Stomatal Responses to Climate Change

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Summary

There remains an ongoing need to make crops more productive in the face of further increases in atmospheric CO_2 concentrations as predicted under climate change, along with higher global surface temperatures and more prolonged, severe and frequent periods of drought. With over 90% of water transpired by plants diffusing through their stomata, studying these small, morphologically varied valves in leaf surfaces remains critical to our understanding the consequences of climate change on stomatal responses, and by extension crop productivity. In the short term, stomata adjust their aperture in response to changes in environmental variables affecting carbon assimilation and water loss. In the longer term, adjustments to stomatal density and size may occur, in conjunction with a range of other responses from the plant.

While increasing CO_2 concentration under climate change had been shown to raise yields and reduce water use by partial stomatal closure, the extent of the fertilisation effect has not been as strong as expected. Meanwhile, higher temperatures and decreasing water availability are likely to have negative yield consequences, with divergent expectations for stomatal aperture and consequently plant water use. However, changes in environmental factors will not occur

in isolation and therefore stomatal and plant responses to a combination of these changes will be hierarchical and involve multiple and possibly unique signalling pathways. Predicting stomatal responses to several simultaneous abiotic stresses such as those outlined above adds a layer of complexity, notably where the stresses produce antagonistic responses in the plant. Targeting steady- state stomatal behaviour has been a successful breeding tactic to date, and continues to generate new insights under interacting stresses. Meanwhile, the emerging field of dynamic stomatal responses to environmental stresses offers new phenotypic targets, and the possibility for enhancing water use efficiency by targeting novel signalling and molecular pathways in stomatal responses.

I. Abbreviations

A	Assimilation rate of CO_2 (μmol m^{-2} s^{-1})
ABA	Abscisic Acid
C_i	Leaf internal CO_2 concentration
C3	Plants exhibiting C3 photosynthetic pathway
C4	Plants exhibiting C4 photosynthetic pathway
$[CO_2]$	CO_2 concentration
$e[CO_2]$	Elevated CO_2 concentration
GC	Guard Cell(s)
GMC	Guard Mother Cell(s)
g_s	Stomatal conductance to water vapour (mmol or mol m^{-2} s^{-1})
ROS	Reactive Oxygen Species
VPD	Vapour Pressure Deficit
WUE_i	Intrinsic Water Use Efficiency ($A\ g_s^{-1}$)

II. General Introduction

The current global population is over 7.2 billion, and is projected to increase to 9.6 billion by 2050 (McGuire, 2013), which means that crop yields need to double in the next 30 years to meet increased demand for plant based products (Ray et al., 2013). In addition to population growth, changing diets and bio-fuel use are further drivers of the need to increase biomass and yield (Amin et al., 2006). The growing population is driving greater urbanisation, (Jones and Kandel, 1992) meaning that the increased demands for food and fuel cannot be met with more land use for crop production and will have to be delivered by greater crop productivity (Ray et al., 2013). World food production is heavily reliant on a small number of crops for food. Rice, wheat and maize are staple foods for 80 % of the world population and rice is consumed by almost 50 % of the global population (Maclean et al., 2013). Against the backdrop of the requirement for increasing productivity with less available land, the changing climate presents additional challenges. The current IPCC 5th assessment (IPCC, 2014) predicts higher concentrations of greenhouse gases leading to higher global mean surface temperatures. In addition, heat waves, drought, heavy and sporadic rain are expected to occur with greater frequency or duration (IPCC, 2014). Atmospheric CO_2 concentration [CO_2] is predicted to rise to 500 - 1000 ppm by the end of the century (Meehl et al., 2007), which could potentially increase yields. Global mean surface temperature is likely to exceed 1.5^{o}C by the end of the century under most climate change projections (IPCC, 2014) and rising temperatures will be detrimental to productivity of C3 crops which face higher rates of photorespiration and a decrease in photosynthesis (Teskey et al., 2015), with further impacts from greater evapotranspiration, leading to reduced soil moisture content, particularly where night temperatures are high (Hatfield and Prueger, 2015).

Higher temperatures are also closely associated with water availability through the impact on transpiration. Transpiration accounts for over 90% of land-based water losses, and therefore, a major goal of crop science is to manage water use more-effectively (Morison et al., 2008, FAO, 2015, Li et al., 2009, Lawson et al., 2003). Stomata - microscopic valves on the leaf surface (Hetherington and Woodward, 2003) - enable water diffusion and are influenced by a number of environmental factors including water availability, leaf temperature and [CO_2] (Lawson and Blatt, 2014). Higher temperatures raise evaporative demand, thus decreasing water availability, driving the need for crops that can tolerate multiple stress conditions (Atkinson and Urwin, 2012). Stomatal pores are at the nexus of the global carbon (Sugano et al., 2010) and hydrological cycles (Betts et al., 2007) and as such are critical to managing the impact of changing temperature, water availability and [CO_2] on crop species.

III. Introduction to stomata

Stomata are microscopic structures found over the predominantly-waterproof and CO_2-impermeable leaf cuticle. Stomata comprise two specialized guard cells (GC) flanking a central pore which facilitate diffusional gaseous flux between the interior of the leaf and the atmosphere (Hetherington and Woodward, 2003, Lawson and Weyers, 1999). When stomatal pores open to maintain CO_2 supply to mesophyll cells for photosynthetic carbon assimilation (A), water is lost through transpiration as a consequence (Morison et al., 2008). Transpirational water loss plays a key role in nutrient uptake from the plant roots as well as evaporative cooling of the leaf tissue (Hetherington and Woodward, 2003, McAusland et al., 2016, Murray et al., 2016, Peterson et al., 2010, Raven, 1977, Raven, 2002). Dynamic stomatal movement acting in response to environmental cues and internal signals in an attempt to optimise the trade-off between A and maintaining plant water status (Farquhar and Sharkey, 1982, Mansfield et al., 1990, Lawson and von Caemmerer, 2010, Buckley and Mott, 2013, Buckley et al., 2017) often referred to as stomatal behavior (Lawson and Blatt, 2014, Zhu et al., 2018). Stomatal behavior is frequently measured as stomatal conductance (g_s), the capacity for the gaseous exchange of water vapor from the leaf to atmosphere, in mole of flux per unit area per second (mol m^{-2} s^{-1}) (Vialet-Chabrand et al., 2017c) and is determined by both stomatal density and behaviour (Lawson and Vialet-Chabrand, 2019). Wong *et al.* (1979) described the relationship of A and g_s as proportional, although the rate of water leaving the leaf through stomata is an order of magnitude greater than CO_2 entering the leaf for A (Drake et al., 2013, Lawson and Vialet-Chabrand, 2019). While Photosynthesis is occurring, there is a constant demand for CO_2 influx into the leaf as chloroplast [CO_2] declines. Carbon dioxide entering the leaf encounters a series of resistance points *en route* to the chloroplast, including boundary layer, stomatal and mesophyll resistance. Of these, stomatal resistance can create the largest impediment to CO_2 influx with g_s being the inverse of stomatal resistance.

A. Factors influencing stomatal responses

Abiotic variables such as light intensity and spectral quality, [CO_2] and temperature are considered to have the greatest direct and indirect impacts on stomatal behaviour (Blatt, 2000), although there is disparity in stomatal sensitivity and responsiveness among different species (Lawson et al., 2012, Lawson et al., 2003, Lawson and Vialet-Chabrand, 2019). Typically, stomata in C3 and C4 species open in response to increasing or high light intensity, low internal [CO_2], high temperatures and low VPD. Conversely, stomatal closure is driven by low or decreasing light, high internal [CO_2] and high VPD as well as hormones such as ABA (Outlaw, 2003, Berry et al., 2010, Elliott-Kingston et al., 2016, Franks and Farquhar, 2001, Inoue and Kinoshita, 2017, Mott and Peak, 2013, Poole et al., 2000, Shimizu et al., 2015, Vialet-Chabrand et al., 2017b, Vialet-Chabrand et al., 2017c, Wang et al., 2008, Woodward, 1987).

The close positive relationship between A and g_s has been well documented in the laboratory (Wong et al., 1979) and a positive correlation between steady-state g_s and yield reported by Fischer *et al.* (1998) in the field. In contrast, in the short term (minutes) stomata can be ‘sluggish’ in their response to environmental factors and internal stimuli leading to nonsynchronous behaviour between A and g_s, which under dynamic conditions can lead to either a limit in A or an unnecessary loss of water (Farquhar and Sharkey, 1982, Hetherington and Woodward, 2003, Franks and Farquhar, 2007, Brodribb et al., 2010, Brodribb et al., 2009, Lawson and von Caemmerer, 2010, Lawson et al., 2012, McAusland et al., 2016, Lawson and Vialet-Chabrand, 2019) leading to sub-optimal water use efficiencies (WUE= A/g_s; (Lawson and Vialet-Chabrand, 2019)). For instance Vialet-Chabrand *et al.* (2017b) reported 18.8% lower than expected A under fluctuating light during the course of a day, which was attributed to stomatal limitation.

B. Stomatal Anatomy: Intra-inter-specific variation

Guard cells (GC) together with the stomatal pore, and if relevant, subsidiary cells make up the stomatal complex (Fig. 1), with stomatal pores ranging in size (10 to 80 μm in length) and density (between 5 and 1,000 mm^{-2}), depending on the species and the environmental growth conditions (Knapp, 1993, Willmer and Fricker, 1996, Beerling et al., 1997, Hetherington and Woodward, 2003). Guard cells are responsible for determining stomatal aperture although the signalling pathways are complex and many remain contentious and are not fully understood (Lawson et al., 2014). By adjusting vacuolar volume through the movement of osmotica including malate, sucrose and K^+, and water, total GC volume is changed (Sussmilch et al., 2019). The change in GC volume and therefore turgor pressure alters stomatal aperture as microfibrils preventing a change in GC width cause curvature of the cell when the pressure potential becomes positive (Willmer and Fricker, 1996, Blatt, 2000, Ziegler, 1987).

