the response of GR activity to combined D&H. This finding is in accordance with the genotypic differences observed in ten wheat cultivars among which GR activity was significantly reduced by heat stress in four cultivars (Wang *et al.*, 2015).

Interestingly, here higher non-enzymatic antioxidant capacity following combined D&H was generally observed in genotypes with reduced GR activity (Fig. 1B), which suggested a potential compensatory non-enzymatic antioxidation in response to reduced enzymatic ROS scavenging activity. This is contrary to the accepted model where we would expect lower concentrations of glutathione and ascorbate, two non-enzymatic antioxidants, when reduced GR activity inhibits regeneration (Supp. Fig 1). Differential sensitivity of SOD and GR activities and the compensatory effect of antioxidant metabolites illustrate the specific modulation of the ROS scavenging system under combined stress in wheat. Specificity of plants' response to oxidative stress under combined D&H has also been reported in other species (Rizhsky *et al.*, 2002; Koussevitzky *et al.*, 2008; Zandalinas *et al.*, 2017). In tobacco, the expression of transcripts encoding antioxidant enzymes Copper/zincCu/Zn SOD, GR, mitochondrial alternative oxidase and glutathione-S-transferase was specifically induced by combined D&H, whereas the expression of transcripts encoding antioxidant enzyme thioredoxin peroxidase, induced under drought, was similar to the control under combined D&H (Rizhsky *et al.*, 2002). In *Arabidopsis*, specific accumulation of ascorbate peroxidase 1 (APX1) proteins in response to combined D&H was accompanied by a significant increase in APX1 activity (Koussevitzky *et al.*, 2008). *Arabidopsis* mutants lacking the cytosolic APX1 gene were significantly more sensitive to combined D&H compared to the wild-type (Koussevitzky *et al.*, 2008). In two citrus species, catalase activity was specifically induced by combined D&H in citrange but was reduced in mandarin. Higher catalase activity under combined D&H was associated with limited oxidative stress (Zandalinas *et al.*, 2017). The specific sensitivity of GR and APX to combined D&H in different species suggests that the glutathione-ascorbate cycle may play a specialized role in plant response to combined D&H. This metabolic pathway involving antioxidant enzymes, GR and APX is the recycling pathway of antioxidant metabolites glutathione and ascorbate, and is essential for maintaining redox homeostasis and scavenging ROS when plants are stressed (Foyer & Noctor, 2011). Further work is needed to monitor other components of the glutathione-ascorbate cycle in response to combined D&H in wheat, for instance, APX, glutathione and ascorbate.

Reactive oxygen species are transient unstable molecules that are difficult to measure due to their short half-lives and high reactivity with other molecules. Measured ROS contents reported in the literature are highly variable due to technical inaccuracies (Queval *et al.*, 2008). Destructive ROS measurements require tissue extraction at low light and temperature, use of a chemical sensor and quantification using spectrophotometers. All these steps introduce artefacts to an already sensitive system. Optical fibres could potentially overcome these errors by bringing the optical system, usually present in the spectrophotometer, to the tissue of interest. We explored the possibility of measuring H_2O_2 *in vivo* using a fibre coupled with a sensor specific to H_2O_2. Preliminary results indicated that this system was sensitive to low ROS concentration and able to detect physiological concentrations of H_2O_2 *in planta*, but it was only able to detect increases in H_2O_2 concentrations. To overcome this limitation, a sensor specific to protein carbonyls was used due to its ability to detect both increases and decreases in protein carbonyls' concentrations. Preliminary results indicated that this sensor was able to detect changes in protein carbonyls' concentrations at high temporal resolution in different plant tissues. Further work will be conducted to optimise the use of functionalised optical fibres as sensors in biological samples; for example we are conducting measurements for a longer period to detect any diurnal variations in the signal. Although the fibre sensor diameter is in the nanoscale range, it is expected that insertion of the fibre in live tissue will induce a ROS response. Long term measurements (over weeks) using the nanophotonic sensors are currently being run and preliminary results suggest that the initial wounding response signal dissipates so that the desired biological signal is detectable. Larger experiments that include controls, replicates and treatments are envisaged. Nonetheless, this system has potential as an alternative to biochemical assays to observe ROS response to various stresses in real time, *in vivo*.

Author contributions

A.E., D.F., R.K. and P.J.T. designed the optical fibre experiment; D.F., R.K. and P.J.T. supervised the optical fibre experiment; A.E. and R.K. performed the experiments and analysed the data. A.E. wrote the article with contributions of all the authors.

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Figure legend

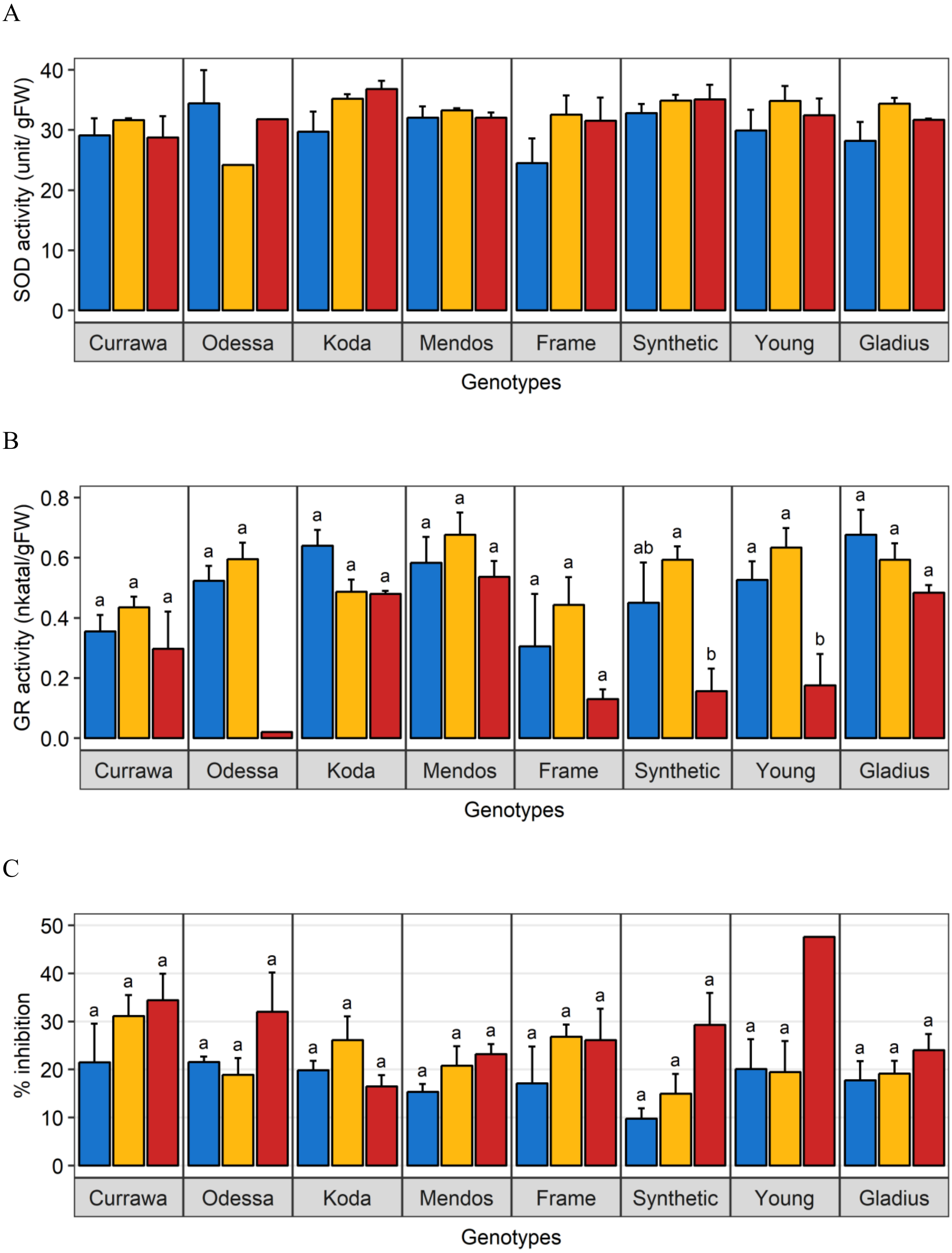
Figure 1. SOD activity (A), GR activity (B) and total antioxidant capacity (C). Plants grown in well-watered conditions (WW, in blue), drought (D, in orange) or combined drought and heat stress (D&H, in red). Values are means and standard error of three replicates ($n = 3$). Letters indicate the results of Tukey's test comparing treatment effect within each genotype ($p < 0.1$). (D) Two-way analysis of variance (ANOVA) showing the statistical significance of antioxidant activity for genotype, treatment and interaction between genotype and treatment.

Figure 2. H_2O_2 and protein carbonyls measurements using an optical fibre. (A) Peak fluorescence intensities at various H_2O_2 concentrations in solution detected with a microfibre coupled with an H_2O_2 sensor. (B) Peak fluorescence intensities at various concentrations of β-hydroxybutyrate in solution detected with a fibre functionalised with a protein carbonyl sensor. (C) Peak fluorescence intensities detected with a functionalised fibre when inserted in the stem and in a developing seed.

Supplemental Figure 1. The ascorbate/glutathione cycle and ROS detoxification process. APX, ascorbate peroxidase; CAT, catalase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; G6P, glucose-6-phosphate; G6PD, G6P dehydrogenase; MDHA, monodehydroascorbate; SOD, superoxide dismutase. Adapted from Foyer & Shigeoka, 2011.

Figures

Figure 1.

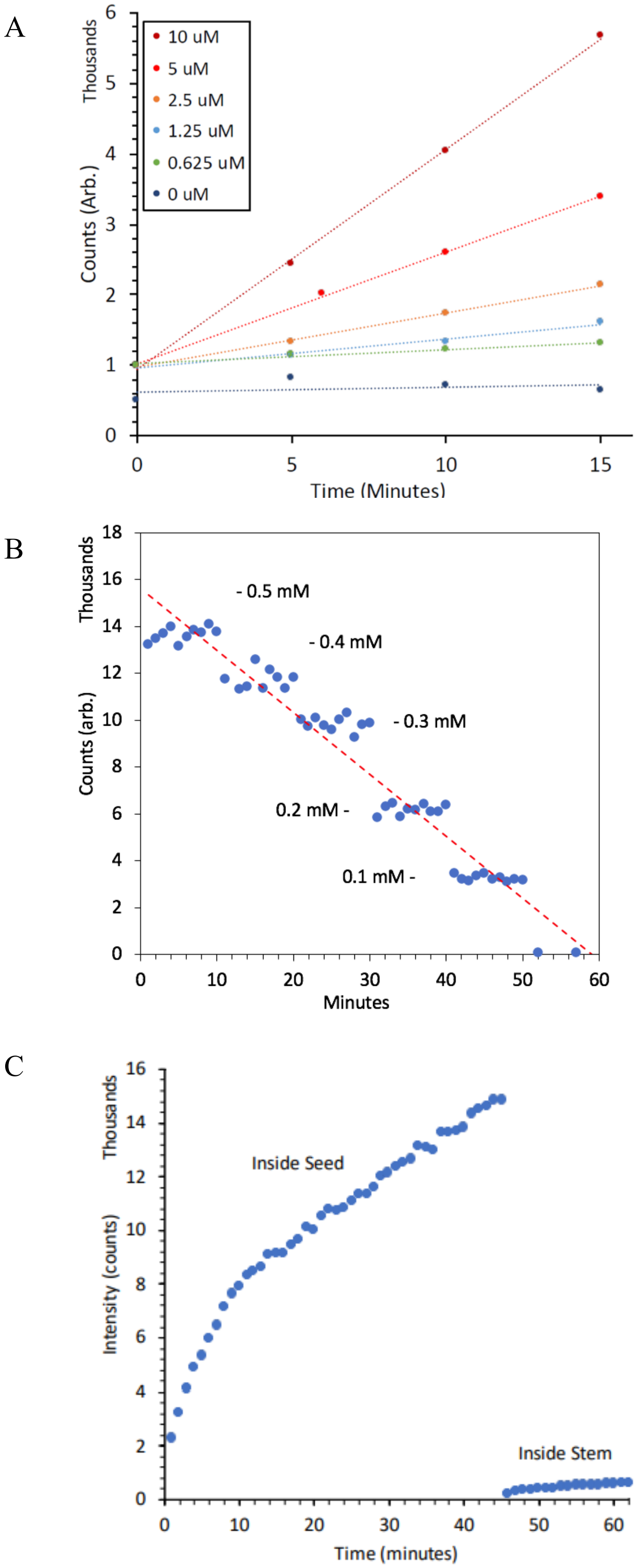


D

Measurement	Genotype	Treatment	Interaction
SOD activity	ns	ns	ns
GR activity	***	***	*
Antioxidant capacity	*	***	*