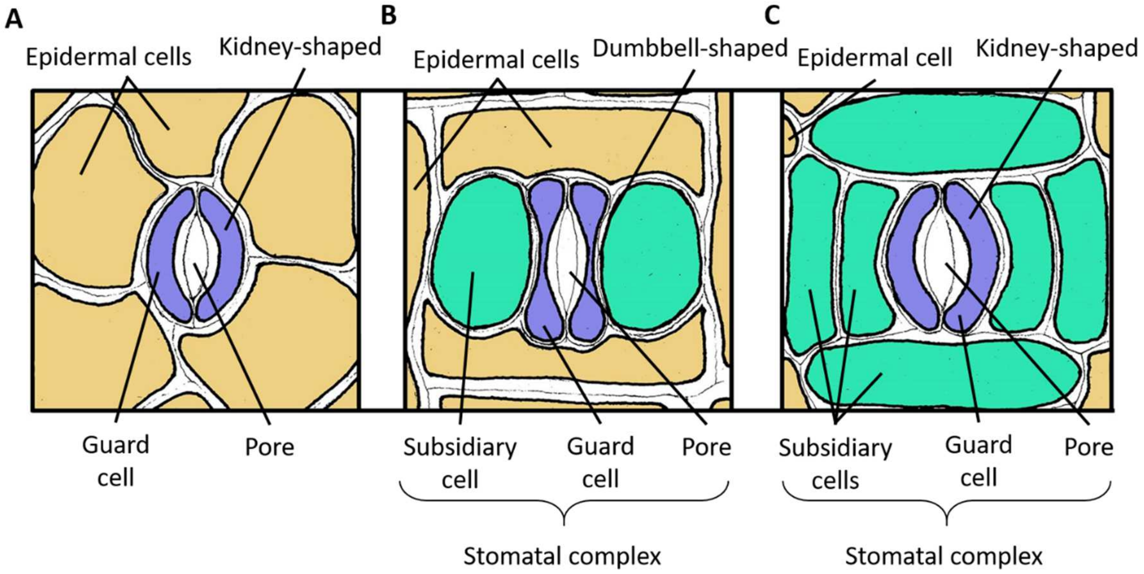

Figure 1: Morphological characteristics of stomatal complexes. A) Elliptical (kidney-shaped – KS) stomatal complex typical of soybean and *A. thaliana* and B) the graminaceous (dumbbell-shaped, DBS) stomatal complex, paired with two main subsidiary cells, typical of rice and wheat. C) Elliptical (KS) stomatal complex with multiple subsidiary cells and kidney-shaped guard cells typical of *Commelina communis*. Adapted and extended from Taiz *et al.* (2008).

Although modern stomata are directly comparable in morphology and function with those in the fossil record from 410 million years ago the density of stomata per leaf area and size has changed considerably through time, as a result of changing climate, most markedly [CO_2] (McElwain and Chaloner, 1995, Beerling and Woodward, 1997, Edwards et al., 1998, Hetherington and Woodward, 2003). Early examples from the Silurian and basal Devonian rock indicate that stomatal complex was either circular or oval in shape (Edwards et al., 1998). Early stomata were anomocytic, i.e. no distinct subsidiary cells (specialised epidermal cells that surround GCs and assist the process of opening and closing stomata), whereas modern species of terrestrial plants can have many subsidiary cells of varying shapes and sizes (Fig. 1) which surround a pair of morphologically and mechanistically diverse guard cells (Edwards et al., 1998, Franks and Farquhar, 2007, Ziegler, 1987). Subsidiary cells differ from epidermal cells due to their ability to shuttle ions (particularly K^+) and water rapidly between themselves and guard cells, modulated by the activity of aquaporins, facilitating a mechanic advantage and increased responsiveness of stomatal movement through rapid changes in turgor pressure (Hachez et al., 2017, Franks and Farquhar, 2007, Raissig et al., 2017, Schafer et al., 2018). The number of subsidiary cells that surround a pair of guard cells differs between species although it is conserved within species, and range from zero (anomocytic - for example sporophytes of some extant hornworts and mosses) to six (hexacytic - for example *Commelina communis*) (Weyers and Paterson, 1987, Rudall et al., 2013, Ziegler, 1987). The significance of subsidiary cells in the efficiency of stomatal functioning in grasses is supported by a recent study by Raissig *et al.* (2017) who manipulated the levels of a transcription factor (BdMUTE) necessary for subsidiary cell formation in *Brachypodium distachyon*. Plants lacking subsidiary cells, (known as subsidiary cell identity defective (*sid*)) had reduced stomatal kinetics, lower g_s and impaired growth (Hughes et al., 2017, Raissig et al., 2017, Hepworth et al., 2018). The evolutionary conservation of the stomatal complex suggests that the pairing of GC and subsidiary cells is integral for the efficiency of stomatal aperture control, highlighting the importance of further studying how the heterogeneity of stomatal complex morphologies affects plant physiology (Raissig et al., 2017, Bertolino et al., 2019, Hepworth et al., 2018).

Two main types of GC distinguished by shape are found in terrestrial plants; graminaceous or dumbbell-shaped (often paired with two main subsidiary cells) (Fig. 1B); and elliptical or kidney-shaped (Hetherington and Woodward, 2003) (Fig. 1A). Dumbbell-shaped GC are typical of grasses and other monocots such as palms, while kidney-shaped GC are found in all dicots, in several monocots as well as mosses, ferns, and gymnosperms (Hetherington and Woodward, 2003). Dumbbell-shaped GC evolved later that kidney shaped GC, between 70 and 50 million years ago, roughly 350 million years after the first perforation that evolved into their kidney-shaped counterparts (Hetherington and Woodward, 2003, Kellogg, 2013). It has been proposed (Hetherington and Woodward, 2003, Franks and Farquhar, 2007) that species such as wheat (*Triticum aestivum*) which possess the characteristic dumbbell-shaped

GC have a faster movement of water across semi-permeable cell-membranes, facilitating superior dynamic performance. The mechanism is thought to be due to the high surface area to volume ratio of the dumbbell-shaped GC's and the close relationship with the adjacent subsidiary cells (Hetherington and Woodward, 2003), with less water needed in order for dumbbell-shaped GCs to increase turgor relative to kidney-shaped GC. As a result, more-rapid opening and closing is facilitated, reducing stomatal response time and supporting higher rates of photosynthetic gas exchange and higher WUE in favourable environments (Johnsson et al., 1976, Willmer and Fricker, 1996, Hetherington and Woodward, 2003, Roelfsema and Hedrich, 2005, Taiz et al., 2018). Two recent reports by McAusland *et al.* (2016) and Lawson &Vialet-Chabrand (2019) support this by demonstrating slower stomatal responses reduce photosynthesis by ca. 10% as well as leading to greater unnecessary water loss during stomatal closure in plants which have kidney-shaped GC's compared with those that exhibit dumbbell-shaped GCs.

C. Stomatal Density and Size

Stomatal anatomical characteristics including the number of stomata per unit area (i.e. density), stomatal size and pore aperture together determine g_s (Lawson and Blatt, 2014) and therefore changes in any one of these variables has a direct influence on g_s. Developmentally, stomatal density differs between and within species and is influenced by a number of environmental variables including light intensity, [CO_2] and water availability (Gay and Hurd, 1975, Woodward, 1987, Gray et al., 2000, Hetherington and Woodward, 2003, Doheny-Adams et al., 2012).

Stomata are morphologically diverse (Willmer and Fricker, 1996, Ziegler, 1987) and species with high densities, often have smaller stomata and *vice-versa* (Franks and Beerling, 2009, Hetherington and Woodward, 2003, Lawson and Blatt, 2014). Hetherington and Woodward

(2003) suggested that smaller stomata have a more-rapid opening and closing time (speed of response) partly due to less water and solute movement between GC and subsidiary cells (osmotic shuttling) (Franks and Farquhar, 2007) and shorter diffusional pathways (Franks and Beerling, 2009). Smaller stomata therefore provide the capacity for rapid increases in g_s, allowing faster diffusion of CO_2 into the leaf for photosynthesis during favourable conditions (Aasamaa et al., 2001). However, species with different stomatal features may have distinct mechanisms influencing the speed of response independently of size (Franks and Farquhar, 2007). Although smaller stomata are not always faster to respond to changing environmental conditions, this relationship typically holds within closely-related species although it is less strong across taxa (Drake et al., 2013, Elliott-Kingston et al., 2016, Lawson and Vialet-Chabrand, 2019, McAusland et al., 2016).