* p < 0.1; ** p < 0.01; *** p < 0.001; ns not significant

Figure 2.


A
Thousands
Counts (Arb.)
6
5
4
3
2
1
0
10 uM
5 uM
2.5 uM
1.25 uM
0.625 uM
0 uM
0
5
10
15
Time (Minutes)
B
Thousands
Counts (arb.)
18
16
14
12
10
8
6
4
2
0
- 0.5 mM
- 0.4 mM
- 0.3 mM
0.2 mM -
0.1 mM -
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10
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30
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Minutes
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Thousands
Intensity (counts)
16
14
12
10
8
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2
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Inside Seed
Inside Stem
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10
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30
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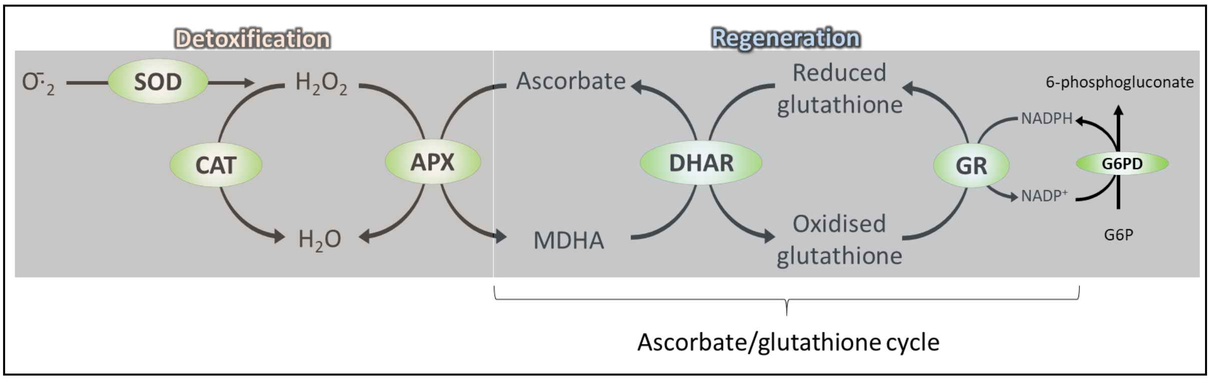
50
60
Time (minutes)

Supplemental Figure 1.



Chapter 6:

General discussion

In a context of increasing frequency and intensity of drought and heat waves, and as concerns about the damage of global warming on wheat production worldwide rise, this project aimed to improve our understanding of wheat plants' response to the simultaneous occurrence of drought and heat stress and to uncover mechanisms underlying wheat tolerance to both stresses, with the goal of identifying physiological traits for wheat tolerance to combined drought and heat stress. Literature on wheat physiology in dry or hot environments highlighted the major roles of plant water use, assimilate partitioning to grains and ROS scavenging for plant productivity. In addition, recent studies on plant response to combined drought and heat stress (D&H) revealed that some of these traits were differently affected by drought or by heat stress (Mittler 2006). Studying the combination of drought and heat stress is required to discover physiological traits for adaptation to hot and dry climates.

Monitoring plant transpiration at high temporal resolution in a diverse set of wheat genotypes allowed the discovery of an important consequence of combined D&H, that is the reduction in plant water use observed in some genotypes (Chapter 2). The experiment clearly demonstrated that differences observed in transpiration response to vapour pressure deficit were the consequence of altered plant ability to use water after a short heat stress in plants already experiencing drought. The results also showed that plants with higher grain weight had higher transpiration rates following combined D&H. Our finding not only emphasized the importance of maintained transpiration for plant productivity following heat stress, but also provided the timing for measuring transpiration when this trait was most informative. We propose the measurement of transpiration immediately following a heat stress as a means to identify genotypes with high productivity in hot and dry environments. This data could be complemented with data on senescence to provide insights on the dynamics of transpiration in relation to senescence. / biomass

To explain the variation in transpiration response to environment, we explored the hydraulic properties of the wheat spike, the peduncle and the flag leaf. Our work provided new insights into how tissue hydraulic properties were affected by drought and heat stress. Results from two genotypes contrasting for tolerance to combined D&H showed that water flow to the spike was not reduced by drought or combined D&H (Chapter 3). Higher resistance to cavitation in the peduncle compared to the flag leaf complemented the experiment and demonstrated a previously unknown hydraulic partitioning in wheat, a potential mechanism for protection of the spike (Chapter 4). It is necessary to repeat these experiments with higher number of replicates to reproduce these results, and more genotypes to confirm these observations in wheat. Future research should evaluate the relevance of vulnerability to cavitation to field conditions, as it is still unknown whether embolism is a common process in crops that is routinely overcome through refilling of embolized conduits, or whether cavitation in crops is as deleterious as it is in trees.

Measurements of water-soluble carbohydrates (WSC) in different parts of the main tiller showed that WSC partitioning differed depending on genotypes and that more recent varieties partitioned more WSC to the spike than older genotypes (Chapter 2). This raises the question on the significance of the contribution of stem WSC to grain filling compared to the spike in modern genotypes in which most of WSC are already present in the spike. If the stem is a major source of carbohydrates reserves for grain filling in hot and dry environments (Blum, 1998, Rebetzke et al., 2008), future studies should investigate the role of WSC accumulated in the spike. If the contribution of stem WSC is less important than currently thought, future studies should include measurements of WSC in the spike and relate it to grain weight as previously done with the stem. This contrasted with the commonly accepted idea that the stem is the main storing organ for WSC. This observation highlighted the importance of exploring traits in diverse genotypes in order to make sound conclusions. Measurements of WSC in grains immediately after heat stress suggested that high WSC concentration in grains was a favourable trait for high grain weight at that stage, and that WSC contents availability was a limiting factor for grain filling under stress. This experiment should be validated on a larger number of diverse genotypes given the variability of results depending on the date of release of genotypes. If these results are confirmed, it could be envisaged to develop a high-throughput method to screen for genotypes with high WSC concentration in grains at early grain filling stage.

We discovered a differential modulation of ROS scavenging enzymes following combined D&H (Chapter 5). The experiment revealed genotypic variation in the response of glutathione reductase to the combined stress only, whereas superoxide dismutase was not sensitive to drought or combined D&H. This finding encourages the exploration of the entire ROS scavenging system under combined D&H in order to find other enzymes specifically sensitive to combination of stresses. This will help in uncovering the ROS metabolic pathways altered by combined D&H and understanding of how these alterations affect ROS concentrations and redox homeostasis. We developed a new tool able to detect physiological H2O2 concentrations and to quantify oxidative stress in vivo. This tool is currently being tested on plants for diurnal measurements of oxidative stress. This technology is very promising and will help exploring this facet of plant stress biology by providing more reliable information on ROS metabolism and by measuring this trait at higher spatial and temporal resolutions.

Overall, the presented work provided new directions for research on wheat's response to drought and heat stress in particular, and plant response to environment in general. This work also demonstrated the relevance of addressing the combination of drought and heat stress as a single stress. In most of the findings, adding heat stress to drought resulted in a different response compared to either individual stress. This highlights a significant gap in knowledge, but also implies that there may be additional opportunities for trait discovery. Genetic diversity proved useful in this work; it allowed us a broad understanding of how traits varied in old and more recent genotypes and to document the effect of breeding on wheat physiology. Genotypes identified in this work as contrasting for combined drought and heat stress response will be a useful resource for further research. The difficulty in including diverse or even exotic genotypes resided in the high variation of morphological traits such as biomass and plant height that limited the interpretation of the results. Nonetheless, wheat genetic diversity is an important, still largely unexploited, source of traits involved in wheat tolerance to D&H (Reynolds et al., 2015).

In addition to the findings arising from these experiments, this work was insightful in the way research on plant response to environment, particularly drought and heat stress, can be conducted in the future. New technologies were an integral part of this work and allowed measuring traits that could not be measured previously. The Droughtspotter platform allowed continuous, real-time quantification of whole plant transpiration and identification of a key physiological trait that was specifically affected by combined D&H. The optical method described in Brodribb et al. (2017) allowed us to visualise cavitation in wheat plants and to observe different timings of cavitation events in the flag leaf and the peduncle. Temporal variation of a biological process is a critical aspect of plant physiology. Temporal measurement of a trait can be insightful but is often time-consuming and new technology can help overcome this limitation. Our work on ROS metabolism involved the unprecedented application of nanophotonics to plant physiology. Using microfibres, we were able to measure H2O2 concentrations and to quantify oxidative damage in vivo. Standard biochemical assays previously used are tedious and often produce variable results. This newly developed nanophotonic technology could not only provide accurate and reproducible data, but also allow real time monitoring of oxidative stress, which is challenging using other assays. Clearly, advances in plant physiology research in relation to abiotic stress and trait discovery depend on emerging technologies (Miflin, 2000, Sinclair, 2011, Sinclair et al., 2004, McDowell et al., 2019).

Finally, this work demonstrated that multidisciplinary research, beyond the various disciplines within plant science, was an effective way to uncover novel aspects of plant physiology. This not only involves biology but also includes physics, chemistry, hydraulics and, ecology and engineering. Plants evolved and grow in complex environments, and only by acknowledging this complexity and integrating its different aspects can researchers significantly improve their understanding of plant response to the environment. By doing so, plant physiology will progress from a tool explaining the improvements due to plant breeding, to a tool directing plant breeding for improvements (Miflin, 2000, Campos et al., 2004).

Appendix

A review paper on the current knowledge and suggested future research on the physiological and genetic basis of tolerance to combined drought and heat stress in wheat.