Manipulating gene expression of key components in the stomatal developmental pathway has proven to be a powerful tool in modifying stomatal density, size and stomatal patterning (Franks et al., 2015). For example, the epidermal patterning factors (EPF) are a family of 11 related small, secreted peptides found to regulate stomatal density in *Arabidopsis thaliana* (Franks et al., 2015). Reducing the expression of EPF1 and EPF2 results in higher stomatal densities while the constitutive overexpression produced a similar phenotype to wild type but with reduced numbers of stomata (Hara et al., 2007, Franks et al., 2015, Hara et al., 2009, Hunt and Gray, 2009). Differences in the spacing of cells or the number of stomatal clusters were also demonstrated. A lack of EPF1, which is expressed in GCs of young stomata and their precursors led to high clustering, whereas plants with EPF2 expressed at slightly earlier stages of stomatal development showed almost no clustering (Hara et al., 2009, Hara et al., 2007, Hunt and Gray, 2009, Franks et al., 2015). These studies also indicated that a strong correlation between stomatal density and size was maintained within these plants and the changes in these parameters have the potential to influence A, g_s, and WUE (Doheny-Adams et al., 2012). Both Doheny-Adams *et al.* (2012) and Mohammed *et al.* (2019) found that plants with reduced density and larger stomata also had reduced g_s yet greater plant biomass. The increase in biomass could be attributed to improved water use and lower metabolic costs associated with GC development, and Lawson and Blatt (2014) suggested that CO_2 influx was not a limiting factor and growth was dominated by leaf water status.

D. Stomatal Development and Patterning

Stomatal initiation is a complex series of patterning events laid down in the leaf primordia, split into two fundamentally different modes of growth and development. Eudicotyledon leaf cells divide at multiple points, with clones of new cells forming throughout leaf growth, adding new cells to the leaf, and leading to subsequent expansion (Croxdale, 2000). Committed cells divide asymmetrically, each with an innate ability to both propagate and limit stomatal development (Dow and Bergmann, 2014, Han and Torii, 2016, Pillitteri et al., 2011, Vaten and Bergmann, 2012). The larger cell undergoes asymmetric (amplifying) divisions before differentiating into a guard mother cell (GMC). The GMC divides symmetrically to create two GC. In dumbbell-shaped species, guard mother cells (GMC – guard cell precursors) recruit neighbouring epidermal cells to form subsidiary cells (Dow and Bergmann, 2014, Peterson et al., 2010).

The leaves of monocotyledons on the other hand, have polarized growth from a single source of cells at the leaf base, creating a leaf blade with the oldest cells at the tip. The epidermis consists of regular longitudinal files of cells along the blade length (Croxdale, 2000). New blade cells and stomatal precursors originate in polarized fashion at the leaf base and are present in a continuum of stages from the immature leaf base to the mature leaf tip (Croxdale, 2000, Dow and Bergmann, 2014). Asymmetric cell divisions produce GMC without a meristemoid stage. Files of protodermal cells flank GMC which polarize towards the GMC and divide asymmetrically producing subsidiary cells. Arrest of the developing stomata is a common phenomenon in monocotyledons and is known to yield more-regularly ordered stomata adhering to the one cell spacing rule (Serna et al., 2002, Bergmann and Sack, 2007). Dicot stomata typically adhere to a one-cell-spacing rule where adjacent stomata are separated by at least one intervening epidermal cell; the rule is thought to be important for efficient gas exchange (Nadeau and Sack, 2002, Sachs, 1991).

E. Steady state and kinetic stomatal responses

Steady state measurements of g_s have remained the core technique for understanding stomatal physiology, (see Ainsworth and Roger 2007). Fischer (1998) for example, made point measurements in the early afternoon over the course of a season, and showed a strong correlation between yield and g_s, underlining the value of steady-state g_s as a measure of breeding success. Successive rounds of breeding had produced wheat cultivars with increased g_s which reduced diffusional constraints, reducing leaf temperature and increasing A (Fischer et al., 1998). Not only is steady-state gs important in determining yield, but also the kinetics and magnitude of change. To explore stomatal responses in more detail, the rate at which stomata open and close under changing environmental conditions has recently been investigated as a novel target for manipulation (Lawson and Blatt, 2014, Lawson and Vialet-Chabrand, 2019, Raven, 2014), with light induced changes in g_s the main focus of this work, but with water availability as well as temperature and VPD also being variables whose manipulation might be of interest. Stomata open and close much more slowly than the rate at which environmental inputs vary and an order of magnitude slower than photosynthetic responses (Lawson and Blatt, 2014, McAusland et al., 2016, Qu et al., 2016), and these response rates can be parameterised and modelled (Vialet-Chabrand et al., 2017c, Vialet-Chabrand et al., 2013, Vialet-Chabrand et al., 2016, Vialet-Chabrand et al., 2017a). Environmental conditions are expected to become more-variable under climate change (IPCC, 2014) driving the need to develop new breeding targets under climate change constraints.

Latterly, there have been attempts to understand the impact not just of simple step changes to changing environmental parameters, but of naturalistic fluctuations in them (Matthews et al., 2018, Vialet-Chabrand et al., 2017b), although this work remains in its infancy.

IV. Stomatal Responses to changing atmospheric CO_2 concentration

A. [CO_2], g_s and yield

A large body of evidence has highlighted the effect of [CO_2] on C3 crop yield, mainly driven by positive effects on photosynthesis (Leakey, 2009, Gray et al., 2016, Hatfield et al., 2011). Several reasons for this enhancement of photosynthesis by elevated [CO_2] (e[CO_2]) have been posited. First, elevated [CO_2] increases C_i and therefore the carboxylation efficiency of Rubisco by reducing photorespiration, adding further to the enhancement of photosynthesis (Leakey, 2009). Second, the rate of electron transport rises, as ATP and NADPH product removal improves reaction kinetics, increasing the efficiency of PSII and PSI and enhancing electron transfer (Zhang et al., 2008). Overall, stimulation of the photosynthetic machinery leads to increases in biomass or yield of between10 and 30% depending on the species, environmental conditions and / or experimental [CO_2] (Leakey, 2009). Additionally, several lines of experimental evidence have demonstrated a mean reduction of g_s under e[CO_2] by 22% as reported by Ainsworth and Rogers (Ainsworth and Rogers, 2007) depending on species and photosynthetic pathway (Fig 2a), with the smallest change half that of the largest. More recently, molecular mechanisms have been explored which go some way to explaining the anatomical and physiological changes triggered by increasing atmospheric [CO_2] and involved in the reduction of g_s which go beyond short term changes in aperture and developmental adjustment of stomatal density in plants (Gamage et al., 2018), and which include impacts on nitrogen metabolism, hormonal regulation and the cell cycle (Gamage et al., 2018, Ainsworth and Rogers, 2007).

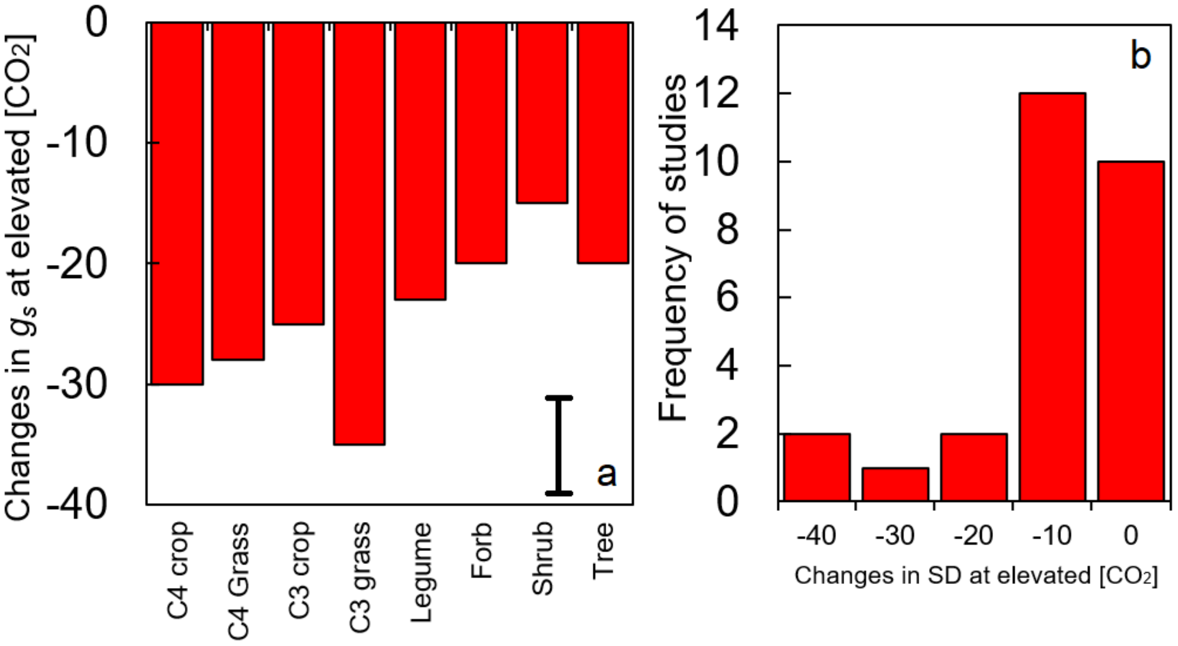

Figure 2. The response of stomatal conductance (g_s) to e[CO_2] in free-air CO_2 enrichment experiments (a) and the histogram of observations from free-air CO_2 enrichment experiments of the change in stomatal density at elevated [CO_2] (b). Data were redrawn from Ainsworth and Rogers (2007).