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Penny Tricker		
Contribution to the Paper	Wrote the manuscript. Edited the figure and table.		
Overall percentage (%)	40%		
Certification:	This paper is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	11/9/19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate;

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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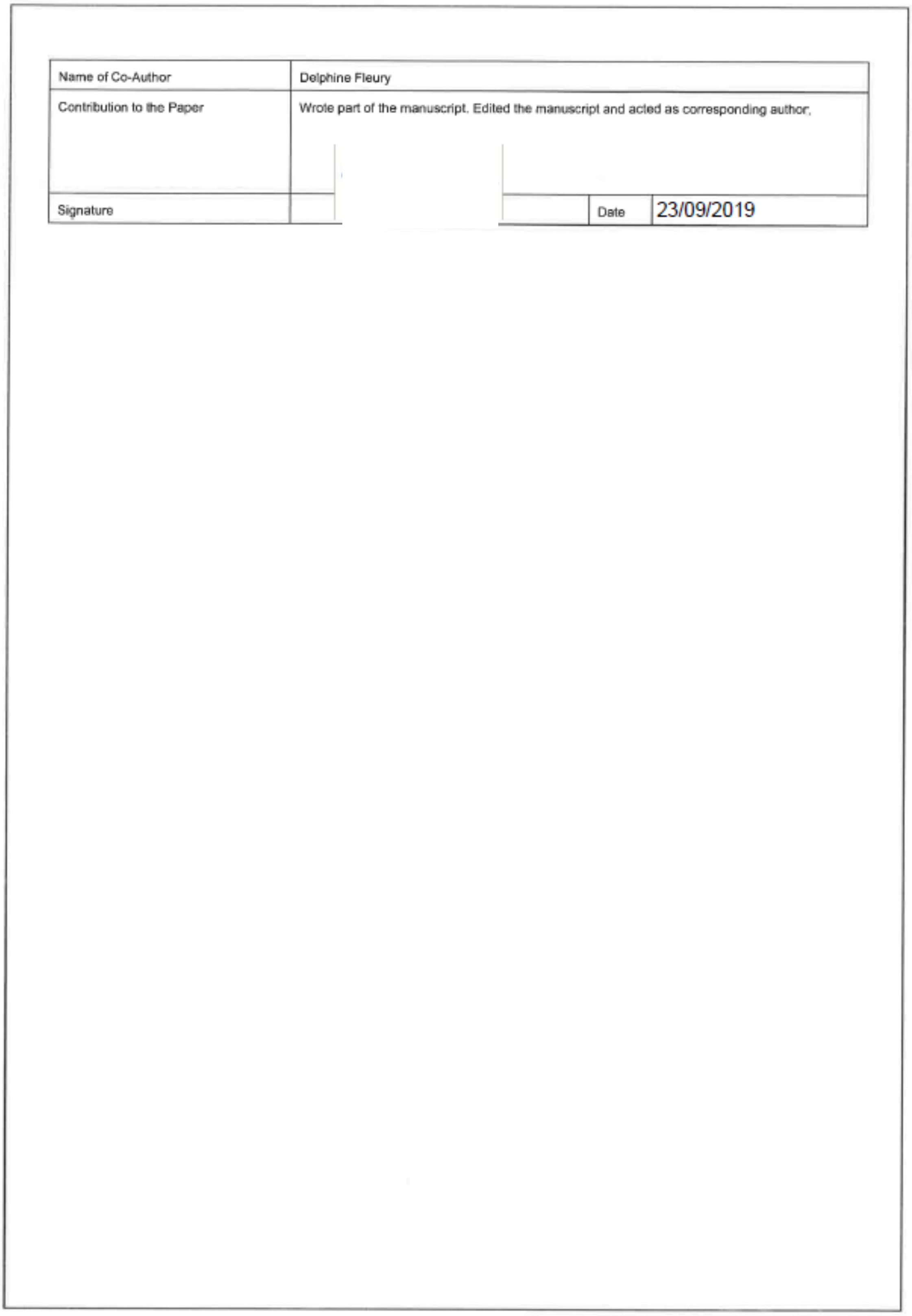

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Contribution to the Paper	Wrote part of the manuscript. Edited the manuscript and acted as corresponding author.		
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REVIEW PAPER

The physiological and genetic basis of combined drought and heat tolerance in wheat

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Abstract

Drought and heat stress cause losses in wheat productivity in major growing regions worldwide, and both the occurrence and the severity of these events are likely to increase with global climate change. Water deficits and high temperatures frequently occur simultaneously at sensitive growth stages, reducing wheat yields by reducing grain number or weight. Although genetic variation and underlying quantitative trait loci for either individual stress are known, the combination of the two stresses has rarely been studied. Complex and often antagonistic physiology means that genetic loci underlying tolerance to the combined stress are likely to differ from those for drought or heat stress tolerance alone. Here, we review what is known of the physiological traits and genetic control of drought and heat tolerance in wheat and discuss potential physiological traits to study for combined tolerance. We further place this knowledge in the context of breeding for new, more tolerant varieties and discuss opportunities and constraints. We conclude that a fine control of water relations across the growing cycle will be beneficial for combined tolerance and might be achieved through fine management of spatial and temporal gas exchange.

Keywords: Cereal, climate, stress, temperature, water, yield.

Introduction

Wheat is the major food for numerous regions around the world, providing approximately 20% of daily calories and protein for 4.5 billion people (Shiferaw *et al.*, 2013). Wheat ranks first in terms of harvested area (223.67 million hectares in 2016) and is the second most produced crop with a global production of 735.3 million tons in 2016 (USDA, 2017). A recent study predicted that wheat yields will decline by 4.1% to 6.4% for each global increase of 1 °C due to climate change (Liu *et al.*, 2016) while wheat consumption is expected to increase by over 30% in the next 40 years (Weigand, 2011). Wheat production would need to reach 858 million tons by 2050 in order to match the predicted global food demand (Alexandratos and Bruinsma, 2012).

Drought and heat are two major abiotic stresses constraining wheat productivity worldwide, causing yield losses of up to 86% and 69%, respectively (Fischer and Maurer, 1978; Prasad *et al.*, 2011). Both stresses are more likely to occur simultaneously rather than separately in semi-arid and hot growing regions in North Africa, Argentina, Mexico, Australia, South Africa, and the Mediterranean countries, and in high latitude, semi-arid growing regions of central and eastern Asia, Canada, the USA, and Kazakhstan (Mooney and Di Castri, 1973; Araus *et al.*, 2002; Pradhan *et al.*, 2012; Tricker *et al.*, 2016). Yield penalty is associated with long periods of drought coinciding with heat waves above 32 °C during heading and grain filling stages (Wardlaw and Wrigley, 1994). In

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Abbreviations: G×E, genotype by environment; GS, genomic selection; HI, harvest index; HSF, heat shock factor; HSP, heat shock protein; QTL, quantitative trait locus; ROS, reactive oxygen species; VPD, vapour pressure deficit; WU, water use; WUE, water use efficiency.

the Australian wheat belt, average daily maximum temperatures and numbers of days over 30 °C during the period of grain filling have been steadily increasing over the past three decades, and further rises are projected with climate change (ABS, 2012). The major decrease in wheat production across central Europe in the exceptionally hot summer of 2003 was likely to be due to short, but severe, heat waves during reproductive development (Wheeler, 2012). Stress tolerance is particularly critical in growing regions where the gap between attained yields and maximum yields is highest, and may have more consequence globally than where differences are lower (Tester and Langridge, 2010). Hence, producing wheat varieties with high and stable yield under these environmental stresses is one of the most important aims of breeding (Gavuzzi *et al.*, 1997; Tilman *et al.*, 2011).

Whereas responses to either drought or heat stress have been studied extensively in wheat, the combination of both environmental stresses has only recently become a matter for research. When irrigated, and with saturated atmospheric humidity (low vapour pressure deficit; VPD) at high temperatures, Australian modern wheat varieties did not show symptoms of heat stress: plants were lush and produced up to 6.8 t ha^{-1} (Parent *et al.*, 2017). This example and others demonstrate that wheat is heat tolerant when water is available. To improve wheat for dual tolerance, plants must be studied under the combination of stresses.

Overall, the combination of both high temperature and drought has a negative, additive impact on plant phenology and physiology, i.e. growth, chlorophyll content, leaf photosynthesis, grain number, spikelet fertility, grain filling duration, and grain yield (Altenbach *et al.*, 2003; Shah and Paulsen, 2003; Prasad *et al.*, 2011; Pradhan *et al.*, 2012; Perdomo *et al.*, 2015, 2017). Although responses to the two stresses share some common mechanisms, other physiological processes are antagonistic (Machado and Paulsen, 2001). For instance, combined drought and heat stress decreases leaf chlorophyll content by 49% while drought or heat alone reduce it by 9% or 27%, respectively (Pradhan *et al.*, 2012; Awasthi *et al.*, 2014). This early senescence of green tissues affects the total amount of carbohydrates transported to the grains and final grain weight. Delayed senescence, a stay-green phenotype, has been associated with drought tolerance (e.g. Pinto *et al.*, 2010) and with heat tolerance in experiments using irrigation (e.g. Shirdelmoghanloo *et al.*, 2016) where water reserves are available and accessible in deep soils for continued water use and transport of assimilates to grains post-anthesis (Reynolds *et al.*, 2005; Christopher *et al.*, 2008). In contrast, a stay-green phenotype is unlikely to contribute to combined drought and heat tolerance where no water reserves are available for continuous water use and might exacerbate the combined stress.

Although plants' responses to the combination of drought and heat have been described (reviewed in Zandalinas *et al.* 2018), few models or explanations are proposed for the physiological traits underlying combined tolerance (Pinto and Reynolds, 2015), and very little is known about genes and loci underlying these physiological mechanisms in wheat. Quantitative trait loci (QTLs) for drought and heat tolerance have, to date, mostly been reported for low-yield field environments where stress is present (such as the mega-environments 1 and 4 defined by CIMMYT, http://wheatatlas.org/), but not controlled and often not measured (Table 1). Complex interactions between QTLs and environments exist that may limit the usefulness of a particular allele. For example, using multi-environment analysis, Bonneau *et al.* (2013) showed that alternative parental alleles of a major QTL for yield in dry and hot environments (*qDHY.3B*) were positive, depending on the severity of the water deficit, soil depth, and co-occurrence with high temperatures.

A greater understanding of the physiology underlying combined drought and heat tolerance should enable researchers and breeders to discriminate between traits and loci useful for improvement. With improving genomic resources and high-throughput phenotyping methods, it becomes possible to identify loci and genes for tolerance and incorporate favourable alleles into breeding programmes. In this review, we outline what is known in wheat of the physiology and genetic variation underlying drought and heat tolerance – defined here as the ability to maintain yield under stress. We propose traits to measure in genetic mapping populations that are likely to prove beneficial for combined tolerance (Fig. 1) and discuss opportunities and constraints for incorporating alleles into breeding for tolerant wheat.