B. Physiological and anatomical changes in stomatal responses to [CO_2]

In the short term, stomatal responses to e[CO_2] are initially seen as changes in stomatal aperture (Bertolino et al., 2019); the physiological mechanisms involved in partial stomatal closure under e[CO_2] (Fig. 2) are relatively well understood and have recently been reviewed (Gamage et al., 2018, Engineer et al., 2016). Reduction of g_s under e[CO_2] is mainly due to increased activity of K^+ channels, the stimulation of Cl^- release from guard cells and increases in Ca^{2+} concentration causing stomatal closure (Brearley et al., 1997). Genes directly affecting signalling pathways under e[CO_2], include the SLAC1 (Slow Anion Channel Associated 1) gene which has been extensively associated with stomatal closure (Laanemets et al., 2013, Vahisalu et al., 2008). Several recent studies have also suggested a central role by carbonic anhydrase as a key regulatory factor in stomatal dynamics, with the bicarbonate ion activating SLAC1 anion channels (Xue et al., 2011, Hu et al., 2010). The hormone ABA is also involved, mainly through triggering the activation of the OST1 (*O*pen *ST*omata 1) gene, a positive downstream regulator of ABA signalling that ultimately modulates ion channel activity in the guard cell (Chater et al., 2015, Merilo et al., 2015) although there is evidence of further ABA-independent pathways (Yamamoto et al., 2016). Other hormones known to be involved in partial stomatal closure under e[CO_2] include jasmonic acid (Geng et al., 2016).

Over developmental timeframes, plants sense their environment to induce a well-established anatomical effect of e[CO_2] on g_s via a reduction in stomatal density (Woodward, 1987). A reduction in density has been reported for a wide range of species grown at e[CO_2] (Casson and Gray, 2008, Casson and Hetherington, 2010, Woodward, 1987, Woodward and Kelly, 1995). Different degrees of reduction in stomatal densities have been also shown (Ainsworth and Rogers, 2007) with some work showing reductions up to 40% (Figure 2b), however species-level responses vary; for example wood species typically demonstrate little change in stomatal density with e[CO_2] (Ainsworth and Rogers, 2007, Xu et al., 2016). Therefore, the mechanisms of e[CO_2] on stomatal density are species-specific and genotype-dependent.

Gray *et al.* (2000) initially explored the genetic mechanism of stomatal density reduction, and demonstrated that the HIC (*HI*gh *C*arbon dioxide) gene downregulated stomatal development and therefore density under e[CO_2]. Elsewhere the SDD1 gene, which encodes for a key protein involved in guard cell development, is also downregulated under e[CO_2] (Kim et al., 2006), reducing stomatal density.

Elevated [CO_2] is known to increase photosynthetic efficiency by increasing C_i and accelerating electron transport (Leakey, 2009, Zhang et al., 2008). On the other hand, there appear to be both symplastic and apoplastic feedback routes by which higher photosynthetic activity under e[CO_2] would initiate a negative feedback loop by concentration at the guard cell along the transpiration stream, limiting guard cell opening, g_s and stomatal dynamics (Kang et al., 2007, Kelly et al., 2013). Translocation of excess sugars via phloem and the apoplast to stomata may be responsible for the feedback phenomenon via increased apoplastic osmolarity (Kang et al., 2007) or via GC sensing of internal sucrose concentrations, and the expression of ABA-related genes (Kelly et al., 2013, Bauer et al., 2013, Waadt et al., 2015). Sucrose may not be the only carbohydrate signal, and a number of other signals originating in the mesophyll may be involved in the process, including malate, pH changes, redox state signalling or a vapor-phase ions (Lawson et al., 2014). Downstream of e[CO_2] are stomatal closure and reduction in density, leading to lower transpiration, higher temperatures and improved WUE, which will also be discussed in following sections.

V. Stomatal Responses to Water Stress

One of the consequences of climate change is the increasing risk of drought and/or heavy precipitation, with the additional possibility of greater contrast between wet and dry regions (IPCC, 2014). Mild to moderate drought stress lacks a consistent definition, but broadly considered, it is the reduction in soil water content to a level at which recovery of plant function is possible post-drought. Harb *et al.* (2010) define the value as 30% of field capacity in Arabidopsis, but this may vary in other species.

A. Phenotypic variability in responses to low water availability

Plant responses to water stress fall into a two categories: conservative or non-conservative (Caine et al., 2019, Chapin, 1980, Valladares et al., 2000). Conservative plants may mature rapidly before late-season drought risks become extreme, thereby *escaping* mortality risks, but there is a cost in terms of potential yield. Alternatively, the plant may *avoid* drought through mechanisms that reduce water loss by closing stomata as well as maintaining turgor (Farooq et al., 2009), with yield consequences. The non-conservative phenotype *tolerate* drought by withstanding lower water potential through detoxification of reactive oxygen species, the production of LEA-proteins (which appear to protect membranes) and producing osmolytes / osmoprotectants such as proline (Claeys and Inze, 2013, Harb et al., 2010). In the context of climate change, the conservative vs non-conservative behaviours are associated with differences in stomatal responses, both within genotypes and across species (Munns et al., 2010). Non-conservatism in the field is closely related to slower opening and closing of stomata and higher biomass under drought in rice, while conservative cultivars that exhibited faster g_s closing responses were better able to manage water deficit (Qu et al., 2016). The conservative/non-conservative paradigm is analogous to isohydry/anisohydry. In the former case, stomata closed rapidly in response to water stress, maintaining higher water potential (Skelton et al., 2015) while anisohydric plants attempt to maintain carbon assimilation by retaining more-open stomata (Skelton et al., 2015). Crops such as wheat and barley are non-conservative under early stress conditions (Munns et al., 2010) (representing early-to-mid season drought) while rice appears highly non-conservative under elevated [CO_2], using more water than it had under ambient [CO_2] (Kumar et al., 2017). Under expected future conditions of greater water stress, the non-conservative phenotype may not be appropriate, and there may be pressure to develop ideotypes that have more-conservative water-use strategies, particularly if yield costs can be minimised (Bertolino et al., 2019).

B. Physiological and genetic consequences of low soil water availability

Stomata close progressively as water stress increases, restricting CO_2 diffusion into the leaf leading to a decline in photosynthetic rate (Farquhar and Sharkey, 1982). Stomatal limitation on A may be small under unstressed conditions, but stomatal closure becomes the major limitation under moderate drought (Farooq et al., 2009). Under mild drought, soybean, winter barley, winter wheat and spring triticale have been reported to respond with a slow decline in transpiration of between 40%-53% with a concurrent decrease in A of 40-54% (Lipiec et al., 2013). In some circumstances, the reduction in biomass under mild drought is not linked to lower A in crops as diverse as barley, rice and maize (Munns et al., 2010, Rollins et al., 2013), but is an adaptive response to stress implying a conservative phenotype (Rollins et al., 2013). To complicate matters, crops such as wheat and barley are generally non-conservative under water deficit, but may switch to conservative behaviour as stress increases in severity (Munns et al., 2010). Furthermore, phenotypes are highly variable across cultivars and within cultivars over time. Stomata in some modern spring wheat cultivars are more sensitive to drought than historical varieties, showing greater stomatal closure than older varieties under drought, with associated reductions in C_i and PSII activity (Guan et al., 2015). Elsewhere, soil moisture can be a stronger driver of stomatal responses than leaf water status in younger leaves, but this situation is reversed as those leaves age (Chen et al., 2013).

There have been a number of successful approaches to the challenge of breeding enhanced drought resistance into crops in order to combat the effects of climate change. Hundreds of genes induced by drought have been identified (Chaves et al., 2003), some of which may relate to stomatal responses. Yet the relationship between drought and stomatal behaviour is complex. GM approaches have had some favourable outcomes, with perennial grass *L. chinensis* incorporating the wheat LEA gene, (Late Embryogenesis Abundant, a family of genes whose products are linked to ABA responses (Hundertmark and Hincha, 2008)) and in oilseed rape, by downregulating ABA sensitivity; in both cases, increased drought tolerance has been achieved (Wan et al., 2009, Wang et al., 2009). One problem for GM approaches has been the multifactorial responses to drought: resistant GM plants may have thicker, smaller leaves and lower g_s overall, leading to yield performance comparable to that of selection-bred cultivars (Lawlor, 2013).

C. Improving dynamic water use efficiency

Productivity gains in crops have already been made by targeting steady-state stomatal responses and overall g_s (Fischer et al., 1998), although steady state conditions rarely persist in nature (Lawson and Blatt, 2014). In this context, short-term stomatal kinetics offers another option by which breeders can seek adaptive responses to climate change. Stomatal kinetics refers to the rate of change in stomatal aperture in response to a change in an environmental variable (in this case, light) (McAusland et al., 2016). Under environmental perturbation there is an order-of-magnitude mismatch between the rate at which the photosynthetic machinery can be activated relative to speed of stomatal opening, leading to potential yield losses or equally, excess water use (Lawson and Blatt, 2014). Kinetic responses to changing light levels are readily modelled by estimating the time taken to reach 63% of maximal g_s (*tau,* Fig. 3). There is wide disparity even between C3 grass crops (Fig. 3) in terms of *tau* for the opening and closing of stomata in response to step changes in light levels. Meanwhile, there is a pronounced difference between the slow speed of opening and faster closing speed for stomata in the case of the legumes, *Vicia faba* and *Pisum sativum* (Fig. 3), suggesting a range of strategies for managing dynamic water use needs.

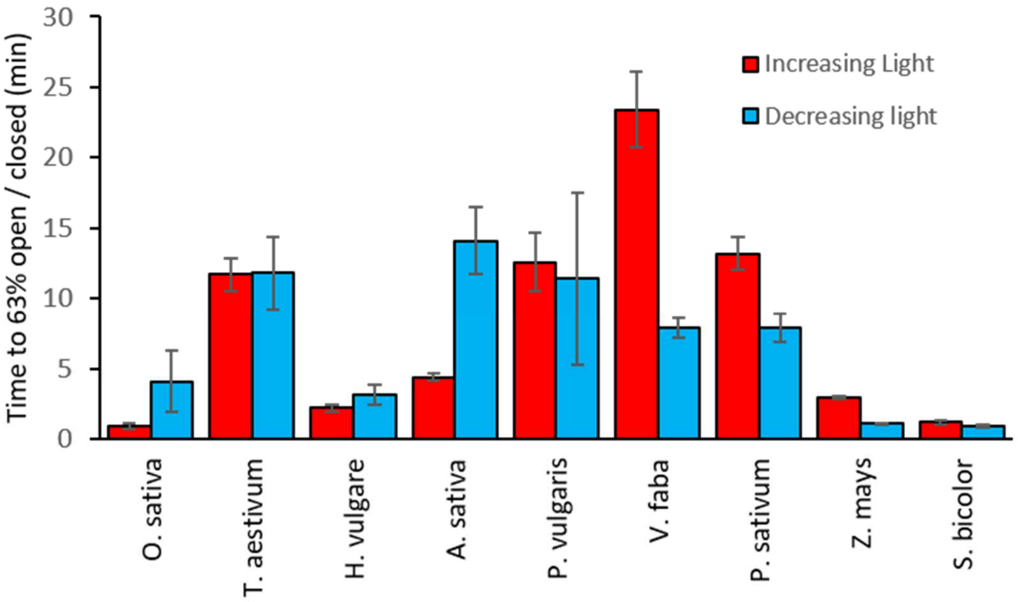

Figure 3: Time constants of kinetic responses of stomata to step changes in light levels for a range of crop species. Data redrawn from McAusland et al. 2016. Means shown +/- se, n=3-5.

D. Breeding for improved performance under drought

Plants show wide variability in the speed of stomatal responses to changing light levels under drought vs well-watered conditions (Fig. 4). Tobacco is notable for its high sensitivity to drought, with large differences between the time constant for opening versus closing depending on the presence or absence of drought. However, barley shows no real change in the speed of g_s response under drought or control conditions under similar conditions (Fig. 4). Rice demonstrated slow closing over slow opening under control conditions, suggesting a prioritising of A over g_s. Under drought, time constants decreased, but the decrease was greater for opening than closing, indicating water was being saved rather than A maintained (Qu et al., 2016). Breeding for g_s kinetic traits should result in increased fitness with respect to drought, but could be at the cost of lower biomass (Lawson and Blatt, 2014, Qu et al., 2016, Faralli et al., 2019a). Therefore, farmers facing water stress will have to trade-off productivity and yield stability when selecting varieties in the face of drought that is likely to increase in severity, duration or frequency (Macholdt and Honermeier, 2016, IPCC, 2014).

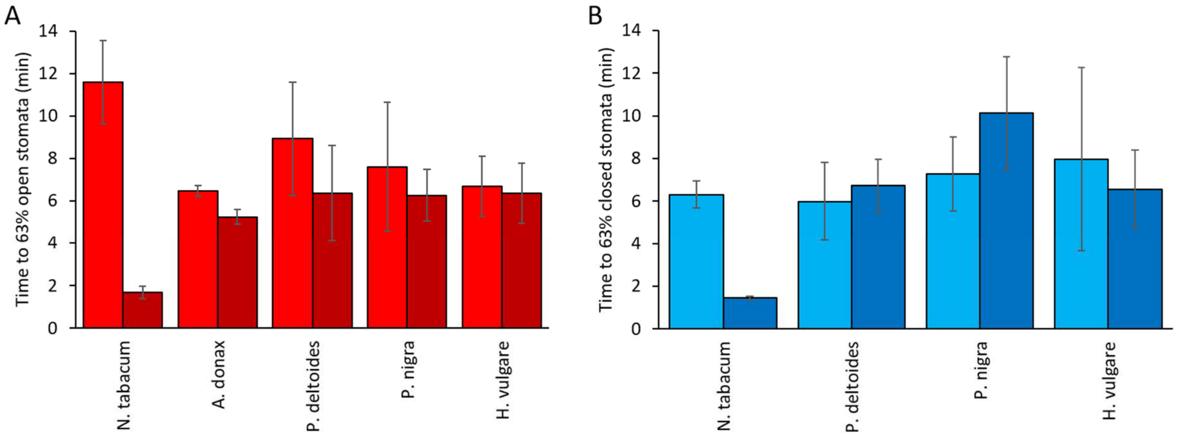

Figure 4: Modelled time constants (tau) to reach 63% of max / min of the increase (A) in stomatal conductance for a step increase in PAR for a range of spp. under control (light red) and drought (dark red) conditions. (B) Time constants for the decrease in stomatal conductance for a step decrease in PAR for a range of spp. under control (light blue) and drought (dark blue) conditions. Data for tobacco from Gerardin et al. 2018, for Arundo from Haworth et al. 2018, for Populus spp. from Durand et al. 2019 for Hordeum from Stevens J. (unpublished data). Means shown +/- error bars

E. Physiological and genetic consequences of excess water availability

Excess water availability (waterlogging) is an important issue in many areas (Box, 1986) and could become a greater problem with climate change, with predictions of greater risks from more frequent and more extreme weather events, including precipitation (Porter and Semenov, 2005, IPCC, 2014). Stomata are known to close in response to waterlogging, reducing g_s by 30-40% with 24 h of stress application (Bradford and Hsiao, 1982) and in legumes, reductions in g_s and A during flooding were correlated (Pociecha et al., 2008). There is clear phenotypic variation across species. Huang *et al.* (1994) reported a range of g_s and A responses to waterlogging in wheat, and noted that reduction in yield potential could be offset by increasing nutrient application

Signalling from root to shoot induces stomatal closure via a mechanism that is not entirely clear (Box, 1986, Bradford and Hsiao, 1982, Chaves et al., 2003, Chaves et al., 2010). Although stress response hormones such as ABA are known to be involved in the process of stomatal closure, alternative pathways exist, including responses mediated by ethylene, cytokinin concentration and xylem pH, while direct hydraulic signals are also thought to be important (Najeeb et al., 2015). Teasing apart gene expression data remains complex, with constitutively expressed gene abundance overlapping with stress response genes under water stress (Chaves et al., 2003).

The timing of waterlogging can affect the severity of the stress, for example in barley g_s was reduced by early (but not late) waterlogging and yield was only reduced by 15%. Late waterlogging reduced barley yield by as much as 62%, mainly through non-stomatal constraints (Ploschuk et al., 2018). A similar pattern was also observed for wheat with between 14% yield reduction under early and 29% under late waterlogging while in peas, waterlogging in general was poorly resisted, with yield declines of up to 92% (Ploschuk et al., 2018). The risk of heavy precipitation events under climate change is a cause for concern for legumes in particular given their susceptibility to waterlogging (Ploschuk et al., 2018), but also for crops such as wheat, which is at risk of significant yield losses under conditions of excess water availability (Herzog et al., 2016). While root-level responses, particularly the formation of aerenchyma, are major drivers of tolerance of waterlogging, stomatal conductance shows wide variability in sensitive (e.g. pea) vs tolerant (e.g. rice, wheat, barley) species (Ploschuk et al., 2017, Herzog et al., 2016, Mohammed et al., 2019). The range of stomatal responses to waterlogging suggest an opportunity for breeders to identify traits for varieties tolerant of the expected increase in duration, severity and frequency of heavy precipitation events (IPCC, 2014).

VI. Stomatal Responses to Temperature Stress

A. The direct impact of increasing temperature

Higher future global temperatures are predicted under all carbon emissions scenarios considered in the most recent assessment of the effects of global climate change (IPCC, 2014),and temperature rises of 1.5^{o}C or more are likely by the end of this century (IPCC, 2014). For every 1°C increase in global temperature, yields of wheat would on average decline by 6%, rice by 3.2%, maize by 7.4% and soybean by 3.1% (Zhao et al., 2017). Thus increasing global temperatures poses a significant risk to global food security.

High temperature can directly affect stomatal behavior and development. Stomatal response to increasing temperature varies depending on species (Sage and Sharkey, 1987) and C_i is an important determinant of stomatal responses (Mott, 1988). Internal [CO_2] varies according to temperature (von Caemmerer and Evans, 2015), and is sensitive to diffusion pathways, encountering diverse gas phase, liquid phase and resistances *en route* from the atmosphere to the chloroplast (Evans et al., 2009).Whether g_s increases, decreases or is unaffected by temperature remains unclear overall (Sage and Sharkey, 1987, Urban et al., 2017b) although the situation is complicated by vapour pressure deficit (VPD, the difference between the water moisture content of air and its saturation point) (Sage and Sharkey, 1987, Merilo et al., 2018). When faced with increasing temperatures, some C3 plants encountered an increase in photorespiration due to a reduction in Rubisco specificity for CO_2 (Peterhansel et al., 2010), an increased affinity for O_2, and stomatal closure (Sage and Sharkey, 1987, Mott, 1988). Yet other species have instead shown increases in stomatal conductance in response to increased temperature (Sage and Sharkey, 1987), which may be attributed to temperature-dependent effects on guard cell metabolism (Lu et al., 2000).