Wheat growth, architecture and biomass partitioning under drought and heat

Water deficit and high temperature affect every aspect of wheat growth from germination to maturity. The impact on yield components depends on the duration and the severity of the stress as well as the stage of plant development when stress occurs (Salter and Goode, 1967; Barnabás *et al.*, 2008; Parent *et al.*, 2017). As water stress reduces plant growth through reduced tillering and leaf expansion (Acevedo *et al.*, 1971), and high temperature accelerates plant growth and shortens developmental stages (Parent and Tardieu, 2012), under combined stress plants flower earlier and produce less biomass than under single stress. Reproductive organs are especially sensitive to drought and heat stress (Stone and Nicolas, 1995; Saini and Lalonde, 1997). Episodes of drought and heat stress around anthesis severely reduce the final number of grains per spike by more than either individual stress due to an increased abortion of ovules (Asana and Williams, 1965; Hochman, 1982; Saini and Aspinall, 1982; Pradhan *et al.*, 2012; Weldearegay *et al.*, 2012). During grain filling, combined drought and high temperature, as frequently occur in major growing regions, reduce the size and weight of individual grains by reducing the division rate of endosperm cells and shortening the duration of grain filling (Jenner, 1994; Barnabás *et al.*, 2008; Prasad *et al.*, 2011; Pradhan *et al.*, 2012).

Complex source–sink interactions underlie tolerance to drought and heat stress, and remobilization of stored assimilates to grain filling following stress at sensitive periods is dependent on sink strength. In maize, grain size, determining

Table 1. *QTL identified in wheat under combined dry and hot conditions, drought or heat stress*

Trait	Chromosome	References
Combined dry and hot conditions		
Grain yield	1AL, 1B, 1D, 2A, 2BL, 3A, 3B, 4AL, 4B, 5A, 6A, 6B, 7A, 7B, 7D	Kirigwi *et al.* (2007)[a], Maccaferri *et al.* (2008)[a,b], Pinto *et al.* (2010)[a], Golabadi *et al.* (2011)[a], Bennett *et al.* (2012)[a], Merchuk-Ovnat *et al.* (2016)[a], Tahmasebi *et al.* (2017)[a]
Thousand grain weight	1D, 2B, 3A, 3B, 4A, 6A, 7A, 7B, 7D	Pinto *et al.* (2010)[a], Golabadi *et al.* (2011)[a], Bennett *et al.* (2012)[a], Tahmasebi *et al.* (2017)[a]
Kernel weight index (large grains–all grains)	1A, 2B, 6A	Pinto *et al.* (2010)[a]
Grain weight spike^{-1}	5B, 6A, 7B	Golabadi *et al.* (2011)[a]
Grain number m^{-2}	1B, 2A, 3B, 3D, 4AL, 6B, 7A	Kirigwi *et al.* (2007)[a], Pinto *et al.* (2010)[a], Bennett *et al.* (2012)[a]
Grain number spike^{-1}	2B, 7B	Golabadi *et al.* (2011)[a], Tahmasebi *et al.* (2017)[a]
Harvest index	1B, 2A, 2B, 3B, 4A, 5A, 5B, 6A, 6B, 7B	Peleg *et al.* (2009)[d], Golabadi *et al.* (2011)[a]
Spike weight	1B, 2A, 4A, 6A, 7A, 7B	Peleg *et al.* (2009)[d], Golabadi *et al.* (2011)[a]
Spike number m^{-2}	2B, 4AL, 5B	Kirigwi *et al.* (2007)[a], Golabadi *et al.* (2011)[a]
Spike harvest index	2B, 3B	Golabadi *et al.* (2011)[a]
Spikelet number spike^{-1}	5A	Tahmasebi *et al.* (2017)[a]
Biomass	2BS, 4AL, 4B, 5A, 7AS	Kirigwi *et al.* (2007)[a], Peleg *et al.* (2009)[d], Merchuk-Ovnat *et al.* (2016)[a]
Plant height	1A, 1B, 2BL, 3AL, 3BS, 4A, 4B, 5A, 7AS	Maccaferri *et al.* (2008)[a,b], Pinto *et al.* (2010)[a], Tahmasebi *et al.* (2017)[a]
Shoot length	2B, 3B, 4A, 4B, 6B, 7A, 7B	Peleg *et al.* (2009)[d]
Peduncle length	3A, 3B	Bennett *et al.* (2012)[a]
Flag leaf width	2B, 3B	Bennett *et al.* (2012)[a]
Days to heading	1A, 1B, 1D, 2AS, 2BS, 2BL, 3A, 3B, 4AL, 4B, 4D, 5A, 6A, 7AS, 7BS, 7D	Kirigwi *et al.* (2007)[a], Maccaferri *et al.* (2008)[a,b], Peleg *et al.* (2009)[d], Pinto *et al.* (2010)[a], Merchuk-Ovnat *et al.* (2016)[a], Ogbonnaya *et al.* (2017)[a], Tahmasebi *et al.* (2017)[a]
Days to maturity	1A, 1D,5A, 7B, 7D	Pinto *et al.* (2010)[a], Tahmasebi *et al.* (2017)[a]
Days from heading to maturity	1B, 2B, 4A, 4B, 5A, 5B, 7A, 7B	Peleg *et al.* (2009)[d]
NDVI at the vegetative stage	1B, 3B, 4A, 7A	Pinto *et al.* (2010)[a], Bennett *et al.* (2012)[a]
NDVI at the grain filling stage	1B, 1D, 2A, 2B, 4A, 4B, 5A, 6A, 6B, 7A, 7B	Pinto *et al.* (2010)[a]
Stem WSC	1A, 1B, 3A, 3B, 4A, 6D	Pinto *et al.* (2010)[a], Bennett *et al.* (2012)[a]
Grain fill rate	4AL	Kirigwi *et al.* (2007)[a]
Grain fill duration	4AL	Kirigwi *et al.* (2007)[a]
Canopy temperature at the vegetative stage	1B, 2B, 3B, 4A, 4B, 6B, 7A	Pinto *et al.* (2010)[a], Tahmasebi *et al.* (2017)[a]
Canopy temperature at the grain filling stage	1A, 1B, 2B, 3B, 4A, 5A, 6B, 7A	Pinto *et al.* (2010)[a]
Canopy temperature depression	1A, 2A, 2B, 3A, 3B, 4A, 4B, 5A, 5B, 6A, 6B, 7A, 7B	Diab *et al.* (2008)[a]
Flag leaf rolling	1A, 2A, 2B, 4B, 5A, 5B, 6B, 7A, 7D	Peleg *et al.* (2009)[d], Tahmasebi *et al.* (2017)[a]
Early vigour	2B, 2D, 3B, 4A	Bennett *et al.* (2012)[a]
Early ground cover	6AS	Mondal *et al.* (2017)[a]
Chlorophyll content	1A, 1B, 3A, 4A, 4B, 4D, 5A, 5B, 6A, 6B, 7A	Diab *et al.* (2008)[a], Peleg *et al.* (2009)[d], Bennett *et al.* (2012)[a]
Chlorophyll fluorescence	1A, 1B, 2A, 2B, 3A, 3B, 4A, 4B, 5A, 5B, 6A, 6B, 7A, 7B	Diab *et al.* (2008)[a]
Carbon isotope discrimination	1B, 2A, 2B, 3A, 3B, 4A, 4B, 5A, 5B, 6B, 7A, 7B	Diab *et al.* (2008)[a], Peleg *et al.* (2009)[d]
Photosynthetically active radiation	1A, 1B, 2B, 3A, 3B, 4A, 4B, 5A, 5B, 6A, 6B, 7A, 7B	Diab *et al.* (2008)[a]
Stomatal density	4AS, 5AS, 7AL	Shahinnia *et al.* (2016)[a]
Stomatal index	2BL, 7BL	Shahinnia *et al.* (2016)[a]
Stomatal aperture area	7AL	Shahinnia *et al.* (2016)[a]
Stomatal aperture length	2BS, 2BL, 7AL	Shahinnia *et al.* (2016)[a]
Guard cell length	1AS, 3BL, 7AL	Shahinnia *et al.* (2016)[a]
Guard cell area	1BL, 4BL, 5AL, 5DL	Shahinnia *et al.* (2016)[a]

Table 1. *Continued*

Trait	**Chromosome**	**References**
Transpiration efficiency	1A, 1B, 2B, 3A, 3B, 4A, 4B, 5A, 5B, 6A, 6B, 7A, 7B	Diab *et al.* (2008)^{a}
Leaf relative water content	1B, 2B, 3A, 3B, 4A, 4B, 5A, 5B, 6A, 6B	Diab *et al.* (2008)^{a}
Water index	1A, 1B, 2A, 2B, 3A, 3B, 4A, 4B, 5A, 5B, 6A, 6B, 7A, 7B	Diab *et al.* (2008)^{a}
Leaf osmotic potential	2A, 2B, 3A, 3B, 4B, 5A, 5B, 6B	Peleg *et al.* (2009)^{d}
Osmotic adjustment	1A, 3A, 3B, 4A, 7A	Diab *et al.* (2008)^{a}
Metabolites (mQTL)	2B, 4A, 5A, 7A, 7D	Hill *et al.* (2015)^{a}
Expression of stress-related genes (eQTL)	6BL	Aprile *et al.* (2013)^{F}
Drought stress		
Grain yield	2D, 3D, 3DL, 4AL, 4BS, 4DL, 5A, 5B, 5DL, 6B, 6D, 7AL, 7BL, 7D	Quarrie *et al.* (2005)^{a}, Czyczyło-Mysza *et al.* (2011)^{d}, Kadam *et al.* (2012)^{F}, Tahmasebi *et al.* (2017)^{a}
Grain weight spike^{-1}	1B, 1D	Xu *et al.* (2017)^{a}
Thousand grain weight	1B, 1D, 2A, 2B, 3A, 3D, 4A, 4D, 5A, 6A, 6D, 7A, 7B	Quarrie *et al.* (2005)^{a}, Dashti *et al.* (2007)^{F}, Yang *et al.* (2007)^{a}, Tahmasebi *et al.* (2017)^{a}, Xu *et al.* (2017)^{a}
Grain number m^{-2}	1B, 5B, 7D	Tahmasebi *et al.* (2017)^{a}
Grain number spike^{-1}	1A, 2A, 2B, 2D, 3A, 3B, 4A, 4B, 5A, 5B, 5D, 6A, 6B, 6D, 7A, 7B	Quarrie *et al.* (2005)^{a}, Czyczyło-Mysza *et al.* (2011)^{d}, Xu *et al.* (2017)^{a}
Harvest index	1B, 2D, 4BS, 5A	Kadam *et al.* (2012)^{F}, Xu *et al.* (2017)^{a}
Spike number plant^{-1}	1A, 2A, 2B, 2D, 4B, 5A, 7B	Quarrie *et al.* (2005)^{a}, Xu *et al.* (2017)^{a}
Spikelet compactness	6A, 7A	Xu *et al.* (2017)^{a}
Spikelet number spike^{-1}	1A, 7D	Tahmasebi *et al.* (2017)^{a}, Xu *et al.* (2017)^{a}
Sterile spikelet number spike^{-1}	7A	Xu *et al.* (2017)^{a}
Fertile spikelet spike^{-1}	2A	Xu *et al.* (2017)^{a}
Biomass	1B	Xu *et al.* (2017)^{a}
Shoot biomass	4B	Kadam *et al.* (2012)^{F}
Root biomass	2D, 4BS	Kadam *et al.* (2012)^{F}
Plant height	1B, 4B, 7D	Tahmasebi *et al.* (2017)^{a}, Xu *et al.* (2017)^{a}
Peduncle length	3B	Dashti *et al.* (2007)^{F}
Coleoptile length	6AS	Spielmeyer *et al.* (2007)^{F}
Spike length	2B, 7A, 7B	Xu *et al.* (2017)^{a}
Root length	2D, 4B, 5D, 6B	Kadam *et al.* (2012)^{F}
Growth rate	5BL	Parent *et al.* (2015)^{F}
Relative growth rate	4AL	Parent *et al.* (2015)^{F}
Inflexion point in growth curves	7DS	Parent *et al.* (2015)^{F}
Leaf expansion rate	5BL	Parent *et al.* (2015)^{F}
Inflexion point in leaf expansion curves	5BL	Parent *et al.* (2015)^{F}
Days to heading	1D, 4B, 7D	Tahmasebi *et al.* (2017)^{a}
Days to flowering	2D	Kadam *et al.* (2012)^{F}
Stem WSC at the flowering stage	1A, 1D, 2D, 4A, 4B, 7B	Yang *et al.* (2007)^{a}
Stem WSC at the grain filling stage	4A	Yang *et al.* (2007)^{a}
Stem WSC at the maturity stage	6B	Yang *et al.* (2007)^{a}
Accumulation efficiency of stem WSC	1A, 2A, 5A, 7B	Yang *et al.* (2007)^{a}
Remobilization efficiency of stem WSC	7A	Yang *et al.* (2007)^{a}
Grain filling efficiency	2A, 4B, 5A,	Yang *et al.* (2007)^{a}
Flag leaf rolling	4B, 5A	Tahmasebi *et al.* (2017)^{a}
Chlorophyll content	1B, 2B, 5B, 7A, 7B	Ilyas *et al.* (2014)^{F}, Tahmasebi *et al.* (2017)^{a}, Xu *et al.* (2017)^{a}
Flag leaf persistence	2D, 3B, 4B, 5A, 6A	Verma *et al.* (2004)^{a}
Net photosynthetic rate	6B	Xu *et al.* (2017)^{a}
Chlorophyll fluorescence	1B, 2A, 2D, 3A, 3B, 3D, 4A, 4B, 4D, 5A, 5B, 6A, 6B, 7A, 7B, 7D	Czyczyło-Mysza *et al.* (2011)^{d}
Stomatal conductance	5A	Xu *et al.* (2017)^{a}