B. Indirect impact of increasing temperature: vapor pressure deficit

While high temperatures *per se* can affect stomata behaviour, responses are often considered indirect, with second-order effects such as transpiration rate, VPD, plant water status and assimilation rate all having an impact on stomatal responses (Urban et al., 2017b). VPD, which is an important consideration in the relationship between leaf temperature and stomata, is a key factor influencing transpiration. An increase in mean temperature, such as that predicted under climate change (IPCC, 2014), is closely associated with increased VPD, and the regulation of transpiration through g_s is a key response to variation in VPD (Lawson and Blatt, 2014). Stomatal responses to VPD are generally well-characterised, with increases in VPD eliciting stomatal closure to conserve water, while decreases in VPD lead to stomata re-opening (Merilo et al., 2018).Stomatal closure through increases in VPD occurs through reduction of leaf turgor and guard cell turgor (Mott and Peak, 2013, Oren et al., 1999). This is also the reason for midday depression in g_s, as high temperatures produce a demand in transpiration that is too great for the plant, and thus a loss of turgor results in the closure of stomata (Balota et al., 2008).

Stomatal responses to VPD are regulated by a variety of mechanisms; for instance, there are active and passive hydraulic responses by stomata to a change in VPD (McAdam and

Brodribb, 2014). Temperature and humidity conditions external to the leaf drive the primary factor regulating stomatal conductance - passive stomatal response to VPD - by affecting leaf and guard cell turgor, and by extension, transpiration (McAdam and Brodribb, 2015). An active response, mediated by ABA, is also present in many species in addition to the passive response. (McAdam and Brodribb, 2015). Guard cells have been shown to autonomously synthesise ABA in sufficient concentrations to induce stomatal closure (Bauer et al., 2013). Knock out of ABA synthesis except at the guard cells maintained stomatal response to increased VPD, suggesting that the stomatal responses to VPD are controlled by guard cell ABA synthesis (Bauer et al., 2013), even where foliar [ABA] is low. However, it has also been shown that ABA promotes stomatal closure through a decrease in water permeability in vascular tissues (Pantin et al., 2013). Recently, it was shown that phloem and guard cell ABA production were functionally redundant, and that ABA is involved in multiple possible pathways in the control of stomatal opening (Merilo et al., 2018). However, the pathway via the protein kinase OST1 remains an important source of stomatal control under high VPD (Merilo et al., 2018, Xue et al., 2011). Increased temperatures due to climate change will result in a higher VPD between leaves and atmosphere, and thus this will decrease stomatal conductance through stomatal closure. This will have an effect on growth and yields, as stomatal conductance is a major limiting factor for carbon assimilation (Farquhar and Sharkey, 1982).

Increased stomatal conductance has been reported under elevated temperature. For example higher temperature increased g_s by 163%, and transpiration by 83% in maize (*Zea mays*) (Zheng et al., 2013) and has also been reported in C3 plants (Crawford et al., 2012, Urban et al., 2017a). The effect of increasing temperature on stomatal anatomy depends on the species and the magnitude of change in temperature. Increased stomatal density at higher temperatures has been reported in soybean (*Glycine max*), oak (*Quercus robur*), tobacco

(*Nicotiana tabacum*), shrubs and grapevines (*Vitis vinifera*) with no effect reported for maize, and a decrease was reported in Arabidopsis (Beerling and Chaloner, 1993, Jumrani et al., 2017, Hu et al., 2014, Hill et al., 2014, Rogiers et al., 2011, Zheng et al., 2013, Crawford et al., 2012, Vile et al., 2012). These findings suggest the employment of different strategies for managing leaf cooling under higher temperatures to counteract the negative impact of heat on photosynthesis and yield. (Crafts-Brandner and Salvucci, 2002, Sage and Kubien, 2007).

C. Night-time temperature increases also affect crop productivity

Global temperature increases due to climate change will also drive greater minimum night time temperature in addition to higher day temperatures, with night time temperature reducing yield in rice and wheat (Easterling et al., 1997, Shi et al., 2016, Narayanan et al., 2015, Prasad et al., 2008, Peng et al., 2004). Although, the effect of night temperature on g_s and the consequences for photosynthesis not fully understood (Peng et al., 2004, Prasad et al., 2008), some reports suggest rice yield declines by 10% for every 1°C increase in minimum temperatures (Peng et al., 2004). While the precise mechanism affecting yield is not clear, it has also been shown that higher night temperatures can affect stomatal opening and photosynthesis during the day perhaps owing to greater water deficit in the leaves (Prasad et al., 2008, Pasternak and Wilson, 1972, Drew and Bazzaz, 1982), highlighting the importance of increases in both the minimum and maximum temperature on crop yield (Welch et al., 2010).

The impact of increased temperature, mediated by behavioural and developmental responses of stomata on crop performance remains a concern under all climate change scenarios, with wheat and maize already showing yield losses (IPCC, 2014, Zhao et al., 2017). There is variability in the consequences of temperature change by region, crop and depending on climate model used, although overall impact assessments remain consistent (Zhao et al., 2017, Iizumi et al., 2017). Furthermore, higher temperatures will drive higher ET rates, which may lead to reduced water availability and greater susceptibility to yield loss in addition to those impacts described above (Condon et al., 2002, Mueller et al., 2012, van Ittersum et al., 2013).

D. The impact of low temperatures on physiology

Stomatal response to low temperatures or chilling depend on whether the species is cold tolerant or cold sensitive. Plants can be considered cold tolerant when stomatal closure occurs before the onset of any water deficit due to decreases in hydraulic conductance and root activity, while cold sensitive plants have less capacity to increase Ca^{2+} uptake by guard cells and therefore stomatal closure is delayed (Hussain et al., 2018, Wilkinson et al., 2001).

Cold sensitive plants, such as *Phaseolus vulgaris* and maize, can have a lowered stomatal conductance after exposure to 24 h of chilling conditions (i.e. less than 8°C day and 4°C night temperatures), or when grown under cool (i.e. 18°C/12°C) conditions (Wolfe, 1991). However in these cold-sensitive plants, which also include cucumber, tomato bean and soybean (Allen and Ort, 2001) stomata can remain open for a period after chilling, which may lead to severe water stress as hydraulic activity in the roots decreases (Eamus et al., 1983). Root temperature, ambient humidity and recovery of hydraulic conductance post-chill appear to be important to the subsequent stomatal response (Allen and Ort, 2001, Bloom et al., 2004), putting these plants at risk of less responsive stomata, lower g_s and by extension, reduced yield.

Cold-tolerant species such as pea and spinach, show 'normal' stomatal opening rates after a period of chilling, and some have been reported to increase overall g_s (Wolfe, 1991). Chilling of Spinach roots to 5°C initially resulted in lower g_s, along with lower root hydraulic conductance, which recovered after a few days of higher temperature to a g_s higher than before the initial chilling, but lower than that observed at higher (i.e. 20°C) root temperatures

(Fennell and Markhart, 1998). Overall, cold-tolerant species exhibit smaller variation in leaf water potential, demonstrating resistance to low-temperature induced water stress (Bloom et al., 2004, Wolfe, 1991), unlike cold-sensitive plants which exhibit decreases in leaf water potential and lower hydraulic conductivity due to their stomatal response (Hussain et al., 2018).

Low temperature affects ABA synthesis (Pardossi et al., 1992) and reduces stomatal sensitivity of guard cells to ABA (Honour et al., 1995, Wilkinson et al., 2001) regardless of whether they are cold-tolerant or cold-sensitive, thereby slowing responses. . However in cold-tolerant species, stomata have been reported to close after a few hours with an increase in root ABA synthesis (Melkonian et al., 2004). Low temperature can affect maintenance of guard cell osmotic potential with stomatal closure the result of increased apoplastic calcium uptake by guard cells (Ilan et al., 1995). Apoplastic calcium influx into the guard cell cytosol has been shown to be responsible for stomatal closure in cold tolerant species, but not in cold sensitive species, through increased sensitivity to Ca^{2+} (Wilkinson et al., 2001). Calcium acts as an intracellular secondary messenger, regulating ion transporter activity in plasma and vacuolar membranes in the guard cell, which determines guard cell turgor (Assmann and Shimazaki, 1999, Wilkinson et al., 2001). Cold hardening, by which process plants become more tolerant of low temperatures through prior exposure, may be due to the cold-induced uptake of calcium into guard cells, which primes the plant for exposure to ABA and thus rapid stomatal closure (Wilkinson et al., 2001). For instance, cold-hardened *Phaseolus vulgaris*, when exposed to low temperatures, reduced stomatal aperture and maintained a positive leaf turgor (Eamus et al., 1983).

The threat from lower temperatures persists even under general conditions of global warming, as increased variability in temperatures is expected (IPCC, 2014). The impact on crop productivity remains uncertain, and will be dependent on both the local temperature history and the sensitivity of the crop species being grown.

VII. Interactions between factors relating to climate change

Under climate change, plants face variability in [CO_2], temperature and water availability. However, the stress caused to plants by these factors does not occur in isolation, and the interaction between them is less predictable. In some circumstances, stresses driving stomatal responses may be additive, in others, antagonistic. Stresses may not combine arithmetically and the impact may be greater than sum of the individual stresses alone (Atkinson and Urwin, 2012). Meanwhile, the genetic regulation of responses varies according to both the nature and extent of the combination of stresses (Vile et al., 2012).

E. CO_2 concentration and drought

The positive impact of e[CO_2] may not only relate to increased photosynthesis, but also to a reduction in g_s and transpiration leading to soil water conservation and water stress mitigation. Many studies have shown that the increased [CO_2] can substantially mitigate the effect of drought, potentially offsetting the reduction in crop productivity relating to climate change. A review by Leakey *et al.* (2009), clearly highlighted that a reduction in ET (followed by reduction in g_s) preserves soil moisture and led to yield maintenance under e[CO_2] in both C3 and C4 crops. Yield maintenance combined with water stress tolerance induced by e[CO_2] was shown in sorghum, wheat, soybean and maize, thus suggesting an optimistic outlook for future crop production under climate change (Kimball et al., 2002). However, recent work focusing on CO_2 x stress interactions has reduced confidence in this prediction. In particular, multi-year and multi-location Free-Air CO_2 Enrichment (FACE) experiments have led to a partial rethinking of earlier beliefs about the positive interaction of e[CO_2] with water stress (Gray et al., 2016, Osborne, 2016). For example, Gray *et al.* (2016), reported that several recent FACE experiments found that the CO_2-stress mitigation hypothesis (mainly driven by the reduction in g_s) is not fully supported. For instance, aerodynamically smooth canopies (such as those seen for soybean and other agriculturally-important species) are likely to see high ET, despite reductions in g_s, arising from a dryer boundary layer driving ET, and an increase in leaf temperature driving higher VPD and thus ET (Gray et al., 2016, Field et al., 1995).

The same study by Gray *et al.* (2016) also showed that e[CO_2] did not stimulate deeper rooting and led to a significant increase in stomatal sensitivity to ABA. The consequence of this was that decreasing g_s response to water stress overrode the stimulative effect of increased sub-stomatal CO_2 concentration. Other long-term FACE and controlled-environment studies supported the lack of [CO_2]-stress mitigation findings in other species (e.g. grasses, canola) with significant negative interactions between leaf area index, g_s, leaf temperature and root-to-shoot signalling for instance (Faralli et al., 2017, McGranahan and Poling, 2018, Osborne, 2016).

A. CO_2 concentration and elevated temperature

The impact of the interaction of e[CO_2] with elevated temperature is controversial with contrasting experimental evidence (Table 1). The expected reduction in g_s under e[CO_2] suggests that crops will inevitably reduce transpirational leaf cooling thus leading to significant increases in leaf temperature (with potential negative downstream effects on photosynthesis). Elevated [CO_2] and temperature can reduce yield by 10-12% in wheat, while for rice the interaction between [CO_2] *and* heat led to modest losses of -1.6% (Hatfield et al., 2011, Cai et al., 2016). Yet in FACE experiments under well-watered conditions, elevated [CO_2] had a positive effect on wheat yield (Fitzgerald et al., 2016), as soil water counteracted the negative effect of e[CO_2] and heat on leaf temperature by supporting high g_s. Therefore, under heat stress the positive effect of [CO_2] on cereals is not only dependent on the timing of stress (i.e. post or pre-anthesis) (Fitzgerald et al., 2016) but also the maintenance of adequate g_s and subsequently optimal leaf temperature (IPCC, 2014). Figure 5 shows the hypothetical pattern of g_s responses (which will vary by species) of conservative and non-conservative crops under varying combinations of environmental stresses over the course of a season, with the overall integral of g_s at the right hand side of the each panel. The latter we link to final yield following Fischer *et al.* (1997). Under developing water stress after establishment (Fig. 5A) g_s responses for conservative genotypes are predicted to involve early stomatal closure (Negin and Moshelion, 2017) compared to non-conservative genotypes (Faralli et al., 2019c), leading to greater integrated g_s over the season for the non-conservative phenotype, and by extension, a yield advantage. Elevated [CO_2] could exacerbate stress-related reduction in yield as described above (Fig. 5B). Yields can fall if no e[CO_2]-induced soil water conservation is achieved and mainly due to the increase in biomass (giving a higher transpirational surface area) and elevated leaf temperature due to reduced g_s (Faralli et al., 2017, Gray et al., 2016, McGranahan and Poling, 2018, Osborne, 2016). Under the drought & elevated e[CO_2] scenario, lower average g_s is achieved, threatening final yields, while the non-conservative phenotype retains a modest advantage in total g_s. The effects of combined water stress and high temperature under e[CO_2] remain to be elucidated by long term FACE experiments although the outcome may be to increase g_s and reduce yield (Fig. 5C) relative to Figure 5B. The consequences of the combined stresses on soil water availability are likely to be exacerbated by the behaviour of the non-conservative plant, which delays g_s responses with serious consequences as the effects of drought are felt. Permanent damage occurs, and the plant is never able to recover to its prior g_s position. Although damage also occurs to the conservative plant, its extent is relatively lower, and post-drought the plant is able to recover to somewhere nearer its previous output. There are yield consequences for both phenotypes, but now the conservative plant outperforms as it is able to deliver higher g_s throughout the season.

Table 1: Example field experiments measuring yield or physiological consequences of an interaction of [CO_2] with water or temperature stress.

Crop	**Stress**	**Physiology or Yield Component**	**% Change in yield vs control**	**Reference**
***Zea mays* (C4)**	Elevated [CO_2] and precipitation	Yield	14% increase from 390ppm to 450ppm, 11% increase from 450ppm to 550pppm. On average, increasing precipitation increases yield by 14.57%	Meng *et al*. (2014) PLoS ONE 9
***Zea mays* (C4)**	Elevated [CO_2] and precipitation	Dry Mass	No significant increase under elevated CO_2. Dry soil results in elevated CO_2 having from 20%-54% higher dry mass.	Samarakoon *et al.* (1996) Australian Journal of Plant Physiology 23
***Zea mays* (C4)**	Elevated [CO_2] and temperature	Grain Yield	Temperature reduces yield by 19% under ambient [CO_2], 38% under e[CO_2]	Abebe *et al*. (2016) Agriculture, Ecosystems and Environment 218
***Zea mays* (C4)**	Elevated [CO_2], well-watered	Assimilation	No significant effect of elevated [CO_2] on photosynthesis under well-watered conditions	Leakey *et al*. (2006) Plant Physiology 140
Triticum aestivum	Elevated [CO_2] and semi-arid precipitation	Yield	13% yield increase	Fitzgerald *et al*. (2016) Global Change Biology 22
***Glycine max* (Soybean)**	Elevated [CO_2], Temperature, Soil Moisture and precipitation	Assimilation	5% increase in assimilation under elevated [CO_2] and temperature, no change under low precipitation/soil moisture	Rosenthal *et al*. (2014) Plant Science
C3 (Mainly Wheat + Soybean)	Elevated [CO_2], Precipitation and Irrigation	Yield Response Ratio	26% increase in Yield Response Ratio with 200mm to 700mm precipitation for FACE experiments, 26% increase in Yield Response Ratio with 1000mm to 200mm precipitation for OTC experiments	Bishop *et al*. (2014) Food and Energy Security 3
Oryza sativa	Elevated [CO_2] + Temperature	Leaf Area, Plant Height	Decreases in leaf area up to 12%, reduced plant height at tillering stage	Liu *et al*. 2017 PLoS ONE 12
Oryza sativa	Elevated [CO_2] and drought stress	Photosynthesis	Higher photosynthesis for elevated [CO_2] plants under drought, than those at ambient [CO_2]	Widodo *et al*. (2003) Environmental and Experimental Biology 49

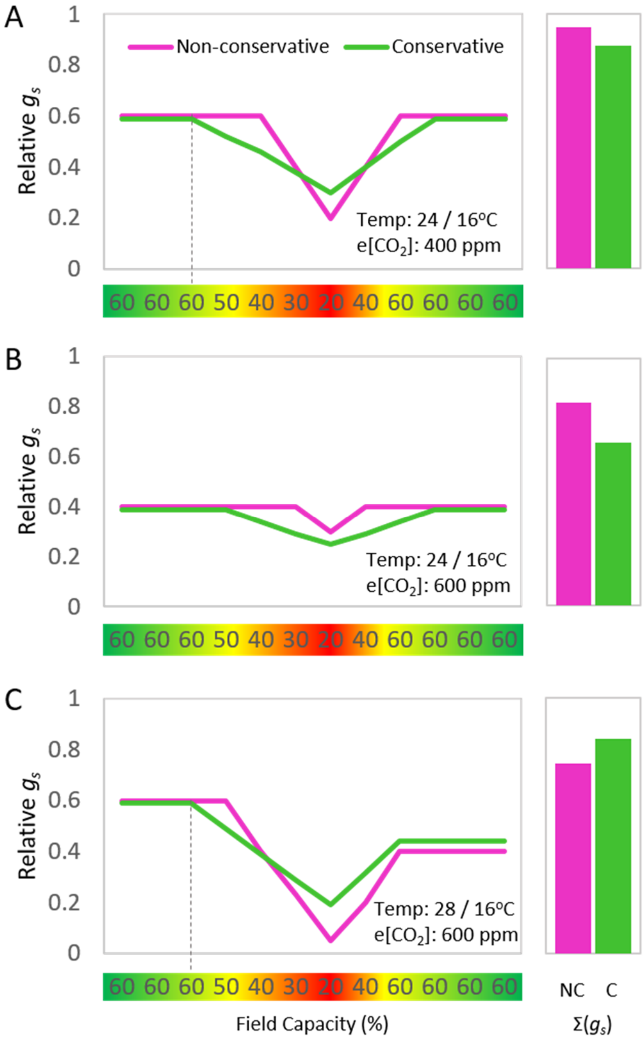

Figure 5. Hypothetical consequences of environmental stress on plants with conservative (green) and non-conservative (magenta) phenotypes during the course of a growing season. In all three instances (A-C) a mid-seasonal drought is applied after establishment progressively reaches 20% of field capacity. For each combination of stresses, the left side describes the relative g_s response to the stress over time, while the bar chart on the right integrates gs over the season and is proportional to final yield. The dotted grey line shows where the conservative plant initiates stress responses. A) Under temperature and e[CO_2] conditions that approximate a Northern European growing season, the conservative phenotype reduces g_s as soil moisture falls while the non-conservative phenotype delays action. As a result, the non-conservative plant is eventually forced to reduce g_s below that of the conservative plant, but at the end of the drought both phenotypes are able to recover gs to the prior level. The non-conservative plant is able to maintain a greater integral g_s and by extension, higher yield. B) Under a situation of e[CO_2], g_s is reduced overall, and the onset of drought responses are delayed. The extent of the reduction in g_s during the drought is affected by other factors (higher total ET from greater biomass, higher leaf temperatures from lower g_s etc.) leading to negative yield consequences overall. C) When both temperature and e[CO_2] are elevated, g_s is initially higher for both phenotypes, perhaps in the range seen in (A) above. Once again, the conservative plant responds early to the changing soil moisture availability, and is able to manage its water needs. The non-conservative plant is rapidly forced to decrease gs to offset declining availability and increased demand. Both phenotypes may sustain damage (reflected in lower post-drought g_s) but the conservative phenotype is likely to recover better from the stress than the non-conservative. Under these condition, it is probable that the conservative plant will be able to achieve greater integrated g_s, and therefore higher yields under possible and imminent climate change scenarios.