Table 1. *Continued*

Trait	Chromosome	References
Stomatal density	5BS	Shahinnia *et al.* (2016)[F]
Stomatal index	5BS, 6DL	Shahinnia *et al.* (2016)[F]
Stomatal aperture length	2BL, 4BS, 7AS, 7DL	Shahinnia *et al.* (2016)[F]
Guard cell area	1BL, 5BS	Shahinnia *et al.* (2016)[F]
Guard cell length	1BL, 4BS, 7AS	Shahinnia *et al.* (2016)[F]
Transpiration rate	3AI, 4BL, 6D	Parent *et al.* (2015)[F], Xu *et al.* (2017)[a]
Water use efficiency	2AL, 4D	Parent *et al.* (2015)[F], Xu *et al.* (2017)[a]
Heat stress		
Grain yield	1A, 1BL, 1D, 2BS, 3A, 3BS, 3BL, 3D, 4A, 4B, 4DL, 5A, 5B, 6A, 6B, 6D, 7AS, 7AL, 7BS, 7BL	Quarrie *et al.* (2005)[a], Maccaferri *et al.* (2008)[a,b], Pinto *et al.* (2010)[a], Golabadi *et al.* (2011)[a], Bennett *et al.* (2012)[a], Paliwal *et al.* (2012)[a], Merchuk-Ovnat *et al.* (2016)[a], Ogbonnaya *et al.* (2017)[a]
Grain weight spike^{-1}	3A, 3BS, 6A, 7A, 7B	Golabadi *et al.* (2011)[a], Shirdelmoghanloo *et al.* (2016)[F], Ogbonnaya *et al.* (2017)[a]
Thousand grain weight	1A, 2A, 2B, 2D, 3A, 3BS, 3D, 4A, 4B, 4D, 5A, 5B, 5D, 6A, 6B, 6D, 7A, 7D	Quarrie *et al.* (2005)[a], Pinto *et al.* (2010)[a], Golabadi *et al.* (2011)[a], Bennett *et al.* (2012)[a], Ogbonnaya *et al.* (2017)[a], Tahmasebi *et al.* (2017)[a]
Single grain weight	2D, 3BS, 5B, 6A	Shirdelmoghanloo *et al.* (2016)[F]
Kernel weight index (large grains–all grains)	1A, 1D, 2B, 3B, 4B, 5A, 5B, 6A, 6B, 6D	Pinto *et al.* (2010)[a]
Grain number m^{-2}	1A, 1B, 1D, 3BS, 3BL, 3D, 4A, 4B, 4D, 5B, 6A, 6B, 6D, 7A	Pinto *et al.* (2010)[a], Bennett *et al.* (2012)[a]
Grain number spike^{-1}	1A, 1B, 2A, 3B, 4B, 4D, 5D, 6A, 7B, 7D	Quarrie *et al.* (2005)[a], Golabadi *et al.* (2011)[a], Ogbonnaya *et al.* (2017)[a], Tahmasebi *et al* (2017)[a]
Threshing index	1A, 1B, 5B	Ogbonnaya *et al.* (2017)[a]
Harvest index	1B, 2B, 3B, 4A, 5A, 5B, 6A, 6B, 7B	Peleg *et al.* (2009)[d]
Spike number m^{-2}	1A, 1B, 3A, 3B, 4B, 5A, 5B, 7B, 7D	Golabadi *et al.* (2011)[a], Ogbonnaya *et al.* (2017)[a]
Spike number plant^{-1}	3A	Quarrie *et al.* (2005)[a]
Spike weight	1B, 2B, 2D, 3D, 4A, 5D, 6A, 7B	Peleg *et al.* (2009)[d], Golabadi *et al.* (2011)[a], Ogbonnaya *et al.* (2017)[a]
Spike harvest index	2B, 5B, 7A, 7B	Golabadi *et al.* (2011)[a]
Spikelet compactness	1A	Tahmasebi *et al.* (2017)[a]
Spikelet number spike^{-1}	1B, 1D, 2B, 4A, 5B, 6A, 6B	Ogbonnaya *et al.* (2017)[a], Tahmasebi *et al.* (2017)[a]
Number of productive tiller	1B	Sharma *et al.* (2016)[a]
Biomass	1BL, 2BS, 7AS, 7BS	Merchuk-Ovnat *et al.* (2016)[a]
Shoot biomass	3BS, 4A, 6B	Shirdelmoghanloo *et al.* (2016)[F]
Plant height	1A, 1B, 2A, 2B, 2D, 3A, 3B, 3D, 4A, 4B, 5A, 5B, 6A, 6D, 7A, 7B, 7D	Maccaferri *et al.* (2008)[a,b], Pinto *et al.* (2010)[a], Ogbonnaya *et al.* (2017)[a], Tahmasebi *et al.* (2017)[a]
Shoot length	1B, 2B, 3A, 3B, 4A, 4B, 5D, 7A, 7B	Peleg *et al.* (2009)[d], Ogbonnaya *et al.* (2017)[a]
Peduncle length	1A, 1B, 2B, 3A, 3B, 5B, 7A	Ogbonnaya *et al.* (2017)[a]
Flag leaf length	3B, 5B	Mason *et al.* (2010)[F]
Flag leaf width	1D, 2B, 3BL, 7A, 3BL	Mason *et al.* (2010)[F], Bennett *et al.* (2012)[a]
Wax score	1B, 2A, 2B, 2D, 3A, 3B, 5A, 6A, 6B, 7B	Mason *et al.* (2010)[F], Ogbonnaya *et al.* (2017)[a]
Days to heading	1BL, 1D, 2A, 2BS, 3B, 3A, 4A, 4B, 4D, 5A, 6A, 7AS, 7BS, 7D	Maccaferri *et al.* (2008)[a,b], Peleg *et al.* (2009)[d], Pinto *et al.* (2010)[a], Merchuk-Ovnat *et al.* (2016)[a], Ogbonnaya *et al.* (2017)[a]
Days to flowering	1B, 1D, 4A, 4B, 4D, 5B	Mason *et al.* (2010)[F], Pinto *et al.* (2010)[a]
Days to maturity	1B, 1D, 2A, 2B, 3B, 4D, 5A, 5B, 5D, 6A, 6B, 6D, 7A, 7B, 7DS	Pinto *et al.* (2010)[a], Bennett *et al.* (2012)[a], Paliwal *et al.* (2012)[a], Ogbonnaya *et al.* (2017)[a]
NDVI at the vegetative stage	1B, 1D, 2B, 2D, 3A, 3B, 4A, 4D, 5A, 6A, 6B, 6D, 7A	Pinto *et al.* (2010)[a], Bennett *et al.* (2012)[a]
NDVI at the grain filling stage	1A, 1B, 3A, 4A, 4B, 5A, 5B, 6A, 7B	Pinto *et al.* (2010)[a]
Stem WSC	1A, 1B, 2D, 3A, 3BL, 5A, 5B, 6A	Pinto *et al.* (2010)[a], Bennett *et al.* (2012)[a]
Grain filling duration	1B, 1D, 2A, 2B, 2D, 3BS, 5A, 6A, 6B, 6D	Mason *et al.* (2010)[F], Shirdelmoghanloo *et al.* (2016)[F], Ogbonnaya *et al.* (2017)[a]
Canopy temperature at the vegetative stage	1A, 1B, 1D, 2B, 3A, 3BL, 4A, 4B, 5B, 6B, 7A	Pinto *et al.* (2010)[a], Bennett *et al.* (2012)[a]
Canopy temperature at the grain filling stage	1A, 1B, 1D, 2B, 3BS, 3BL, 4A, 4D, 5A, 5D, 7A, 7B	Pinto *et al.* (2010)[a], Bennett *et al.* (2012)[a]
Canopy temperature depression	7BL	Paliwal *et al.* (2012)[a]
Flag leaf rolling	1A, 2A, 2B, 2D, 3D, 4B, 5A, 5B, 6A, 6B, 7A, 7B	Peleg *et al.* (2009)[d], Ogbonnaya *et al.* (2017)[a], Tahmasebi *et al.* (2017)[a]
Early vigour	2B, 2D, 3BL	Bennett *et al.* (2012)[a]

Table 1. *Continued*

Trait	**Chromosome**	**References**
Chlorophyll content	1A, 1B, 1D, 2B, 3A, 3BS, 4A, 4D, 5A, 5B, 6A, 6D, 7A, 7B, 7D	Peleg *et al.* (2009)[d], Pinto *et al.* (2010)[a], Bennett *et al.* (2012)[a], Tahmasebi *et al.* (2017)[a]
Flag leaf persistence	1B, 1D, 2A, 3A, 3BS, 6A, 6B, 7A,	Vijayalakshmi *et al.* (2010)[c], Talukder *et al.* (2014)[c], Shirdelmoghanloo *et al.* (2016)[c]
Chlorophyll loss rate	3BS, 6BL	Shirdelmoghanloo *et al.* (2016)[c]
Chlorophyll fluorescence	7A	Vijayalakshmi *et al.* (2010)[c]
Carbon isotope discrimination	1A, 2A, 4A, 5B, 6A, 6B, 7B	Peleg *et al.* (2009)[d]
Leaf osmotic potential	2A, 3A, 3B, 5A, 5B, 6A, 6B	Peleg *et al.* (2009)
Plasma membrane damage	1D, 2B, 7A	Talukder *et al.* (2014)[c]
Thylakoid membrane damage	1D, 6A, 7A	Talukder *et al.* (2014)[c]

Dry and hot field conditions are defined using the CIMMYT mega-environments 1 and 4 (Rajaram *et al.*, 1994). NDVI, near differential vegetative index; WSC, water-soluble carbohydrates

[a] Field conditions.