In general, the overall inconsistencies in interactions between elevated [CO_2] and environmental stresses are probably derived from variability in treatment applications (e.g. [CO_2] and the timing, degree and length of applied stress). It appears likely that responses are species-specific and that further large-scale multi-year experiments are required to fill this knowledge gap.

B. Drought and heat stress

There has been fairly extensive work on interactions between water deficit and high temperature (Table 1). The impact varies according to the species, growth conditions, conservatism or non-conservatism of the plant, and degree, extent and timing of the stress. One of the difficulties plants face, in particular with reference to combined heat and drought stress is that the initial (and ongoing) plant response – adjustment of stomatal aperture – may involve countervailing and mutually antagonistic hormone pathways (Atkinson and Urwin, 2012). In general, combined stresses lead to lower g_s as minimising water loss is prioritised over heat damage. Transpiration was reduced by 82% under combined heat and drought stress in spring wheat, and A was reduced by 69% (Lipiec et al., 2013). Combined water and heat stresses reduced A regardless of the impact on g_s, with drought, and combined drought & heat affecting yield more than heat alone as total light interception is reduced over the shortened life cycle, although gs is also expected to decline (Lipiec et al., 2013). A similar outcome has been reported in tobacco, where stomata opened under heat stress but closed under drought and the combined stresses (Rizhsky et al., 2002). It appears that drought is the 'greater risk' to the plant, and hierarchically dominates responses. Work in barley has underlined the difficulty in comparing across species, showing a reduction in biomass and yield but not photosynthesis under drought, while heat or combined drought and heat stresses led to lower photosynthesis (Rollins et al., 2013). Morphological changes such as reduced spike number were more evident under drought, while under heat treatment, physiological effects dominated (Rollins et al., 2013), notably in terms of grain yield relative to water use. Underlying these mechanisms are pathways and the expression of cascades of genes, for instance, *Arabidopsis* subjected to combined heat and drought stress had 454 transcripts only elicited under the combined stress compared to the individual stresses alone, and understanding the interaction of multiple abiotic stresses on physiology remains a complex endeavour (Rizhsky et al., 2004, Bechtold et al., 2018). Gene expression had remarkably little overlap under combined heat and drought stress, underlining the validity of the different physiological pathways in a comparison of two durum wheat cultivars (Aprile et al., 2013, Zandalinas et al., 2018). Combined heat and drought stress elicits differential responses of pathways involved in photosynthesis and antioxidant mechanisms, hormone signalling and transcription factor abundance, meaning the link to stomatal responses remains complex and dependent on the balance of stresses (Zandalinas et al., 2018).

There has been relatively little published on responses to a combination of excess water availability and heat stress. In general, flooding and low temperature responses appear aligned (Klay et al., 2018), and can lead to stomatal closure and photosystem damage in wheat (Li et al., 2014), although at lower levels of waterlogging, *A* was relatively improved (Li et al., 2014). Responses to flooding and high temperatures were antagonistic in some genes but aligned in others (Klay et al., 2018).

Multi-location and multi-year field trials will remain of critical significance (Ainsworth and Long, 2005, Gray et al., 2016, Osborne, 2016) in the unpacking of stomatal phenotypes and in the understanding of GxE interactions (Claeys and Inze, 2013). A desire to understand ever more complex combinations of factors – such as [CO_2] x Water stress x Heat stress – will gain in importance as climate change will bring about a wide range of stressors that will vary by region (IPCC, 2014). Current understanding accepts that stomatal responses are complex and possibly hierarchical (Lawson and Blatt, 2014), and much more work needs to be done to understand which stomatal response will dominate and under what set of circumstances.

VIII. Conclusion

Stomata are the gatekeepers of gas exchange and the primary determinants of ET and [CO_2] assimilation rates (Hetherington and Woodward, 2003). They have complex developmental histories, and are under genetic control that is heavily influenced by environmental conditions experienced during that development (Doheny-Adams et al., 2012). From day-to-day, stomata respond to changing variables such as temperature and water availability, as well as atmospheric [CO_2] concentrations (Blatt, 2000). Thus managing the size, number and responsiveness of stomata offers breeders the potential to manage the interaction of g_s and A; by extension, there will be an impact on yield (Lawson and Blatt, 2014).

For these reasons, climate change offers potential opportunities as well as threats to plant productivity, and these threats will vary by region across the world. Climate change is expected to drive higher average temperatures. Heat waves are more likely to occur, droughts and heavy rain more often and for greater duration. The exact extent of the combination of these factors for any given location remains highly uncertain (IPCC, 2014).

In general, the action of a single factor, as mediated by stomata, on plant productivity is well understood (Gray et al., 2016). Rising [CO_2] should lead to higher yields with less water use as stomata can reduce aperture while maintaining internal [CO_2] (Gamage et al., 2018), and over longer periods, reduce stomatal density (Woodward, 1987). Higher temperatures are likely to result in stomatal closure as a result of higher internal [CO_2] from photorespiration (Mott, 1988). Lower water availability is likely to force stomata to reduce aperture on average (Farooq et al., 2009), although varieties and cultivars that adopt a non-conservative approach may maintain yield better than conservative genotypes (Bertolino et al., 2019).

Meanwhile the link between stomatal density and drought tolerance is contested (Jones, 1977); developmentally, altered stomatal size and density under water stress may be offset by other changes such as to leaf size and thickness, and total leaf area (Lawlor, 2013).

Elevated [CO_2] was thought to offset the negative implications of drought (Leakey, 2009). Recent work puts this conclusion in doubt, particularly in terms of unintended consequences such as shallower roots or stomatal sensitivity to ABA (Gray et al., 2016). The interaction between higher temperatures and e[CO_2] might also be expected to lead to higher leaf temperatures, as g_s declines, with a knock-on consequence for photosynthesis and hence yield (Cai et al., 2016). However, in well-watered plots, high temperatures can be overcome and yield maintained under elevated [CO_2] (Fitzgerald et al., 2016). The combination of drought and heat stress involves potentially antagonistic pathways of stomatal responses (Atkinson and Urwin, 2012), with water stress dominating heat stress, and with unique transcription pathways evoked under the combined stresses (Rollins et al., 2013, Zandalinas et al., 2018). However, A is not always affected, even if g_s and yield are (Rollins et al., 2013). In a three-way interaction, the situation is more-complicated again, and there is little clarity over whole-plant and field-level responses as mediated by stomata. We might expect these responses to be hierarchical, and in the context of genetic control, to be epistatic. This is an area that certainly needs more work if we are to understand better the link between expression and response to multiple interacting factors.

Meanwhile, expression patterns and physiological and anatomical responses to single stresses in stomata are relatively well-understood. Measuring kinetic rather than steady-state responses clearly needs more work, with limited understanding to date of the rate of stomatal response to fluctuating light, let alone diurnal patterns of fluctuating temperature (Vialet-Chabrand et al., 2017c, Faralli et al., 2019b) or water availability. Furthermore, one of the predictions of climate change is an increased variability in environmental stressors such as water availability or temperature (IPCC, 2014). Once again, the acclimation of stomata in responding to these fluctuations is poorly understood, and is an active area of research. Overall, there is a great heterogeneity of research, and we call for some agreement between groups in defining protocols and experimental conditions which probe some of these complicated interactions in a consistent and reasoned manner.

There has been widespread realisation in recent years that plant physiology, notably in stomatal responses, is critical to driving ongoing increases in yield in crop species under climate change. Much work remains to be done to understand the genetic and metabolic pathways underpinning responses to such complex interactions. We have discovered that stomatal responses to changing environmental variables appeared straightforward, but like Churchill's comments on pre-war Russia, remain 'a riddle wrapped in a mystery inside an enigma'. We look forward to unravelling this conundrum.

IX. Outlook

FACE, greenhouse and growth chamber experimental work are now giving detailed insights into our understanding of stomatal responses to climate change, and forcing some revision of earlier expectations. The emerging study of stomatal kinetics under varying environmental conditions in addition to steady-state measurements gives additional opportunities to discover phenotypes of interest. The interaction of multiple abiotic stresses such as e[CO2], heat and drought simultaneously remain difficult to model and poorly understood, with a lack of clarity over experimental methods. An agreement among researchers over definitions of stress levels and applications (e.g. mild drought, high temperature) that allow consistency across studies and greater confidence in the robustness of results would be extremely beneficial.

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