[b] Trials in Italy, Tunisia and Morocco with maximum temperature at grain filling ≤26.1 °C.

[c] Controlled conditions.

[d] Semi-controlled conditions.

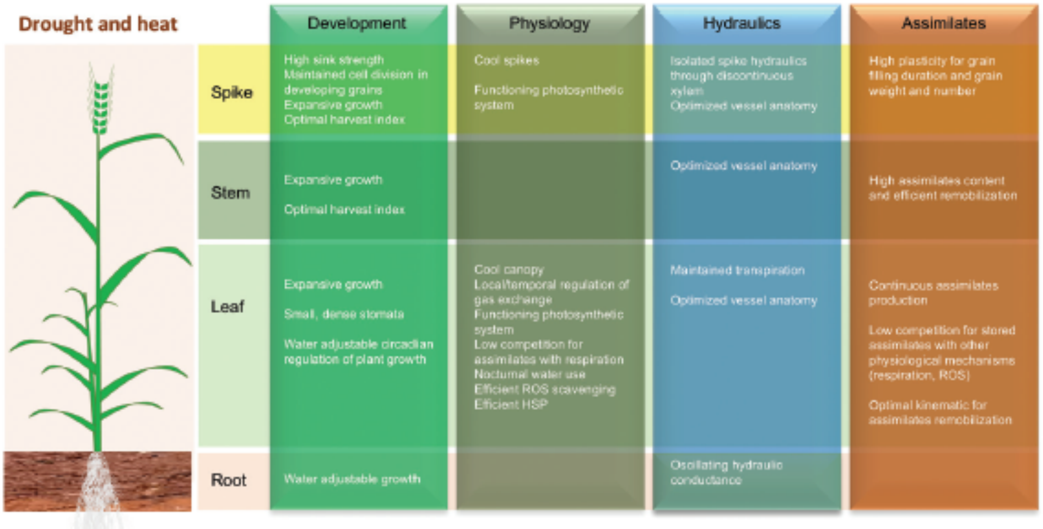
sink strength for grain filling, is determined by expansive plant growth, which is the increase in volume due to water entry into growing cells (Tardieu *et al.*, 2014). There is limited evidence for differences in carbon metabolism or status in ovules under stress, but many studies demonstrate reductions in organ elongation rates at sensitive periods with either drought or heat stress. In maize, silk growth and leaf elongation rate are highly correlated (Parent and Tardieu, 2012; Tardieu *et al.*, 2014). When the *PLASTOCHRON1* (*ZmPLA1*) gene was expressed in maize, increasing the length of the cell division zone, the duration of cell division, the duration of leaf elongation, kernel number, and size were increased in field experiments under mild drought (Sun *et al.*, 2017). QTLs for organ size and growth and expansion rates have been identified in wheat under drought (Table 1) but have not been studied under combined drought and heat stress, and no studies of genetic variation for the expansive growth trait have yet been carried out. Theoretically, increased expansive growth will be beneficial for combined drought and heat tolerance where loss of grain number is due to reduction in spike growth and development. Expansive growth will increase sink strength and be beneficial for remobilization of assimilates to the grain during filling.

Traits that increase overall assimilation should increase drought and heat tolerance when partitioned beneficially to the grain. Several QTLs for harvest index (HI) have been reported (Table 1). Meta-analysis of reported QTLs for drought or heat stress revealed meta-QTLs for spike weight/density and plant height were significantly (at $P<0.1$) associated with meta-QTL regions for yield under drought or heat in wheat (Acuña-Galindo *et al.* 2015). Major clusters were located at the *Rht-B1* and *Rht-D1* dwarfing loci. Plant height restriction due to the *Rht-B1* allele increases HI and is due to gibberellin insensitivity (Peng *et al.*, 1999). In barley, exogenous gibberellin application increases sensitivity to high temperature stress (Vettakkorumakankav *et al.*, 1999), so it is possible that widely used dwarfing alleles in modern, semi-dwarf wheat varieties already contribute to heat tolerance through the gibberellin pathway. Modern, semi-dwarf phenotypes are already widely used to prevent undesirable lodging, but there are alleles that appear more or less beneficial in particular environments. For example, Wang *et al.* (2014*b*) suggested that the *Rht13* or combination of *Rht13* + *Rht8* alleles could be favourable in water-limited environments. Thus, there is scope to study and improve wheat drought and heat tolerance through the deployment of new combinations of dwarfing alleles, identification of genes controlling the gibberellin pathway, and optimization of expansive growth (Fig. 1).

Breeding for canopy temperature and evapotranspiration under drought and heat

The main mechanism wheat plants use to decrease their internal temperatures under heat stress is evaporative cooling, driven by transpiration. Under drought, plants close their stomata to avoid excessive water loss; this reduces transpiration and evaporative cooling and, as a result, drought-stressed plants display higher leaf and canopy temperatures than well-watered plants (Reynolds *et al.*, 2009). Cool canopies were always associated with better yield performance (Pinto and Reynolds, 2015). Several QTLs have been reported for canopy temperature depression under drought and heat in wheat grown in deep soils of northern Mexico (Pinto *et al.*, 2010; Pinto and Reynolds, 2015). The major QTLs on chromosome 2B were shown to be associated with root distribution, with cool canopy genotypes able to extract more water at depth under water stress due to a greater proportion of deeper roots (Pinto and Reynolds, 2015). The deep root trait was not recapitulated under heat stress alone (with irrigation) (Pinto and Reynolds, 2015). This suggested that the beneficial physiological trait conferred by the 2B QTL was not a different root system architecture or distribution *per se*, but the ability to optimize root distribution to capture water for continued cooling dependent on water distribution in the soil.

Transpiration efficiency is a ratio between biomass and transpiration, while water use efficiency (WUE) is the biomass



	Development	Physiology	Hydraulics	Assimilates
Spike	High sink strength Maintained cell division in developing grains Expansive growth Optimal harvest index	Cool spikes Functioning photosynthetic system	Isolated spike hydraulics through discontinuous xylem Optimized vessel anatomy	High plasticity for grain filling duration and grain weight and number
Stem	Expansive growth Optimal harvest index		Optimized vessel anatomy	High assimilates content and efficient remobilization
Leaf	Expansive growth Small, dense stomata Water adjustable circadian regulation of plant growth	Cool canopy Local/temporal regulation of gas exchange Functioning photosynthetic system Low competition for assimilates with respiration Nocturnal water use Efficient ROS scavenging Efficient HSP	Maintained transpiration Optimized vessel anatomy	Continuous assimilates production Low competition for stored assimilates with other physiological mechanisms (respiration, ROS) Optimal kinematic for assimilates remobilization
Root	Water adjustable growth		Oscillating hydraulic conductance	

Fig. 1. Beneficial traits for combined drought and heat tolerance in wheat.

produced per unit of water used, at the whole plant level or whole plot in the field. Carbon isotope discrimination ($^{12}C/^{13}C$ ratio) in dry matter is negatively correlated to transpiration efficiency in wheat and a surrogate for this trait (Condon *et al.*, 1990). It has been successfully used for breeding water use efficient wheat for dry regions in Australia (Condon *et al.*, 1990, 2002). Increased transpiration efficiency alone might not improve tolerance. The equation for grain yield in water-limited environments includes harvest index (HI) and water use (WU) as well as WUE (Passioura, 1977; Passioura, 1996): GY=HI×WU×WUE. The theoretical physiology underlying this relationship has been extensively explained and reviewed (Ehrler *et al.*, 1978; Araus *et al.*, 2002; Blum, 2005; Reynolds *et al.*, 2007; Fischer, 2011; Vadez *et al.*, 2014). It has been argued that, if transpiration efficiency is increased by a reduction in the transpiration term of the equation, a low intrinsic stomatal conductance and transpiration reduces growth, biomass accumulation and light interception. Therefore, selecting plants with high transpiration efficiency might select for smaller plants (Blum, 2009). When small plants are selected, sink strength is lost and fewer assimilates are mobilized to the grain. Under the combination of drought and heat, low intrinsic transpiration could, additionally, penalize evaporative cooling. Reynolds *et al.* (2007) found that carbon isotope discrimination, together with canopy temperature linked to water uptake, was associated with improved performance in drought-stressed environments. Diab *et al.* (2008) found QTLs associated with tolerance in wheat for canopy temperature depression, transpiration efficiency, water index, and grain carbon isotope discrimination in dry and hot field conditions (Table 1).

Evaporative demand, or VPD, which depends on the amount of moisture in the air and the air temperature, also plays a critical role in transpiration and transpiration efficiency. Different sensitivities of transpiration to high VPD have been found amongst wheats and its genetic control described in the Australian wheat population RAC875/Kukri (Schoppach *et al.*, 2016). Six QTLs were identified for transpiration response to VPD, with one QTL on chromosome 5A individually explaining 25.4% of the genetic variance (Schoppach *et al.*, 2016). A study of 23 Australian wheat varieties released from 1890 to 2008 showed that whole-plant transpiration rate in response to VPD was limited at VPD above a breakpoint of about 2 kPa (Schoppach *et al.*, 2016). The breakpoint and transpiration response at VPD>2 kPa were correlated with the year of release indicating that breeders, by selecting for yield in the hot and dry climate of southern Australia, selected lines with limited whole-plant transpiration rate.

Transpiration rate might also be moderated by patchy stomatal closure and the threshold for closure might differ in sensitivity between VPD and soil moisture deficit (Vadez *et al.*, 2014). In maize, the relationship between expansive growth (leaf expansion rate; LER) and stomatal conductance was rapid and linear in contrast to the relationship between LER and transpiration rate (Caldeira *et al.*, 2014*b*). Tardieu *et al.* (2014) suggest that this is because increases in biomass and in expansive growth in volume are under different genetic controls and that, under water deficit, they are uncoupled over time. Because of the dependence of transpiration efficiency on both the biomass term and VPD, transpiration response traits should be evaluated in QTL studies. To keep an optimal balance between evaporative cooling and water saving, plants with fine adjustment of transpiration should have an advantage under combined drought and heat (Fig. 1).

Temporal regulation of gas exchange

Vadez *et al.* (2014) have argued that the total plant water use over the growing season and WUE for yield depend on available water and use at critical stages. Plants can increase effective use of water by timely modifications of water uptake at critical stages. Timely modifications in stomatal conductance, transpiration, and water use might include different patterns of stomatal opening with developmental stage, time of the day, time of season, and microclimate VPD driven by differences in plant architecture.

High stomatal densities and conductance are associated with increased yield potential in both well-watered and water-limited environments (reviewed in Roche, 2015). High stomatal density could give more flexibility to the plant to adjust stomatal opening depending on the local environmental conditions and ensure continued water uptake and use under favourable conditions. For example, the Australian line RAC875, which is drought and heat tolerant, has many small stomata by contrast with the susceptible Australian variety Kukri with fewer large stomata (Shahinnia *et al.*, 2016). QTLs for stomatal size and density have been identified in dry and hot field conditions in wheat (Table 1). While no correlation was found between yield and stomatal traits in the RAC875/Kukri population, we found a locus for stomatal density and size on chromosome 7A that overlaps with QTLs for grain number per spike, normalized difference vegetation index, harvest index, and yield in the same population (Shahinnia *et al.*, 2016).

When heat stress is severe, leaf stomata will open to allow evaporative cooling despite water limitation. At very high temperatures, the photosynthetic machinery is damaged (Berry and Bjorkman, 1980) and leaf or other vegetative tissues may be sacrificed (Lohraseb *et al.*, 2017). Under combined drought and heat stress, this balance between open stomata and damaged photosynthetic machinery can become critical to allow continued assimilation and can depend on the fine spatiotemporal regulation of gas exchange. That is, continued assimilation in periods of lower stress, as temperatures rise and cool diurnally, may make a plant more tolerant (Richards *et al.*, 1986). Diurnal regulation of gas exchange will make a difference during stress exposure and circadian use of water and regulation of transpiration may both alleviate combined drought and heat stress and be a source of tolerance. A shift in transpiration to cooler times of the day could confer tolerance.

Nocturnal water use, particularly night-time transpiration, is of increasing interest for its role in sustaining sugars export at night (Marks and Lechowicz, 2007) and its potential role in drought tolerance in wheat (Schoppach *et al.*, 2014; Resco de Dios *et al.*, 2016; Sadok, 2016). Genotypic variation for night-time transpiration and its sensitivity to VPD has been documented in wheat and influences the next day's gas exchange under normal conditions and drought (Schoppach and Sadok, 2013; Schoppach *et al.*, 2014; Claverie *et al.*, 2017). Night-time transpiration rate in response to VPD varied consistently with the sensitivity of the genotypes to drought and increased under soil water deficit (Claverie *et al.*, 2017). The effect of night-time temperature was also significant, with an increase in transpiration with increasing temperature observed, as well as genotypic variation. Despite the importance of nocturnal water use for potential drought and heat stress tolerance, no genetic studies have yet been carried out in wheat and no QTLs are known. The interplay between night-time export of assimilates and day-time gas exchange is also yet to be explored. Supply and demand ratios are likely to play a role in determining assimilation and export and, as yet, no studies of circadian regulation in wheat have been carried out in plants during grain filling when grains determine sink strength. With the development of non-destructive phenotyping methods, it will become possible to collect plant data over time and examine the kinematics of plant physiology.

Optimal hydraulic conductance for drought and heat tolerance

Hydraulic conductance is a measure of the flow induced by a pressure or water potential gradient normalized to the plant/organ geometry. Caldeira *et al.* (2014*b*) proposed that circadian oscillations of hydraulic conductance accounted for fluctuating growth (leaf elongation rates) in Arabidopsis. The degree of oscillation was highly dependent on evaporative demand and water stress. High root hydraulic conductance oscillation under water deficit likely led to the ability to control water uptake in response to available soil water when needed. Soil water status regulates the root hydraulic conductance of maize (Caldeira *et al.*, 2014*a*) adjusting growth to water availability. Maintenance of high hydraulic conductance in spikes of long-awned cultivars of wheat significantly reduces spike temperature during grain filling (Maydup *et al.*, 2014). The end of grain filling correlates with a loss of hydraulic conductance at the rachis-xylem conduit (Neghliz *et al.*, 2016). Thus, we hypothesize that by maintaining optimal hydraulic conductance in the different tissues under drought and heat stress (Fig. 1), wheat plants could extend grain filling duration, cool down grain and spike, and optimize water uptake for expansive growth.

In grapevine, soil–leaf differences in water potential among genotypes were shown to be less related to sensitivity of transpiration to soil water deficit than to change in soil–leaf hydraulic conductance, likely due to rapid changes in water transport within the plant (Scharwies and Tyerman, 2017). The ability to partition and channel water between stem, leaf, tillers, and spikes determines both expansive growth in these tissues and remobilization of assimilates following stress. Differences in hydraulic resistances in different tissues influence water transport capacity and drought and heat tolerance (Coupel-Ledru *et al.*, 2014; Bramley *et al.*, 2015). Hydraulic resistance may be determined by differences in structure and architecture of stems, peduncles, and rachis, and differences in xylem vessel diameter and leaf venation (Scharwies and Tyerman, 2017). Vessel structure has an important role in the control of water conductivity in plants in water-limited environments (Tixier *et al.*, 2013; Caringella *et al.*, 2015; Kadam *et al.*, 2015). In wheat, Barlow *et al.* (1980) demonstrated that a xylem discontinuity at the base of the peduncle permitted the isolation of spike hydraulics from the rest of the plant, and that this anatomical feature was crucial during water scarcity, resulting in the independence of water relations in the spike from the rest of the plant. The xylem in wheat is also discontinuous between rachis and grains, isolating grains and, potentially, preventing water loss during stress (Zee and O'brien, 1970). Photoperiod response (*Ppd* loci) genes have pleiotropic effects on plant growth and development (Cockram *et al.*, 2007) that can modify plant hydraulics. The photoperiod sensitive allele *Ppd-D1* increases daytime and night-time transpiration while decreasing whole-plant leaf area in response to VPD increase in wheat (Schoppach *et al.*, 2016). This suggests that whole-plant hydraulics are developmentally controlled. Deciphering the relationship between vessel structure and plant hydraulics and the genetic control of plant development in wheat will provide a better understanding of the involvement of these physiological mechanisms in tolerance to combined drought and heat stress and their potential for breeding tolerant varieties.

Competition for assimilates under drought and heat stress

Redox balance is crucial for the normal function of many cellular processes. Its fine control is essential for a proper integration of environmental and developmental stimuli and signal transduction (Choudhury *et al.*, 2017). Recent studies demonstrated the important role of photorespiration in maintaining redox homeostasis (Scheibe and Dietz, 2012), mitigating oxidative stress and protecting the photosynthetic apparatus from photoinhibition (Rivero *et al.*, 2009; Peterhansel and Maurino, 2011; Voss *et al.*, 2013). With either drought or heat stress, net photosynthesis is reduced and photorespiration increased (Long and Ort, 2010), but the relative contributions of photorespiration and mitochondrial respiration to combined drought and heat stress tolerance in wheat are unknown and genetic variation for this ratio has not been explored.

Heat stress affects membrane stability and the quantum efficiency of photosystem II, which can be measured, respectively, as cell viability and chlorophyll fluorescence (Blum, 1988; Mohammed and Tarpley, 2009). Drought stress also affects chlorophyll fluorescence with a dramatic decrease of F_v/F_m ratio in susceptible wheat compared with tolerant lines (Izanloo *et al.*, 2008). QTLs have been reported for chlorophyll fluorescence in drought- or heat-stressed wheat (Table 1), but studies in other species suggest that responses to combined drought and heat stress are unique in comparison with either individual stress (Mittler, 2006). At the ecosystem level, drought may actually reduce heat-driven increases in plant respiration due to reduction in carbon substrates available (Schauberger *et al.*, 2017). However, if stored carbohydrates are used for respiration and less available for remobilization following heat stress, drought may exacerbate the effect of heat stress-induced increases in respiration. The rate of grain filling from stem reserves is increased with increasing temperature, reducing grain filling duration (Blum *et al.*, 1994). Tolerance to drought and heat stress will then depend on both the initial concentration of remobilizable carbohydrates and the use of these reserves for respiration. Genetic variation for stem water-soluble carbohydrate content has been explored with known QTLs in drought or heat stress and in combined drought and heat stress (Table 1). Yang *et al.* (2007) also investigated genotype × environment (G×E) interactions for QTLs for stem water-soluble carbohydrate content and remobilization efficiency under water stress in wheat and found significant interactions for all traits. They showed that not all reserves were translocated to grain following water stress and suggested that losses due to respiration could be significant. Zhang *et al.* (2014) explicitly investigated water-soluble carbohydrate QTLs under drought, heat, and combined drought and heat stress and were able to identify additive effects and combinations of favourable alleles for both content and remobilization, suggesting that the genetic mechanisms underlying tolerance will not depend purely on accumulation of stored carbohydrates. QTLs for respiration are now being studied in wheat for the first time under the International Wheat Yield Partnership umbrella (http://iwyp.org/wp-content/; accessed 5 February 2018).

Under prolonged stress exposure, photosynthetic activity is further inhibited by excessive accumulation of reactive oxygen species (ROS), causing damage to the membranes, proteins, and chlorophyll molecules of the photosynthetic apparatus (Price and Hendry, 1991; Jiang and Huang, 2001; Allakhverdiev *et al.*, 2008; Silva *et al.*, 2010; Redondo-Gómez, 2013; Awasthi *et al.*, 2014; Das *et al.*, 2016). Plants use a complex antioxidant system to regulate ROS levels and avoid toxicity, but changes in redox status are also perceived by plants as a signature of a specific stress that will result in a corresponding acclimation response (Foyer and Noctor, 2005; Choudhury *et al.*, 2017). The balance between accumulation of ROS in response to stress and their signalling role under stress is yet to be defined. ROS scavenging is generally induced under drought and heat stress, and higher antioxidant capacity is generally correlated with tolerance to stress (Koussevitzky *et al.*, 2008; Suzuki *et al.*, 2014; Wang *et al.*, 2014*a*). In some wheat genotypes, tolerance to drought or heat stress was associated with increased antioxidant capacity and reduced oxidative damage (Sairam and Saxena, 2000; Sairam *et al.*, 2000; Lascano *et al.*, 2001; Almeselmani *et al.*, 2006; Sečenji *et al.*, 2010; Lu *et al.*, 2017; Zang *et al.*, 2017; Zhang *et al.*, 2017). The effects of combined drought and heat on the ROS system in wheat are unknown, but recent studies highlight the importance of modulation of ROS scavenging, some pathways being specifically induced by combined stress (Rizhsky *et al.*, 2002; Koussevitzky *et al.*, 2008; Demirevska *et al.*, 2010; Zandalinas *et al.*, 2017). The alleles that regulate photorespiration, membrane stability and antioxidant capacity under drought and heat are yet to be discovered in wheat.

As genomics and phenomics advance, the ability to analyse differences in physiological traits in empirical experiments has improved. Important advances in phenotyping with imaging or other equipment mean that it is possible to, for example, measure senescence or canopy temperature in real time in fields (Araus and Cairns, 2014). Further advances that allow, for example, field-scale simultaneous measurements of gas exchange, or non-destructive measurements of water-soluble carbohydrate movement can be anticipated. For researchers, these will provide a wealth of previously unquantifiable data for physiological traits.

Breeding for stability, plasticity, and G×E interaction under drought and heat

In past breeding of tolerant varieties, efforts have been concentrated on the search for stable QTLs that show the same allelic effect across environments to produce generalist, high-yielding varieties (Eberhart and Russell, 1966). Acuña-Galindo *et al.* (2015) conducted a meta-QTL analysis of 24 genetic studies where QTLs had been mapped for drought, heat, or combined stress in wheat. Co-localization with meta-QTLs for yield was only significant (at $P<0.1$) for the maturity/date of anthesis, spike weight/density, plant height, and canopy temperature depression QTLs. This analysis underscored the pleiotropic effects of phenology and dwarfing alleles on wheat stress response. These generalist QTLs are already bred for with *Ppd* and *Vrn* alleles routinely used in marker-assisted selection. Other stress tolerance QTLs are not generalist and have strong G×E interaction.

In wheat, directional selection (Chapman *et al.*, 2012) has been used to breed varieties that respond consistently to the target environment and management practice. Whilst this approach has been successful in achieving yield gains in some tested environments, strong G×E interactions mean that it is difficult to identify genotypes responding consistently positively in a range of stressful environments, even for a single physiological trait (Reynolds *et al.*, 2009; Lopes *et al.*, 2012). When testing lines bred in high- and low-moisture and reciprocal environments at different sites, Kirigwi *et al.* (2004) found significant environment × selection regime interactions. In this study, development in alternating high-to-low or low-to-high-moisture regimes facilitated the selection of lines that performed well for yield in both, whereas lines selected in either continuous high- or continuous low-moisture regimes had lower yields in these respective environments. The authors suggested that selection under these alternating environmental conditions favoured retention of both high yield under stress and high responsiveness to water input.

In a changing environment, trait plasticity is theoretically beneficial (Bradshaw, 1965; Aspinwall *et al.*, 2015). Plasticity can be defined as the variance in genotypic response across an environmental gradient – that is the slope of its reaction to change, with a steeper slope indicating higher plasticity (Nicotra *et al.*, 2010). Plasticity can be measured as phenotype versus an environmental range for any trait and considered as a trait in itself (Sadras and Slafer, 2012), i.e. it has its own genetic variation and underlying QTLs. Phenotypic plasticity should be advantageous for fitness in variable environments and neutral in stable environments (Bradshaw, 1965; Nicotra *et al.*, 2010). It can be argued that selection for plasticity QTLs, against the background of the increased pace of climate change, will prove beneficial for maintaining or improving agricultural yields (Aspinwall *et al.*, 2015). However, plasticity is particular to the trait. For example, Sadras *et al.* (2009) found that high yield plasticity in wheat was disadvantageous in low-yield environments when it was associated with low plasticity of post-anthesis development. Breeding for plasticity in grain yield components coupled with plasticity for the length of the grain-filling phase will be useful but is limited due to a trade-off between low plasticity in grain size and high plasticity in grain number during this stage.

Many QTLs have been found for grain production in dry and hot climates (Table 1), but very few (possibly none) are used in breeding programs. The main limiting factor to the deployment of these QTLs in breeding is the inconsistency in performances of the introgressed lines due to the strong QTL×E interaction. Although only field experiments are relevant for evaluating crop tolerance to stress as performance in an agricultural system, most studies fail to explain why a QTL is significant in one environment and not in another. Field trials are usually considered as a qualitative factor, which enables detection of G×E interactions but not its measurement (Acuña-Galindo *et al.*, 2015). Recent development in phenomics and sensors means that we can now continuously measure soil water potential and air temperature across the crop cycle in field conditions. But how can we use these data to understand G×E?

Uncoupling responsive and adaptive physiological traits is often complex and disentangling the effect of a specific environmental condition is not simple in experiments and often requires complex analysis and modelling (reviewed by Parent and Tardieu, 2014). Parent *et al.* (2017) described new models that exploit such data and measure a plant's response to quantitative variations in drought and heat stress. Applied to lines that segregated for specific yield QTLs, such models revealed, in Australian wheats, that a QTL on chromosome 1B was constitutively expressed under various combinations of soil water potential and high temperature, while a QTL on chromosome 3B was heat responsive with a positive effect of the drought-tolerant parental line RAC875 when temperature was above 23 °C around flowering stage (Parent *et al.*, 2017). This information is highly valuable as it enables us to understand a QTL's function and use it in appropriate environments. By equipping national variety trials with sensors to measure soil moisture and air temperature, such models could also predict the level of tolerance of new varieties to quantified drought and heat. Combined with whole genome genotyping, this would provide information on the effects of haplotypes on yield in response to specific environmental conditions.

Find the drought and heat tolerance genes and design the genome

Another obstacle in using yield QTLs in breeding programmes is the small effect of a single QTL and the need to introgress several QTLs to gain a significant increment in yield improvement. As breeders can only recombine as many loci as the size of their breeding programmes allows, they prioritize those with strong and stable effects, such as phenology, plant height, and disease resistance, and select for yield under dry and hot environment empirically or, more recently, by genomic selection (GS). So, were the efforts in finding QTLs for drought and heat tolerance wasted? The answer is probably yes, unless we carry on the positional cloning of these QTLs and find the genes controlling combined drought and heat tolerance. Gene-level knowledge of the control of drought and heat tolerance will enable the identification and creation of new sequence variants.

Although many QTLs have been found for drought or heat tolerance (Table 1), little is known about the genes underlying these effects in wheat. The molecular network of drought and heat stress response in model species includes heat shock proteins (HSPs, chaperone proteins that protect the cell machinery), a number of drought stress response or heat stress transcription factors (DSF, HSF), and signal transduction proteins (Mittler *et al.*, 2012). A study in adult durum plants identified genes that respond specifically to combined drought and heat including a chaperone homologous to a putative t-complex protein 1 theta chain (Rizhsky *et al.*, 2002, 2004; Rampino *et al.*, 2012). Two classes of heat shock factors, A6 and C2, have been shown recently to enhance heat tolerance in transgenic wheat (Xue *et al.*, 2014; Hu *et al.*, 2018). Overexpression of TaHsfC2a-B in transgenics up-regulated a cascade of HSP genes in grains during grain filling under heat and also in leaves under drought stress. Combining positive alleles of HSF and DSF such as dehydration-responsive element-binding (DREB) proteins (Morran *et al.*, 2011) might be a way to enhance wheat tolerance to simultaneous stress, but the positive effects will need to be tested in the field in dry and hot climates and redundancy and interactions measured. The forward genetics approach starting with a locus with a demonstrated yield effect is attractive but, to date, none of the QTLs for drought and heat tolerance (Table 1) has been cloned in wheat.

While GS is an efficient tool to quickly identify the best haplotypes, it still requires the incorporation of new alleles into the breeding programme New alleles can also be found in wild relatives of wheat and landraces well adapted to local environments (Lopes *et al.*, 2015), including hot and arid environments. Natural diversity encompasses adaptive mechanisms that wheat plants developed to cope with harsh conditions (Huang and Han, 2014). Emmer wheat and cultivated wheat's wild relatives are sources of tolerance to high temperature or water limitation that could be used to overcome the bottleneck in genetic diversity within the cultivated wheat genepool (Feuillet *et al.*, 2008). The usefulness of a wider germplasm is illustrated by the QTLs deriving from wild emmer wheat for drought (Peleg *et al*, 2005; 2009) and QTLs for salinity tolerance from *Triticum monococcum* (Munns *et al.*, 2012). This is a rare example of successful introgression of a locus (*Nax2*) for abiotic stress tolerance in wheat, following both physiological characterization (James *et al.*, 2006) and positional cloning of the causative gene (*TmHKT1;5-A*) and demonstrates the power of this approach.

New alleles of known genes can also be created by deliberate mutagenesis or genome design (E. Buckler, Plant and Animal Genome conference XXVI, 2018). The ability to efficiently screen for mutations by sequencing (TILLING (Targeting Induced Local Lesions IN Genomes) by sequencing) is quite recent in wheat (Tsai *et al.*, 2011) and is based on both an increased understanding of genomics and advances in next generation sequencing and analysis. Using this approach, Simmonds *et al.* (2016) were able to rapidly identify the causative mutation for the locus *TaGW2-A1* and cross the mutant allele into durum and bread wheat to develop isogenic lines with increased grain weight. The advantage of a mutant collection over wild germplasm is that the new alleles are in agronomically relevant backgrounds where their effect can be readily measured. As the current sequenced collections are in English and US genetic backgrounds, namely Kronos and Cadenza (Tsai *et al.*, 2011), the sequencing of new TILLING collections in varieties that are locally relevant to hot and dry climates is urgently needed.

An alternative method is to specifically edit genes for drought and heat tolerance in a modern, relevant variety. The ability to specifically edit the wheat genome using CRISPR-cas9 ribonucleoproteins has been demonstrated in bread wheat (Liang *et al.*, 2017). This technique promises transgene-free modification of the genome to enhance traits of agronomic interest including abiotic stress tolerance. The use of this technique, however, depends on a detailed knowledge of the sequences underlying tolerance and is likely to require cassettes of sequence edits in the case of editing for combined drought and heat tolerance for wheat. With three highly similar sub-genomes, the majority of wheat gene sequences have homeologues and the contributions of these homeologues to copy number variation and dosage-dependent expression as well as functional redundancy are often unknown in wheat but will influence the success of gene editing approaches. In some cases, a gene/QTL effect could be increased if we were to combine the positive alleles of the three homeologous copies. On a whole genome level, pan-genome data are now being used to understand and mark structural variation of this kind, for instance in maize (Lu *et al.*, 2015). The coming together of advances in genome editing and pan-genomics in wheat should facilitate editing for the future.

Conclusions

Because wheat is heat tolerant when water is available (Parent *et al.*, 2017), to improve wheat for dual tolerance, plants must be studied under the combination of stresses. Results from experiments with heat treatments and well-watered conditions are unlikely to be relevant when water is limiting in the field. There is a large body of evidence showing that water use is essential for either drought or heat tolerance and that, for tolerance of the combined stress, fine control of water relations across the growing cycle will be beneficial. This might be achieved through fine management of spatial and temporal gas exchange. For a wheat plant to be drought and heat tolerant, beneficial traits likely include the following: finely regulated transpiration through small, dense stomata, able to respond to the micro-environment (shade, water, VPD, radiation); maintenance of optimal hydraulic conductance in different tissues; a root system able to grow fast in response to water availability; water-adjustable circadian regulation of plant growth; ability to retain water in essential organs to avoid tissue dehydration; efficient HSPs to protect enzymes and membranes against high temperature; efficient carbohydrate synthesis, export, and remobilization; and an efficient ROS scavenging system (Fig. 1).

The rationale for identifying and deploying alleles for combined drought and heat tolerance in wheat breeding is compelling. Improvements in phenotyping of physiological traits and genomic information are particularly encouraging as we seek to discover and incorporate, possibly, rare, novel tolerance alleles in breeding programmes. Improvement of methods capturing plant and environmental data over time will enable us to phenotype genetic populations for kinematic traits, and this will help us unravel the genetic basis of complex biological processes. Although wheat physiology under drought and heat stress is complex, this complexity and plasticity in itself provides sources of tolerance and hope.

Modifying a single trait might not have a significant effect on yield under stress as some of these traits are co-dependent and would be effective only in combination. Rather than improving a single trait at a time, we might need to combine them in order to increase yield. With underscoring genetic resources and a clear picture of valuable physiological traits, combined drought and heat tolerance in wheat can now be realized in research for use in breeding programmes.

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